PHYSIO-BIOCHEMICAL RESPONSES OF *LABEO GONIUS* (HAMILTON, 1822) FINGERLINGS EXPOSED TO MULTIPLE STRESSORS AND ITS MITIGATION STRATEGY

Thesis submitted in partial fulfillment
of the requirements
for the degree of

Ph.D. (Aquaculture)

by

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SEPTEMBER 2014

Das, P., 2014. Physio-biochemical responses of *Labeo gonius* (Hamilton, 1822) fingerlings exposed to multiple stressors and its mitigation strategy. Ph.D. Thesis, Central Institute of Fisheries Education, Mumbai, p. 285.

ISBN 978-93-81550-02-1

Dedicated to my beloved parents,
wife & daughter

भाकृ अनुप
ICAR

केन्द्रीय मात्स्यिकी शिक्षा संस्थान
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Indian Council of Agricultural Research,
Ministry of Agriculture Govt. of India

Dated: 25 September 2014

CERTIFICATE

Certified that the thesis entitled **"PHYSIO-BIOCHEMICAL RESPONSES OF *LABEO GONIUS* (HAMILTON, 1822) FINGERLINGS EXPOSED TO MULTIPLE STRESSORS AND ITS MITIGATION STRATEGY"** is a record of independent bonafide research work carried out by **Mr. Pronob Das** during the period of study from October 2008 to September 2014 under our supervision and guidance for the degree of **Doctor of Philosophy (Aquaculture)** and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar titles.

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I hereby declare that the thesis entitled **“PHYSIO-BIOCHEMICAL RESPONSES OF *LABEO GONIUS* (HAMILTON, 1822) FINGERLINGS EXPOSED TO MULTIPLE STRESSORS AND ITS MITIGATION STRATEGY”** is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma , associateship, fellowship or any other similar title.

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ACKNOWLEDGEMENTS

It is exquisitely a jubilating occasion and unique opportunity to express my deepest sense of gratitude and indebtedness to my esteemed guide Dr. Neelam Saharan, Principal Scientist & Head, Division of Aquaculture, CIFE, Mumbai and chairman of the advisory committee, for suggesting this research and for her indefatigable effort, constructive criticism, inspiring suggestions, benign attitude and sagacity coupled with patience in guiding me at every stage of this investigation.

I owe a deep sense of reverence to Dr. A. K. Pal, Joint Director, CIFE, Mumbai and member of advisory committee for his erudite counseling, instinctive attention and constant encouragement without whose august supervision it was just about impossible to complete the experiment. His innovative guidance, suggestions strengthened me for executing the research work.

I owe my obligations to Dr. N. P. Sahu, Principal Scientist & Head, Division of Fish Nutrition Biochemistry and Physiology, CIFE, Mumbai and member of the advisory committee for his timely help, cordial co-operation, encouragement and valuable guidance given at various stages for the improvement of this study.

I am indebted to Dr. V. K. Tiwari, Principal Scientist and Dr. Chandra Prakash, Senior Scientist, Division of Aquaculture, CIFE, Mumbai and members of the advisory committee, for their cordial co-operation and encouragement given at various stages for the improvement of this study.

I profess my heartfelt gratefulness and sincere regard to Dr. S.C. Mukherjee, Ex. Joint Director, Dr. Dilip Kumar, Ex. Director, CIFE and Dr. W.S. Lakara, Director, for providing me all the facilities, CIFE could afford during the course of my study.

I must express my sincere thanks to Dr. S. V. Ngachan, Director, ICAR Research Complex for NEH Region, Umiam, Meghalaya, Dr. N. Prakash, Joint Director, ICAR Research Complex for NEH Region, Manipur Centre, Imphal and Dr. B. K. Bhattacharjya, Head of Centre, CIFRI Regional Centre, Guwahati for their help, kind co-operation and constant encouragement for completion of this work.

I owe a deep sense of reverence to all my respected teachers for their valuable advice and unconditional support, which enforced me to specially thank them forever.

I sprightly acknowledge all the faculty members of Aquaculture Division and Fish Nutrition Biochemistry and Physiology Division, for their kind co-operation and affectionable dealing during the investigation. I am really indebted to all of them.

I shall be grateful to all the staff members of CIFE library and librarian for their substantial assistance in compiling the manuscript.

I wish to place on record, my deep sense of honor, love, humble regards and gratitude to all the staff members of CIFE, Mumbai.

I also wish to express my sincere thanks to the Director, CIRCOT, Mumbai for permitting me to carryout electron microscopy analysis at their institute.

I am at loss of words to express my special thanks to my friends Akhtar, Ciji, Shailesh, Ashish, Kundan, Khogen, Sipra, Arup, Prusty, Tapas, Sanjay, Janmajay, Nilesh, Gyan, Soma, Sreenath, Kiruba, Satheesa, Chandan, Alam, Tarku, Srijala, Chanu, Aritra, Barun, Neeraj, Akhlak, Siddaya, Remeya, Laly, Ratheesh, Jesmi, Viji, Vivek, Kashyap, Moina, Saurav, Kaustav, Trincy, Jyotish da and Dalvi Sir for their help and maintaining friendly atmosphere during my entire period of work.

A special note of thanks to Pravin, Charu, Abhay, Santosh, Saroj, Ashish and Sharma for their help and co-operation.

I express my special thanks to Bhagabati sir, Munil sir, Aruna mam, Rupam da, Vasati bau, Bhaskar da and Mandal da for providing homely environment during my stay.

I am also thankful to Dipesh da, Sona mam and Anjil ji of CIFRI Regional Centre, Guwahati for their kind support and encouragement. A special thanks to Pranab and Amulya of CIFRI for making all necessary arrangements for sending the experimental animals from Assam to Mumbai through air.

A special note of thank also to Jungalbalahu Fish Farm, Nagoan district, Assam for providing experimental animals for my research.

The financial assistance provided by the Indian Council of Agricultural Research (ICAR), New Delhi and Central Institute of Fisheries Education (CIFE), Mumbai for my research is gratefully acknowledged.

I fell short of words to express my wholehearted gratitude to my parents, family members and beloved wife whose selfless, unfeigned love, empathy, Sky high inspirations has been the guiding force of my life propelling me towards brighter horizons of happiness.

Lastly, I bow my head before the Almighty who is most benevolent, beneficent and who's blessing have solely contributed for my success till this phase of life and future.

Place: Mumbai
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सारांश

Labeo gonius के अंगुलिकाओं में तनाव का मूल्यांकन कई तनावकारकों (तापमान एवं पीएच) के चार अलग प्रयोगों के माध्यम एवं इसका शमन L-tryptophan (L-TRP) का प्रयोग कर आयोजित की गई । इन सभी चार सम्मिलित प्रयोग को 60 दिन प्रत्येक अवधि तक आयोजित किया गया । पहला प्रयोग चार अलग तापमान (27, 30, 33 और 36^0C) के संपर्क में *L. gonius* अंगुलिकाओं के शारीरिक विकास एवं फिजियो-जैव रासायनिक प्रतिक्रियाओं का मूल्यांकन करने के लिए आयोजित किया गया था । प्रयोग के परिणामस्वरुप पता चला कि 27 और 30^0C में उच्च विकास दर और सामान्य शारीरिक गतिविधियों और 33 और 36^0C पर तनाव का कारण बनता है । दूसरा प्रयोग, कई तनाव के संपर्क में *L. gonius* अंगुलिकाओं के विकास, जैव रासायनिक और रक्त-प्रतिरक्षा प्रतिक्रियाओं को स्पष्ट करने के लिए आयोजित किया गया था । मछलियों को तापमान एवं पीएच के संयुक्त प्रभाव T_1 (27^0C एवं पीएच 7.5), T_2 (27^0C एवं पीएच 5.5), T_3 (27^0C एवं पीएच 9.0), T_4 (33^0C एवं पीएच 7.5), T_5 (33^0C एवं पीएच 5.5) और T_6 (33^0C एवं पीएच 9.0) से अवगत कराया गया । परिणाम यह बताता है कि मिश्रित तनाव, एकल तनाव कारकों से अधिक गंभीर होता है । हालांकि, तापमान और अम्लीय पीएच तनाव के संयुक्त प्रभाव, क्षारिय समकक्ष से अधिक गंभीर था । एकल तनाव कारकों में अम्लीय पीएच, तापमान और क्षारिय तनाव से अधिक गंभीर पाया गया । तीसरा प्रयोग आहारिय L-tryptophan का मूल्यांकन *L. gonius* अंगुलिकाओं के विकास एवं immunomodulatory को दर्शाने के लिए आयोजित किया गया । मछलियों को पाँच iso-nitrogenous आहार L-tryptophan के श्रेणीबध्द स्तर (0, 0.35, 0.7, 1.4 एवं 2.1%) का भोजन खिलाया गया । विकास और immunomodulatory प्रतिक्रिया (पी<0.05) नियंत्रण की तुलना में L-TRP के साथ खिलाया गया समूहों में काफी उच्च था । चौथा प्रयोग, आहारिय L-tryptophan का तनाव के संपर्क में *L. gonius* अंगुलिकाओ के तनाव शमन पर की संभावित मिश्रित भूमिका का अध्ययन करने के लिए आयोजित किया गया था । मिश्रित तनाव (33^0C एवं पीएच 5.5 और 33^0C एवं पीएच 9.0) का समापन करने हेतु मछलियों को L-TRP के श्रेणीबध्द (0, 0.7, 1.4 एवं 2.1%) iso-nitrogenous का शुध्द आहार खिलाया गया । इस अध्ययन से पता चला कि आहारिय L-TRP प्रभावकारी तरीके से प्रतिरक्षा को बढ़ाता है एवं cortisol को कम करके मिश्रित तनाव को कम करता है । *Aeromonas hydrophila* के संक्रमण का चुनौती अध्ययन के परिणामस्वरुप पता चला कि आहारिय L-TRP का आहार *L. gonius* अंगुलिकाओं में संक्रमण विरोधी एवं जीवित रहने की दर एवं प्रतिरक्षा में सकारात्मक प्रभाव उत्पन्न करता है । वर्तमान अध्ययन यह दर्शाता है कि आहारिय 1.4% L-TRP के न्यूनतम स्तर से मिश्रित तनाव कारकों (तापमान एवं पीएच) का निदान करने में फायदेमंद होता है । इस महत्वपूर्ण अध्ययन से यह कहा जा सकता है कि शोधकर्ताओं को इससे मिश्रित तनाव के जुड़े विभिन्न कारकों एवं तनाव के खिलाफ शमन रणनीति विभूषित करने में मदद कर सकते हैं एवं *L. gonius* एक वैकल्पिक उम्मीद प्रजाति के रुप में उध्दीमान एवं सहायक हो सकता है ।

ABSTRACT

Four separate experiments were conducted to evaluate the stress responses of *Labeo gonius* fingerlings exposed to multiple stressors (temperature and pH) and its mitigation through dietary L-tryptophan (L-TRP). All the four experiments were conducted for a period of 60 days each and they are interrelated. First experiment was conducted to evaluate the growth and physio-biochemical responses of *L. gonius* fingerlings exposed to four different temperatures (27, 30, 33 and 36^0C). The results of the experiment showed higher growth rate and normal physiological activities at 27 and 30^0C and causes stress at 33 and 36^0C. Second experiment was conducted to elucidate growth, biochemical and haemato-immunological responses of *L. gonius* fingerlings exposed to multiple stressors. Fishes were exposed to combined effect of temperature and pH *viz.*, T_1 (27^0C x pH 7.5), T_2 (27^0C x pH 5.5), T_3 (27^0C x pH 9.0), T_4 (33^0C x pH 7.5), T_5 (33^0C x pH 5.5) and T_6 (33^0C x pH 9.0). Results revealed that the stress incurred on exposure to multiple stressors was more severe than the single stressor. However, combined effect of temperature and acidic pH stress was higher than the alkaline counterpart. Among the individual stressor, effect of acidic pH stress appeared to be more severe than the temperature or alkaline pH stress. Third experiment was conducted to evaluate the growth and immunomodulatory effect of dietary L-tryptophan on *L. gonius* fingerlings. Fishes were fed with five iso-nitrogenous diets containing graded level of L-TRP (0, 0.35, 0.7, 1.4 and 2.1%). Growth and immunomodulatory response were significantly ($p<0.05$) higher in the groups fed with L-TRP compared to control. Fourth experiment was conducted to study the possible role of dietary L-tryptophan on stress mitigation of *L. gonius* fingerlings exposed to multiple stressors. Fishes were fed with iso-nitrogenous purified diets containing graded levels of L-TRP (0, 0.7, 1.4 and 2.1%) to mitigate the effect of multiple stressors (33^0C x pH 5.5 or 33^0C x pH 9.0). It was found that dietary supplementation of L-TRP significantly reduced the effect of multiple stressors by triggering the immunity and reduces the stress induced cortisol levels. Challenge study also confirmed that supplementation of L-TRP in diet positively influence the survival rate of *L. gonius* fingerlings by resisting the *Aeromonas hydrophila* infection and resulted better immunity. Results of the present study suggest that dietary supplementation of L-TRP at a minimum level of 1.4% is beneficial to mitigate combined effect of multiple stressors (temperature and pH). This basic information will be useful for researchers to understand the interactions of multiple stressors, which can subsequently help to develop mitigation strategies against such stressors and also to establish *L. gonius* as an alternative candidate species in aquaculture.

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Introduction

1. INTRODUCTION

Aquaculture is one of the fastest growing sectors in the world, providing food and nutrition security to millions of people. In India, freshwater aquaculture is dominated by Indian major carps, which contribute around 85% of the total freshwater production. India is known for its rich diversity of carps in its freshwater ecosystems. The country is blessed with 15-20 varieties of minor and medium carps that have a high potential for freshwater aquaculture (Mohanta *et al*., 2008). These carp species can be considered as an alternative to the cultivable major carp species, for diversification in aquaculture. In India, attempts have also been made for diversification of the carp polyculture system through inclusion of new candidate species (Jena *et al*., 2011). In this context, *Labeo gonius* can be considered as one of the potential candidate species for aquaculture (Mohanta *et al*., 2008; Jena and Das, 2011; Jena *et al*., 2011).

Labeo gonius is an important medium-sized carp species once well distributed in the warm water rivers of south-east Asian countries (Talwar and Jhingran, 1991; Petr, 1999). It is commonly known as 'Kuria labeo' or 'Gonius' (Mohanta *et al*., 2008) and Khursa bata (Chattterji, 1992). Rahman (1989) reported that the species was once abundant in the rivers, natural depressions and floodplains all over Bangladesh. In India, it is well distributed in Assam, West Bengal, Uttar Pradesh, Bihar, Rajasthan, Madhya Pradesh and Punjab in the major freshwater rivers, reservoirs, lakes, jheels and tanks (Chattterji, 1992; Mohanta *et al*., 2008). Munilkumar and Nandeesha (2007) also mentioned that the species is well distributed in different parts of Northeast India. The fish is easily recognized by the characteristic silvery scales, the upper part of the body is covered with greenish and olive-coloured fine scales and the lower part with smaller-sized shiny silvery scales and lateral line with 75-80 scales (Rahman *et al*., 2008). The fish has considerable scope to be component of carp poly-culture system. Since the fish is herbivorous bottom feeder, it can be cultured as a bottom feeding substitute in composite fish culture. The species has been widely accepted as cultivable fish in northeastern region of India, especially in Assam (Jena and Das, 2011). Webber and Riordan (1976) also reported that ultimate use or consumer acceptance criteria are considered as essential requirements for a candidate species for aqua-farming.

However, information on several aspects of the fish is scanty. So, generating research information on different aspects of the species will definitely help to establish the species to a greater extent.

During the culture period, fishes are exposed to various environmental changes, which cause degree of trauma (stress) in aquatic organism's results detrimental consequences (Reubush and Heath, 1996). Stressful condition in aquaculture causes growth reduction, reproduction inhibition, abnormal behavior, immune-depression, decrease in disease resistance and even death of the fish (Pankhurst and Van der Kraak, 1997; Schreck *et al.*, 1997), which affect both economic and socio-economic status of the aquaculturist. Stress can be defined as an assumption of stimulus acting on biological system and the subsequent responses to that system, and the stimulus is normally referred to as the stressor, which induces stress and creates the stress responses. Seyle (1950) gave the most widely accepted definition of stress as, "the sum of all physiological responses by which an animal tries to maintain or re-establish a normal metabolism in the face of physical or chemical force".

The stressful condition in fish is not due to a single stressor but is a combined effect of many stressors like natural and anthropogenic heavy metals, radionuclide, temperature, crowding, pesticides, pH, low dissolved oxygen, etc. Rearing of aquatic organisms in man-made environment has resulted in exposure to the stressors, which may not be experienced to the same degree in natural environments (Donaldson, 1981). However, information on the combined effect of different stressors on growth and physio-biochemical response of fishes is limited. Understanding the interactions of multiple stressors is the biggest challenge to the researchers which subsequently help to develop mitigation strategies during aquaculture practices.

Among the different stressors, temperature and pH are of prime concern due to their practical utility in aquaculture. Higher temperature can alter the physiological functions such as thermal tolerance, growth, metabolism, food consumption, reproduction and to ability to maintain internal homeostasis (Fry, 1971). Similarly, change in water pH causes stress in fish affecting its physiology and growth (Pickering, 1981). However, there is no information on the combined effect of temperature and pH stress on growth, biochemical and haemato-immunological response of *L. gonius* fingerlings.

Temperature is a major factor, which directly influences metabolism affecting all physiological processes in ectotherms such as food intake, metabolism and nutritional efficiency (Brett, 1979; Burel *et al*., 1996). Thus, water temperature directly affects the growth of fish. Therefore, knowledge of suitable temperature range at which fish have a faster growth rate is very important for effective management of aquaculture systems (Cui and Wootton, 1988). Improved growth and survival were recorded in composite culture of Indian major carps when water temperature was 28^0C (Chakraborty *et al*., 1976). However, carps are reported to thrive well between 18.3 and 37.8^0C (Jhingran, 1975). Indian carps are eurythermal in nature (Kasim, 2002).

Thermal stress is considered as one of the most important aspects in current aquaculture scenario. It is defined as, "any temperature change that produces a significant disturbance in the normal functions of a freshwater teleost and thus decreases its probability of survival" (Elliott, 1981). Thus, thermal stress is the temperature change that causes disturbances in the homeostasis of an animal. All definitions of stress, however, share the common premise of stimulus acting on a biological system and the subsequent reaction of the system (Pickering, 1981). Water temperature influences oxygen concentration, metabolism, reproduction and growth of fishes (Fraser *et al*., 2002). Induction of stress by environmental temperature variation determines whether an organism adapts to changed conditions and survives or suffers from physiological disturbances (Peuranen *et al*., 2003). Rising temperature up to certain limits favours aquaculture by reducing the time required to produce marketable sized animals and producing more generations per unit of space and time. On the contrary, higher temperature adversely affects the health of aquatic animals by increasing metabolic rates and subsequent oxygen demand and assisting proliferation, and virulence of bacteria and other pathogens that cause a variety of patho-physiological disturbances in the host (Wedemeyer *et al.,* 1999). It has been shown in animal that extreme temperature changes induce stress in organism and that is characterized by changes at the physiological (Trot, 2011) as well as the cellular architectural levels (Egginton and Sidell, 1989).

Water pH is also considered as one of the important parameters for successful aquaculture production. The optimum pH range for culture of carps is 7.5-8.5 (Banerjea, 1967) and the majority of the Indian carp culture ponds have a pH in this range. However, fluctuations in water pH can cause stress in fish affecting its body physiology and growth (Pickering, 1981). A change in water pH exerted stress in Indian major carps characterized by swelling of erythrocytes, production of immature erythrocytes and reductions in the total erythrocyte counts, haemoglobin and serum protein content and also increase in blood glucose (Das *et al*., 2006). Water pH plays an important role in maintenance of the homeostasis in aquatic animals and fluctuations in pH are reported to cause disturbances in acid-base and ion regulation, and ammonia excretion (Wilkie and Wood, 1996). Brown *et al*. (1984) recorded the alteration in cortisol level in rainbow trout exposed to acid treated water. Li and Chen (2008) also reported that variations in pH can also be acute toxic to crustaceans, resulting in reductions in rate of survival and growth among them, sometimes accompanied by serious diseases or mass mortality. However, the degree of these responses got varied among species.

In this context, application of natural/ synthetic compounds as stress mitigators in many fish species has been found to be beneficial to overcome this problem. Some of them are vitamin C, vitamin E, pyridoxine, methyl donor, levan, etc. These have been proven to be beneficial for their multiple functions. Chung *et al*. (2005) mentioned that nutraceuticals can be used to counter stress. Several studies have indicated that immunostimulants can trigger defense system, even in stressful conditions and therefore reduces the deleterious effects mediated by stress (Ishiibashi *et al*., 1992; Ortuno *et al*., 2003; Manush *et al*., 2005). Considering L-tryptophan function as a cortisol blocker (Lepage *et al*., 2003; Tejpal *et al*., 2009), it can be evaluated as a dietary supplement to mitigate combined effect of multiple stressors in diverse species to improve protein utilization efficiency and hence to increase aquaculture production.

Tryptophan is an essential amino acid, which cannot be synthesized in the body and thus must be obtained from food or supplements. In addition to being a constituent of protein, it acts as a precursor of neurotransmitter serotonin (5-hydroxytryptamine/ 5-HT) and nicotinic acid, whereas serotonin is precursor of melatonin and act as a regulator polysome function and carbohydrate metabolism.

Elevated dietary intake of TRP has been reported to result in increased brain levels of TRP and elevated rates of 5-HT synthesis and metabolism (Lepage *et al.*, 2002). Dietary supplementation of TRP, more or less blocked the increase in plasma cortisol induced by stress (Lepage *et al.*, 2003). Tejpal *et al.* (2009) also reported that L-tryptophan supplemented diet mitigated crowding stress and enhanced growth of *Cirrhinus mrigala* fingerlings. However, no attempt has been made to evaluate the possible role of dietary L-TRP supplementation on stress mitigation of *L. gonius* fingerlings exposed to multiple stressors.

With these backgrounds, the present study was intended with the following three objectives:

1. To study the growth and physio-biochemical responses of *Labeo gonius* fingerlings exposed to four different temperatures.
2. To elucidate the growth, biochemical and haemato-immunological responses of *Labeo gonius* fingerlings exposed to multiple stressors.
3. To evaluate the possible role of dietary L-tryptophan on stress mitigation of *Labeo gonius* fingerlings exposed to multiple stressors.

Review of Literature

2. REVIEW OF LITERATURE

2.1 Diversification of Species in Aquaculture

To cope up with the growing demand for food, there is a tremendous pressure on aquaculture production. In this context, diversification of species has to play a vital role in days to come. In India, freshwater aquaculture is dominated by Indian major carps of Cyprinidae Family, which contributes about 87% of the total freshwater production (ICLARM, 2001). However, India is known for its rich diversity of carps in its freshwater ecosystems. The country is blessed with 15-20 varieties of minor and medium carps that have a high potential for freshwater aquaculture (Mohanta *et al*., 2008). These carp species can be considered as alternative to the cultivable major carp species for diversification in freshwater aquaculture. In India, attempts have also been made for species diversification of the carp polyculture system through inclusion of new candidates (Jena *et al*., 2011), which mainly includes fringe-lipped carp, *Labeo fimbriatus*; kuria labeo, *Labeo gonius*; kalbasu, *Labeo calbasu*; olive barb, *Puntius sarana*; silver barb, *Puntius gonionotus*, etc. In this context, *L. gonius* can be considered as one of the potential candidate species for aquaculture (Mohanta *et al*., 2008; Jena and Das, 2011; Jena *et al*., 2011).

2.1.1 ***Labeo gonius*** **as an alternative candidate species**

L. gonius is a medium carp (Talwar and Jhingran, 1991) of Cyprinidae Family, commonly known as 'Kuria labeo' or 'Gonius' (Mohanta *et al*., 2008) and Khursa bata (Chattterji, 1992). It is one important medium-sized carp species once well distributed in the warm water rivers of South-east Asian countries (Talwar and Jhingran, 1991; Petr, 1999). Rahman (1989) reported that the species was once abundant in the rivers, natural depressions and floodplains of all over Bangladesh. In India, it is well distributed in Assam, West Bengal, Uttar Pradesh, Bihar, Rajasthan, Madhya Pradesh and Punjab in the major freshwater rivers, reservoirs, lakes, jheels and tanks (Chattterji, 1992; Mohanta *et al*., 2008). Munilkumar and Nandeesha (2007) also mentioned the well distribution of the species in North-eastern states. The fish is easily recognized by the characteristic silvery scales, the upper part of the body is covered with greenish and olive-coloured fine scales and the lower part with smaller-sized shiny silvery scales and lateral line has 75-80 scales (Rahman *et al*., 2008).

2.1.1.1 Culture potential of *L. gonius*

The fish has been identified as an important species for diversification of aquaculture practices in India (Mohanta *et al*., 2008; Jena and Das, 2011). It has a greater scope as a component of carp culture system (Jena and Das, 2011). In a culture system, it attains more than 500 g in a year, showing its potential as an important candidate species for culture. Since the fish is herbivorous bottom feeder, so it can be cultured as a bottom feeding substitute in composite fish culture. It competes with *C. mrigala*, *L. calbasu*, *L. fimbriatus* and *L. bata* in composite fish culture. The species has been widely accepted as cultivable fish in Northeastern India, especially in Assam because of its high market value and better consumer preference. Webber and Riordan (1976) also reported that ultimate use or consumer acceptance criteria are considered as essential requirements for a candidate species for aqua-farming. Munilkumar and Nandeesha (2007) also reported the culture of the species in different parts of the North-eastern region. It is a highly valued fish in North-eastern India (Jena and Das, 2011). *L. gonius* is also treated as ornamental fish in its juvenile stage and termed as non-classified ornamental fish (Mandal *et al*., 2007).

2.1.1.2 Growth performance of *L. gonius*

The maximum reported size of *L. gonius* is 150 cm (Day, 1878; Talwar and Jhingran, 1991) in natural water bodies, however around 50 cm is more typical in commercial catches. Rahman (1989) and Hussain and Mazid (2001) reported that the fish can attain a weight of more than 1.0 kg, with a maximum size recorded to be 1.4 kg and 61.0 cm. In culture system, *L. gonius* can reach upto 40 cm in size and 750 g in weight in one year period. However, small sizes of 100-300g are easily marketable as compared to 700-800g of Indian major carps. Even though the growth of the species is little slower compared to *L. rohita* (rohu) and *C. catla* (catla) but it fetches better prices, which compensates the low growth rate. However, its growth rate is more or less similar to *C. mrigala* (mrigal) and other bottom feeding fishes. The culture of the fish has been well-established in North-eastern states, especially in Assam, as a component of composite fish culture. Since the fish is herbivorous bottom feeder, there is a feasibility of substituting *C. mrigala* with *L. gonius* in grow-out carp culture system without compromising the yield, which has been already proved by Jena and Das (2011). Silver barb (*P. gonionotus*) and kuria labeo (*L. gonius*) showed higher growth potential than rohu (*L.* rohita) and fringe-lipped carp (*L. fimbriatus*) under polyculture in concrete tanks (Jena *et al.*, 2011). Stocking density of 0.6 million hatchling/ha appears to be the most efficient stocking density for rearing of *L. gonius* fingerlings in earthen nursery ponds (Rahman *et al.*, 2008).

2.1.1.3 Significance of *L. gonius*

The species is having high consumer preference due to its good taste. Kalita *et al*. (2005) reported the medicinal properties of the fish to cure disease. It is a high valued fish especially in North-eastern states of India (Jena and Das, 2011) and the growth of the species is comparable with Indian major carps. It fetches better prices than the other carp species in domestic markets of North-eastern India. The price of the species is much higher than the Indian major carps, providing better profit to the farmers. As the fish is bottom feeding in nature, it can be considered as an alternative to other bottom feeding fishes in composite fish culture, without compromising the yield and profit (Mohanta *et al.*, 2008).

2.1.1.4 Researchable issues on *L. gonius*

Even though, *L. gonius* is considered as a potential candidate species for diversification of freshwater aquaculture in India, the information on several aspects like thermal tolerance limit, nutrition and growth, stress and physiology, health status, etc. are scanty. So, a study on the effect of temperature, multiple stressors and stress mitigation on the species will be useful in the context of today's climate change scenario.

2.2 Stress in Fish Culture: An Emerging Issue

During the culture period, fishes are exposed to several stressors, such as faulty practices and environmental changes. These frequently cause various degree of trauma (stress) in aquatic organisms resulting in detrimental consequences (Reubush and Heath, 1996). Stress is considered as one of the major problems in aquaculture, where it has been related to growth reduction, reproduction inhibition, abnormal behaviour and immunodepression, the last frequently associated with infectious diseases and death (Pankhurst and Van der Kraak, 1997; Schreck *et al.*, 1997).

2.2.1 Definition of stress

Stress can be defined as an assumption of stimulus acting on biological system and the subsequent responses to that system and the stimulus is normally referred to as the stressor, which induces stress and creates the stress responses. Stress is a confounding term (Schreck, 1981) and the concept (as applied to biological systems) has long and controversial history primarily because of difficulties in formulating a universally acceptable definition (Pickering, 1981). However, Seyle (1950) gave the most widely accepted definition of stress as, "the sum of all physiological responses by which an animal tries to maintain or re-establish a normal metabolism in the face of physical or chemical force".

2.2.2 Type of stressors in fish culture

The cultured organisms are exposed to many stressful activities during routine aquaculture practices. The commonly encountered stressors in aquaculture are physical, chemical, biological and procedural stressors (Wedemeyer *et al.*, 1999). Physical stressors include temperature, light, D.O., sound, etc. Chemical stressors include water quality, pesticides, pollution, diet, metabolic waste, etc. Biological stressors include stocking density, microorganisms (pathogenic and non-pathogenic), macroorganisms (parasites), lateral swimming requirements, etc. Procedural stressors include handling, hauling, stocking, disease treatment, feeding (manual and automated), etc.

2.2.3 Concept of multiple stressors in Aquaculture

In fish, the stressful condition is not due to a single stressor but in fact, it is a combined effect of many stressors like natural and anthropogenic heavy metals, radionuclide, temperature (low and high), crowding, pesticides, heavy metals, pH (low acidic and high alkaline), low dissolved oxygen, etc. These cumulative stressors may lead to economic loss to the fish culturists attributed to reduction in growth rate, decrease in disease resistance, immunosuppression and even death of the fish. Rearing of aquatic organisms in manmade environment has resulted in exposure to a number of stressors, which may not be experienced to the same degree in natural environments (Donaldson, 1981). Ortuno *et al*. (2002) reported higher cortisol level due to combined effect of multiple stressors.

However, there is very limited research on the combined effect of different stressors on growth and physio-biochemical response of fishes. Crain *et al*. (2008) also mentioned that cumulative effect of multiple stressors on ecological communities remains largely unknown. Understanding the interaction of multiple stressors is the biggest challenge to the researchers which subsequently help to develop mitigation strategies during aquacultural practices.

2.2.4 Stress responses in fishes: An overview

Stressor is a causative factor and stress is a response. Stress responses in fish have been well documented by Wendelaar Bonga (1997) and Barton (2002). The stress response of fish follows the general vertebrate pattern (Weytes *et al*., 1999). A key element in the response is a switch from anabolism to catabolism, the animal uses up the energy mobilized as it attempts to avoid or overcome the immediate threat (Pickering, 1992). The quantum of response may vary with nature and factors such as age, sex, maturation stage, environmental temperature, species and strain of fish. Physical, chemical and perceived stressors can all evoke non-specific responses in fish, which are considered adaptive to enable the fish to cope with the disturbance and maintain its homeostatic state (Barton, 2002). There are differences in the generalized stress responses among different fish species (Vijayan and Moon, 1994) and different stocks or races of the same species (Iwama *et al*., 1999) differ in their tolerance to applied stressors. Primary stress responses as well as secondary vary in different fish species, their developmental stages as well as in individuals within the same species (Barton and Peter, 1982; Pottinger, 1992). Changes in stress responses may depend on the type of stress and stress duration. The stress response is therefore an expression of both genetic and environmental factors (Iwama *et al*., 1992).

When fish and other aquatic organisms experience environmental disturbances that lie outside the normal range, the effect may be drastic. At the organism level, a series of physiological changes occur following stressful challenges, which are adaptive in nature. These physiological responses are termed as ‘General Adaptation Syndrome’ (GAS), Seyle (1950). It consists of (1) an alarm reaction in which catecholamine and corticosteroids “stress hormones” are released, (2) a stage of resistance during which adaptation occurs and (3) a stage of exhaustion if adaptation is lost because the stress was too severe or long lasting (Wedemeyer and Mcleay, 1981). Only a general understanding exists of the sequence of physiological, behavioural and genetic alterations that occur as fishes attempt to maintain themselves in the stressful environmental changes.

The physiological systems of fish can be challenged or stressed, by a wide array of biological, chemical and physical factors. Stress produces effects that threaten or disturb the homeostatic equilibrium and elicits a coordinated set of behavioural and physiological responses thought to be compensatory and or adaptive, enabling the animal to overcome the threats. However, during chronic stress, the stress response may lose its adaptive value and become dysfunctional, which may result in inhibition of growth, reproductive failure and reduced resistance to pathogens (Wendelaar Bonga, 1997; Jobling *et al*., 1995). It is conventional to classify stress response as primary (neural and neuro-endocrine responses), secondary (physiological consequence of such primary response) (Mazeaud *et al.,* 1977). Wedemeyer and Mcleay (1981) expanded this system to include tertiary changes; changes in behaviour, growth rate and increased susceptibility to diseases. Stress response in all vertebrates including fish results in the activation of the neuro-endocrine system, which brings about changes in metabolism, osmoregulation and haematology (Barton, 2000). Stress responses in fish have been broadly categorized into the primary, secondary and tertiary responses (Barton, 1997; Wendealaar Bonga, 1997).

2.2.4.1 Primary stress responses in fishes

Primary stress responses (Wendealaar Bonga, 1997) include release of adrenocorticotropic hormones (ACTH) from the adenohypophysis and release of “stress hormones” (catecholemines and corticosteroids) from the interrenal. In animals, sensory perception of stress is a prerequisite for stress response. In fish, an adverse condition stimulates the afferent neural pathway that run in the synaptic nervous system from the hypothalamus to the chromaffin tissue of the head of kidney. Direct stimulation of the chromaffin tissue leads to the release of catecholamines. Other neurons, within the hypothalamus that run to the adenohypophysis of the pituitary glands, secrete a neuropeptide that stimulates the pituitary to produce and release of adrenocorticotropic hormone (ACTH). ACTH released in the blood stimulates the interrenal cells of the head of the kidney to produce corticosteroid hormone particularly cortisol. This results in rapid elevation of plasma catecholamines and cortisol that leads to secondary stress responses.

2.2.4.2 Secondary stress responses in fishes

Secondary stress (Wendealaar Bonga, 1997) responses include changes in osmoregulatory capacity and ionic balance; blood chemistry and haematological; metabolic and cellular changes. In fish, cortisol enters liver cells where it binds to nuclear receptor, resulting in activation of genes that produce a series of enzymes that have a range of metabolic effects. This results in a suite of biochemical and physiological changes, which may include hyperglycemia, hyperlacticaemia, depletion of tissue glycogen reserves, lipolysis and inhibition of protein synthesis. Other changes may include the osmotic and ionic disturbances, due to diuresis and loss of electrolyte from the blood and change in haematology with erythrocytic changes and reduction of white blood cells. Catecholamines in particular have marked influence on cardiovascular functions; leading to change of blood circulation, gill perfusion and oxygen carrying capacity of blood. Corticosteroids, on the other hand, are known to stimulate the ion-transport mechanism in the gill and kidney. These secondary stress responses are believed to be adaptive mechanisms and are particularly important for fish to recover from stress, by maintaining oxygen supply to the tissues, to regain osmotic and ionic equilibrium and to meet the increased energy demands imposed by exposure to environmental stressor. Typically these changes persist only for few hours or days, following acute exposure to the stressor and do not therefore result in any deleterious effect to the animal.

2.2.4.3 Tertiary stress responses in fishes

Chronic exposure to stressors provokes tertiary stress responses (Wendealaar Bonga, 1997) that result in a number of pathological changes and reduction in reproductive success, depression of growth rate and decreased disease resistance. Tertiary stress response represents whole animal and population level changes (Barton, 1997) associated with stress. Thus, when fish are exposed to environmental stressor, a hierarchy of responses is initiated and if the stress is severe or long-lasting, successively higher levels of biological organisation get affected. This signifies that the primary responses are the changes at the endocrine level; whereas the tertiary responses refer to those changes that can be easily seen by observing the animal (Jobling *et al.*, 1995). A fish's or fish population's tolerance to environmental alterations thus depends at least in part, upon the individual fish's ability to regulate stabilizing processes so as to accomplish the required physiological or behavioural adaptation (Elliott, 1981).

2.3 Combined Effect of Multiple Stressors

Combined effect of stressors led to deterioration in surface water quality and the aquatic habitat (Hinton, 1998). Hilton (1998) also mentioned that the interaction of multiple stressors, coupled with invasions of foreign species and the export of juvenile fish into aqueducts, has driven several species of fish to near extinction. Schindler (2011) reported that cumulative effects of climate warming will affect both water quality and quantity. Climate warming will exacerbate the effects of acid precipitation. Water resources globally are affected by a complex mixture of stressors resulting from climate change (Hering *et al.*, 2014). Global warming are characterised by temperature more frequent extremes (IPCC, 2012) and these are likely to have negative impact on aquatic organisms (Jentsch *et al.*, 2007).

During the culture, fishes are exposed to number of stressors, which alter the physiology of fish and ultimately result in death of the fish. However, the degree of these responses varied among the different species. Among the different stressors, temperature and pH are of prime concern due to their practical utility in aquaculture. Higher temperature can alter the physiological functions such as thermal tolerance, growth, metabolism, food consumption, reproduction and ability to maintain internal homeostasis (Fry, 1971). Similarly, change in water pH cause stress in fish affecting its body physiology and growth (Pickering, 1981).

Verma *et al.* (2007) reported that increasing acclimation temperatures alters the immune status of *Cyprinus carpio* advanced fingerlings and persistent sub-lethal exposure to chlorine augments this temperature induced immunosuppression. On exposure to salinity and temperature imparts stress and significantly influences growth performance and anti-oxidative potential (Akhtar *et al.*, 2013). Kumar *et al.* (2012) reported that concurrent exposure of temperature and endosulfan stress significantly affects the growth performance of *L. rohita* fingerlings. Reduced growth rate and food conversion efficiency have been reported in fish at combined stress of salinity and higher temperature (Likongwe *et al.*, 1996). Janssens *et al.* (2014) mentioned that combined effect of extreme temperature and pesticide stress interacted across metamorphosis on populations of the damselfly, *Ischnura elegans*. Concurrent exposure of organisms to contaminants and warming there is increasing concern that stressors may interact in a delayed way (Segner, 2011). Such delayed effects are easily overlooked in the many animals that have a complex life cycle. In rainbow trout, combined effect of high temperature and high pH reported to increase mortality of the fishes (Wanger *et al.*, 1997). Dockray *et al.* (1998) reported that combined effect of warmer temperatures and sub-lethal low pH appeared to be slightly more costly than either stressor alone. However, there is no information on the combined effect of high temperature and pH (low and high) stress on physio-biochemical and haemato-immunological responses of *L. gonius* fingerlings.

2.4 Temperature Stress in Fish

Temperature is a major factor, which directly influences metabolism affecting all physiological processes in ectotherms such as food intake, metabolism and nutritional efficiency (Brett, 1979; Burel *et al.*, 1996). Thus, water temperature directly affects the growth of the fish. Therefore, knowledge of suitable temperatures at which fish have a faster growth rate is very important for effective management of aquaculture systems (Cui and Wootton, 1988). Improved growth and survival were recorded in composite culture of Indian major carps when water temperature was 28^{0}C (Chakraborty *et al.*, 1976). However, carps are reported to thrive well between 18.3 and 37.8^{0}C (Jhingran, 1975). Indian carps are eurythermal in nature (Kasim, 2002).

Thermal stress is defined as, “Any temperature change that produces a significant disturbance in the normal functions of a freshwater teleost and thus decreases its probability of survival” (Elliott, 1981). Thus, thermal stress is the temperature change that causes disturbances in the homeostasis of an animal. All definitions of stress, however, share the common premise of stimulus acting on a biological system and the subsequent reaction of the system (Pickering, 1981). Water temperature influences oxygen concentration, metabolism, reproduction and growth of fishes (Morgan *et al*., 1999; Fraser *et al*., 2002). Induction of stress by environmental temperature variation determines whether an organism adapts to changed conditions and survives or suffers from physiological disturbances (Peuranen *et al*., 2003). Thermal stress can adversely affect fish health by increasing metabolic rate and subsequent oxygen demand, invasiveness and virulence of bacteria and other pathogens that cause a variety of pathophysiological disturbances (Wedemeyer *et al.,* 1999).

2.4.1 Thermal tolerance in fishes

Thermal tolerance limit of Indian major carps has been well defined by several workers (Chatterjee *et al*., 2004; Das *et al*., 2004, 2005). However, no attempt or study has been made so far to evaluate the thermal tolerance limit of the *L. gonius*. Critical thermal methodology (CTM) is used to estimate thermal tolerance as the mean temperature at which fish get exposed to slow, constant changes in water temperature and reach a predefined nonlethal (but near lethal) end point (Cox, 1974). This method can be used as reliable to envisage the suitability of any species for aquaculture in different agro-climatic regions (Debnath *et al*., 2006). The area of the tolerance zone is a useful index of thermal tolerance (Elliott, 1981). Oxygen consumption is an index of metabolism in freshwater fish (Kutty and Peer Mohamed, 1975) and is dependent on the acclimation temperatures (Kita *et al*., 1996). In fishes, the metabolic responses that are quantified in terms of oxygen consumption show a linear correlation to temperature due to its direct effect on the kinetics of the enzyme reactions involved (Hazel and Prosser, 1974; Hochachka and Somero, 1971). The final preferred temperature may be estimated indirectly based on the relationship between oxygen consumption and acclimation temperature (Kita *et al*., 1996).

Temperature adaptation is an essential physiological phenomenon in fishes and is dependent on the acclimation temperature (Beitinger and Bennetta, 2000). Over the years many workers have studied the thermal tolerance limit of the variety of fishes. Some of them are, Indian major carps (*L. rohita, C. catla and C. mrigala*) advance fingerlings (Das *et al.*, 2004), Rohu (*L. rohita*) fry (Das *et al.*, 2005), Rohu (*L. rohita*) and common carp (*Cyprinus carpio*) fingerlings (Chatterjee *et al.*, 2004), yellowtail catfish (*Pangasius pangasius*) fingerlings (Debnath *et al.*, 2006) and climbing perch (*Anabas testudineus*) (Sarma *et al.*, 2010).

2.5 pH (Acidic and Alkaline) Stress in Fish

The water pH is the measure of the hydrogen ion (H^+) concentration. The pH scale ranges from 0 to 14.0 with a pH of 7.0 being neutral. A pH below 7.0 is acidic and a pH of above 7.0 is basic. Water pH is considered as one of the important parameters for successful aquaculture production. The optimum pH range for culture of these carps is 7.5-8.5 (Banerjea, 1967) and the majority of the Indian carp culture ponds have a pH in this range. However, a change in water pH can cause stress in fish affecting its body physiology and growth (Pickering, 1981). The disturbances in the gas exchange, nitrogenous waste excretion, acid-base and ionic balance due to the change in water pH causes stress in fish affecting its body physiology and growth (Pickering, 1981). The gill is the primary interface between fish and its environment for gas transfer, acid-base balance, ion regulation and ammonia excretion. Water pH plays an important role in maintenance of the homeostasis in aquatic animals and increases or decreases in pH are reported to cause disturbances in acid-base and ion regulation and ammonia excretion (Wood, 1989; Wilkie and Wood, 1991; Wilkie *et al.*, 1993; Jensen and Brahm, 1995).

The fish gill is highly permeable to hydrogen ions. The significant influx of H^+ at low environmental pH has also marked effect upon the respiratory homeostasis and osmoregulatory process. The effect of low environmental pH on fish includes increased branchial loss of ions, reductions in plasma pH, bicarbonate, Na^+ and Cl^- concentrations, slowing of apical H^+/Na^+ exchanger process (Ultsch *et al.*, 1981), increased urinary excretion of surplus H^+ ions for compensation of the net base loss at branchial epithelium (McDonald and Wood, 1983). Besides increases in blood pH and higher plasma ammonia concentrations, exposure to high water pH also reduces plasma electrolytes such as Na^+ and Cl^-, a little can increase a reduction in the blood protein and haemoglobin concentration (Wood, 1989; Wilkie and Wood, 1996; Wilkie *et al.*, 1993).

Several authors have reported that variations in pH can also be acute toxic to crustaceans, resulting in reductions in rates of survival and growth among them, sometimes accompanied by serious diseases or mass mortality (Chen and Chen, 2003; Li and Chen, 2008). Brown *et al*. (1984) studied the alteration in cortisol level in rainbow trout exposed to acid treated water for 21 days. Plasma cortisol level got increased at pH 5.2. They opined that to increase cortisol level, 8 days of acid treated water at pH 4.7 would be required. Acharya *et al*. (2005) reported that exposure of *L. rohita* fingerlings to to acidic pH 5.5 and alkaline pH 9.0 affects physiological process and structural changes in gill tissue.

A change in water pH exerted stress in Indian major carps (*L. rohita*, *C. catla* and *C. mrigala*) characterized by swelling of erythrocytes, production of immature erythrocytes and reductions in the total erythrocyte counts, haemoglobin and serum protein content and also increase in blood glucose (Das *et al*., 2006). However, the degree of these responses varied among the three Indian major carps. The leucocyte count decreased significantly in *C. mrigala* at acidic and alkaline pH, most likely due to the greater stress level at such higher pH change. Das *et al*. (2006) confirmed that chronic exposure of Indian major carps to acidic pH 5.5 and alkaline pH 9.0 is harmful as it imparts stress by affecting haemato-immunological parameters.

2.6 Stress Mitigation in Fish

Stress is considered as one of the major problems in aquaculture, where it has been related to growth reduction, reproduction inhibition, abnormal behaviour and immunodepression, the last frequently associated with infectious diseases and death (Pankhurst and Van der Kraak, 1997; Schreck *et al*., 1997). Application of different stress mitigators is found to be beneficial over the years to overcome these problems. A number of natural and synthetic compounds have been applied as stress mitigator in many fish species, which have been proven to be beneficial for their multiple functions. Some of them are vitamin C, vitamin E, beta glucan, pyridoxine, methyl donors and microbial levan, etc.

Several studies have indicated that immunostimulants can trigger defence system, even in stressful conditions and therefore reverse the deleterious effects mediated by stress. Nutraceuticals are used to counter thermal stress

(Chung *et al*., 2005). It is reported that supplementation of high protein and vitamin C reduced the bioaccumulation and stress responses in *Channa punctatus* (Sarma *et al*., 2009). Dietary high protein and vitamin C were supplemented for ameliorating stress (Manush *et al*., 2005) in *Macrobrachium rosenbergii*. Vitamin C was demonstrated to enhance tolerance to environmental stressors, hypoxic stress (Ishiibashi *et al*., 1992) and increase in immunoresistance (Orbach and Laurencin, 1992). Vitamin C and E are used as dietary supplements to overcome stress in gilthead seabream (*Sparus auratus*).The results showed that all stress-induced increase in blood glucose and cortisol levels were lower in fish fed with vitamin C and/or E-supplemented diet than in fish fed the control diet (Ortuno *et al.,* 2003). Recent studies have indicated that immunostimulants can trigger defence systems, even in stressful conditions, and can reverse the harmful effects mediated by stress (Meena *et al*., 2012; Wang *et al*., 2012; De *et al*, 2013).

L-tryptophan has been used as a growth promoter and also as a stress mitigator in several fishes. However, responses varied from species to species. Tejpal *et al*. (2009) successfully used to mitigate crowding stress in *C. mrigala* fingerlings by reducing cortisol level. However, there is no information on the possible role of dietary L-tryptophan as growth and immuno-modulator and also as stress mitigator on *L. gonius* fingerlings.

2.6.1 L-Tryptophan (L-TRP)

Tryptophan is an essential amino acid, which cannot be synthesized in the body of fishes and thus must be obtained from food or supplements. In addition being a constituent of protein, it acts as a precursor of neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) and nicotinic acid, whereas serotonin is precursor of melatonin and acts as a regulator polysome function and carbohydrate metabolism (Wittman, 1976; Sidransky *et al*., 1981). Deficiency of L-TRP causes scoliosis, lordosis and renal calcinosis in rainbow trout (Kloppel and Post, 1975).

2.6.1.1 Physiological metabolism of tryptophan

Tryptophan is the precursor of serotonin (5-hydroxytryptamine, 5-HT) and its availability is a limiting factor for the synthesis of brain 5-HT, both in mammals (Fernstrom and Wurtman, 1972) and in fish (Aldegunde *et al*., 1998, 2000). The first and rate-limiting step in the 5-HT biosynthesis is the hydroxylation of L-tryptophan (TRP) to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase (TPH) (Boadle-Biber, 1993). The enzyme tryptophan hydroxylase adds a hydroxyl group to tryptophan's benzene ring at position 5, creating 5-hydroxytryptophan. Another enzyme, amino acid decarboxylase, removes a carboxyl group from 5-hydroxytryptophan forming 5-hydroxytryptamine which is more commonly known as serotonin. Tryptophan hydroxylase (TPH) does not appear to be subjected to any inhibition of 5-HT (serotonin), the end product of the reaction pathway. Hence elevation of brain TRP levels results in an increase in rate of 5-HT synthesis. Since the enzyme TPH catalyzing the hydroxylation of TRP does not seem to be saturated by TRP *in vivo*, the rate of this reaction appears to be restricted by TRP availability both in mammals (Boadle-Biber, 1993) and in teleost fish (Aldegunde *et al.,* 1998, 2000; Winberg *et al.,* 2001).

Elevated dietary intake of TRP has been reported to result in increased brain levels of TRP and elevated rates of 5-HT synthesis and metabolism (Johnston *et al.,* 1990; Aldegunde *et al.,* 1998, 2000; Winberg *et al.*, 2001; Lepage *et al.,* 2002). In mammals, increased functional release of 5-HT following elevated dietary intake of TRP has been confirmed using microdialyses and *in vivo* voltametry (Boadler-Biber, 1993; Fernstrom, 1983; Fernstrom and Wurtman, 1972). Serotonin seems to inhibit aggressive behaviour in all vertebrates and the fact that elevated dietary intake of TRP suppresses aggressive behaviour in rainbow trout, *Oncorhynchus mykiss* (Winberg *et al.*, 2001) reveals that elevated dietary intake of TRP in fish also results in increased synaptic 5-HT release.

One important physiological function of tryptophan is its use in protein synthesis. Since mammals cannot synthesize tryptophan, the proportion that is metabolized into serotonin and kynurenine is lost for protein synthesis (Floc'h *et al.*, 2010). In most animals, TRP oxidation is usually incomplete and the enzyme thought to initiate the principal catabolic pathway is tryptophan pyrolase (EC1.13.11.11) which occurs mainly in liver (Bender, 1978). In warm-blooded animals, major proportion (75%) of tryptophan in plasma is bound to protein whereas in trout very little, if any, tryptophan was found in the bound state. Fish is a cold-blooded species and similar lack of binding has been found in frog blood by Fuller and Roush (1973) and Badaway and Evans (1975). There was no relation between dietary TRP level and activity of hepatic tryptophan pyrolase (Walton *et al.,* 1984). In rat liver, the enzyme activity in liver is induced by hydro-cortisone or tryptophan (Feigelson and Greengard, 1962). However, Brown and Dodgen (1968) found that administration of ACTH or three glucocorticoids or repeated doses of tryptophan into channel cat fish failed to induce the liver enzyme.

Badaway and Evans (1975) observed that in some animal species such as rat, pig and chicken, the enzyme exists as the holoenzyme and apoenzyme whereas in other species such as cat, sheep and rabbit the apoenzyme is absent. When the apoenzyme is absent there is no hormonal induction, no stimulation by haematin and the animals are more sensitive to tryptophan toxicity. Similar observations were made in trout (no stimulation by haematin or apparent response to increased tryptophan levels) by Walton *et al.* (1984) and they also concluded that control of tryptophan catabolism is more likely to be related to tissue substrate concentration than levels of tryptophan pyrolase. Alternatively, TRP may be catabolized by other routes such as indoleamine pathway found in cattle (Yang and Carlson, 1972). At suboptimal dietary intake, TRP would be required only mainly for protein synthesis, but once this demand had been met any additional tryptophan would be oxidized (Walton *et al.,* 1984).

2.6.1.2 L-Tryptophan and Large neutral amino acids

The carrier transporting TRP across the blood-brain barrier is non-specific, also transporting other large neutral amino acids (LNAA; *i.e.*, tyrosine, phenylalanine, leucine, isoleucine, valine). Brain levels of TRP will thus not only depend on plasma levels of TRP, but also on plasma levels of other LNAA competing for the same carrier (Boadle- Biber, 1993; Aldegunde *et al.*, 1998, 2000). However, Aldegunde *et al.* (2000) obtained results suggesting that the competition between TRP and other LNAA for uptake into the brain is less important in rainbow trout than in mammals. One reason for this could be that the total plasma pool of TRP is directly available for uptake to the brain, since TRP is largely found in the free-state in rainbow trout plasma (Rozas *et al.*, 1990).

In mammals, on the other hand, TRP in plasma is primarily bound to albumin and only the small fraction of free TRP is directly available for uptake into the brain. The fact that TRP is primarily albumin-bound in blood plasma of mammals which spares it from insulin-mediated uptake by muscle and as a consequence, a meal rich in carbohydrate will increase brain 5-HT synthesis by inducing insulin secretion, which lowers the blood concentration of LNAA other than TRP (Fernstrom, 1983). Markus *et al*. (2000) found that a carbohydrate-rich, protein-poor (CR/PP) food diminished the depressive mood and cortisol response to controllable as well as uncontrollable laboratory-induced stress in highly stress prone human subjects. Assuming that the central 5-HT system is involved, Markus *et al*. (2000) hypothesised that the effect of CR/PP food on the stress-induced cortisol response in stress prone subjects is mediated by a stimulation of the 5-HT pathway connecting the raphe nucleus to the hippocampus, which inhibits HPA activation (Deakin and Greaff, 1991).

2.6.1.3 Serotonin and its mode action

The organisation and function of the brain serotonergic system seems to be highly conserved across the vertebrate subphylum (Parent *et al*., 1984). Serotonin (5-hydroxytryptamine, 5-HT) is involved in the regulation of the hypothalamic-pituitary-adrenocortical (HPA) axis in mammals (Chaoulof, 1993; Dinan, 1996) as well as in the control of the hypothalamic-pituitary-interrenal (HPI) axis (the teleostean homologue of the HPA axis) in fish (Winberg and Lepage, 1998; Overli Ø *et al*., 1999; Hoglund *et al*., 2000).

In several studies, the ratio of 5-hydroxyindoleacetic acid (5-HIAA, the major 5-HT metabolite) to 5-HT brain concentrations has been found to correlate with plasma levels of cortisol, suggesting that the action of brain 5-HT on the HPI axis is stimulatory (Overli *et al*., 1999; Hoglund *et a*l., 2000; Winberg and Lepage, 1998). Moreover, treatment with 8-OHDPAT, a selective 5-HT1A receptor agonist, elevates plasma levels of cortisol in cannulated rainbow trout (Winberg *et al*., 1997). However, the role of the brain 5-HT system in the control of the HPI axis is not clear. Brain serotonergic pathways in mammals are important when coping with stress, not only by initiating the adrenocortical stress response but also terminating it (Markus *et al*., 2000). The brain 5-HT system is not a unitary system and, in mammals, 5-HT pathways terminating in the hypothalamic paraventricular nucleus stimulate HPA axis activity, whereas those terminating in the hippocampus inhibit it

(Jacobson and Sapolsky, 1991; Deakin, 1991; Deakin and Graeff, 1991; Maes and Meltzer, 1995; Summers *et al*., 1998; Markus *et al*., 2000).

In mammals, 5-HT terminals make synaptic contact with corticotrophin releasing hormone immunoreactive neurons within the hypothalamic paraventricular nucleus (Liposists *et al*., 1987) and treatment with 5-HT precursors such as TRP or 5-HT receptor agonists (8-0H-DPAT) has been reported to stimulate HPA axis activity, elevating plasma levels of gluco-corticoids (Chauloff, 1993). Thus, the elevation of plasma cortisol in non-stressed fish fed TRP-supplemented feed could have been mediated by stimulation of the brain 5-HT system. In this regard, it is contradictory that dietary TRP reduces stress induced elevation of plasma cortisol, but the reason for this could be elevated negative feedback as a result of increased basal plasma cortisol by elevated dietary L-tryptophan in non-stressed group.

Serotonin may also suppress HPI axis activity by inhibiting central norepinephrine (NE) activity. In mammals, NE stimulates hypothalamic corticotropin-releasing hormone which in turn has a stimulatory effect on NE activity (Huether, 1996), creating a positive feedback loop, which seems to be counterbalanced by an inhibition of the NE system by 5-HT (Aston-Jones *et. al*., 1991; Engberg, 1992). The central NE system has been suggested to stimulated HPI axis activity in teleost fish (Overli Ø *et al*., 1999; Hoglund *et. al*., 2000). Consequently, by elevating brain 5-HT activity, increased dietary intake of TRP may suppress the stress-related activation of brain NE system and by that inhibits the stressed-induced activation of the HPI axis.

Dopamine (DA) effect may be opposite to 5-HT (Winberg and Nilsson, 1993) and L-dopa treatment, which elevates brain DA activity has been reported to induce social dominance (Winberg *et al*., 1992) to counteract the stress-induced elevation of plasma cortisol & brain 5-HT activity in Arctic char, *Salvelinus alpinus* (Hoglund, 2001). These results suggest that brain catecholamine systems are interacting with the 5-HT on the HPI axis. Moreover, catecholamines are synthesized from L-tyrosine, another essential large neutral amino acid (LNAA), competes with TRP for uptake to the brain (Fernstrom, 1983). Thus, elevated dietary intake of TRP may also affect brain NE and DA activity.

2.6.1.4 L-tryptophan in the diet and growth in fishes

The quantitative requirement of L-tryptophan appears to vary from species to species. Several studies have been conducted by numbers of workers on dietary requirement of L-TRP in many species for their growth and other multiple functions. However, very limited studies on role of L-TRP on growth performance carps have been conducted over the past. In fact, no attempt has been made to evaluate the possible role of L-TRP on growth of the *L. gonius* fingerlings.

Halver (1965) reported 2 g/kg diet as the requirement for Chinook salmon (*Oncorhynchus schwytscha*). Walton *et al.* (1984) found that the dietary requirement of tryptophan is 2.5 g/kg diet for rainbow trout (*Salmo gairdneri*). Based on growth, Wilson *et al.* (1978) suggested tryptophan requirement for fingerling channel catfish (*Ictalurus* punc*tatus)* is about 0.12% of the diet (dry weight basis) or 0.5% of the dietary protein. Nose (1979) reported the dietary requirement of L-tryptophan for Japanese eel (*Anguilla japonica*) is 4 g/kg diet. On the basis of the growth response, the TRP requirement of milkfish juveniles was estimated to be 3.1 g/kg diet (Coloso *et al*., 1992). Fish fed with low levels of TRP exhibited low weight gains and poor feed conversion ratios (Coloso *et al*., 1992).

Diets of *C. mrigala* fingerling should contain tryptophan at 0.38 g/100g dry diet, corresponding to 0.95 g/100 g dietary protein for optimum growth and efficient feed utilization (Ahmed and Khan, 2005). Tryptophan requirement values reported for *L. rohita* fingerling is 0.59% (Khan and Jafri, 1993) and 1.13% of protein (Murthy and Varghese, 1997) which was based on growth data only. Based on the second-degree polynomial regression analysis of the WG%, FCR and PER data, the optimum requirement of fingerling *L. rohita* for L-tryptophan is recommended in the range of 0.36-0.38% of the diet, corresponding to 0.90-0.95%, respectively, of the dietary protein for growth and efficient feed utilization (Abidi and Khan, 2010). Tejpal *et al*. (2009) also reported the improved growth performance in *C. mrigala* fingerlings fed with dietary L-TRP.

Akhtar *et al*. (2013) reported that dietary supplementation of 1.4% TRP augments growth in *L. rohita* juveniles. They also observed higher RNA and RNA/DNA ratio in L-TRP supplemented groups. Naatjes *et al*. (2014) reported increase in body weight gain of pig with supplementation of dietary L-TRP along with lysine. Kumar *et al.* (2014) also mentioned that dietary supplementation of either 0.7 or 1.4% L-TRP in diet improved growth in *L. rohita* fingerlings.

2.6.1.5 L-tryptophan and stress mitigation in fishes

Amino acid tryptophan is essential for fish and precursor of serotonin (5-hydroxytryptamine, 5-HT) with stress-releasing effect (Winberg *et al.*, 2001; Lepage *et al.*, 2002; Hseu *et al.*, 2003; Hoglund *et al.* 2005). Fish fed with L-tryptophan supplemented feed showed elevated plasma and brain levels of L-tryptophan (Winberg *et al.*, 2001; Lepage *et al.*, 2002).

Feeding the fish with L-tryptophan-supplemented feed for 3 days had no effect on aggressive behaviour, whereas feeding the fish L-TRP supplemented feed for 7 days significantly suppressed aggressive behaviour in the fish, an effect seen at both levels of L-tryptophan supplementation (Winberg *et al.*, 2001). Feeding rainbow trout with TRP supplement diet for 7 days resulted in slight elevation of basal plasma cortisol in non-stressed fish. In response to the stress, fish that had been fed control feed showed elevated plasma cortisol levels, but fish fed with the TRP-supplemented feed displayed a significant reduction in this stress-induced elevation of plasma cortisol levels (Lepage *et al.*, 2002).

Feeding the rainbow trout with 3-different levels of (2, 4, 8 times the TRP of commercial trout feed) TRP supplemented feed for 7-days resulted in dose dependent decrease in post-stress plasma cortisol levels along with a dose-dependent elevation of basal plasma cortisol (Lepage *et al.*, 2003). Feeding the fish with same diet for 3 days had no effect. However, on post-stress plasma cortisol even though basal plasma cortisol was slightly elevated as compared to fish fed control feed (Lepage *et al.*, 2003). Following 28 days of elevated dietary intake of TRP there was no effect on either post-stress or basal plasma cortisol (Lepage *et al.*, 2003), this may be due to that long term dietary TRP that may activate compensatory mechanisms, normalizing brain TRP and cortisol release.

TRP supplementation resulted in slightly lower cannibalism than in controls. However, body weight and total length of TRP groups were significantly smaller than those of the control. The results indicated that cannibalism among juvenile groupers could be mitigated by the oral administration of TRP, in addition or in place of environmental factors and the recommendation on supplementary TRP level to be used is 0.5% of dry diet (Hseu *et al*. 2003).

Aggression is an important part of the behavioural repertoire of juvenile cod and feed supplemented with TRP suppresses this behaviour (Hoglund *et al*. 2005), an effect that seems to be mediated by the central serotonergic system. Furthermore, TRP-induced decrease in aggression is likely to be most pronounced in dominant individuals, who consume the larger part of the feed offered. Thus, decreased size heterogeneity and cannibalism could be expected in association with TRP supplementation. This effect could be even more pronounced when combined with other methods for decreasing cannibalism, such as feeding strategy and optimal stocking densities (Hecht and Pinienaar, 1993).

Dietary supplementations of L-tryptophan have shown mitigation in crowding stress response in *Cirrhinus mrigala* fingerlings (Tejpal *et al*., 2009). The LDH, MDH, AST and ALT activities were found to be significantly higher in the control groups and decreasing trend of the enzymes were observed with the increasing level of dietary L-tryptophan (Tejpal *et al*., 2009). L-tryptophan supplemented groups were found to have higher weight gain %, SGR and PER. Gradual supplementation of L-tryptophan in diet significantly reduced the cortisol and blood glucose level in stress group (Tejpal *et al*., 2009).

Control and TRP supplemented groups showed increasing and decreasing pattern in cortisol and glucose values when experiment progressed after osmotic challenge (Hoseini and Hoseini, 2010). Both the groups showed increasing pattern in sodium and chloride values when experiment progressed after osmotic challenge. Results indicated that TRP supplementation enhanced salt water tolerance of carp, due to increase in basal cortisol and anti-stress effect of TRP and possibly increase in serotonergic activity (Hoseini and Hoseini, 2010).

The aggressive behaviour of juvenile mud crab can be suppressed by supplementation of L-TRP. The survival of juvenile mud crab can be improved with TRP to 0.5-1% (Laranja *et al*., 2010) in diet. TRP supplementation resulted to a significant increase of 5-HT concentration in the haemolymph which was clearly observed after the fight suggesting that 5-HT plays an important role in suppressing the agonistic behaviour of mud crab during aggressive encounters (Laranja *et al*., 2010). The juvenile of *Brycon amazonicus* have aggressive and territorial behaviour and that a diet containing TRP of 9.4 g/kg feed alter their aggressiveness, without affecting the stress-related physiological parameters (Wolkers *et al.*, 2011).

Hoseini *et al.*, (2012) reported that dietary supplementation of L-TRP can reduced the negative effects of stress, minimized toxicity and increased immune response in fish. Tryptophan is capable to reduce stress response in common carp by reducing cortisol levels and the effect was more pronounced in 9.9g tryptophan/kg diet (Hoseni and Hoseini, 2013). Akhtar *et al.* (2013) reported that dietary supplementation of TRP augments growth, lowers energy demand and helps in mitigating individual or combined effect of temperature and salinity stress in *L. rohita* juveniles. Martins *et al.* (2013) observed significant reduction in cortisol level in unstressed fish fed with TRP-supplemented diet.

Ciji *et al.* (2013a) reported that dietary supplementation of L-TRP modulates growth, immuno-metabolic status and combat nitrite stress in *Labeo rohita* juveniles. Ciji *et al.* (2013b) also reported enhanced non-specific resistance factors *viz.*, NBT, lysozyme activity and WBC count in vitamin E and L-TRP diet fed groups compared with control upon challenge with *A. hydrophila*, which revealed the role of these two additives in boosting non-specific immunity in *L. rohita* juveniles. Dietary supplementation of 1.5% L-TRP, overcome the negative impact of nitrite stress on steroidogenesis and thyroid hormone (Ciji *et al.*, 2013c).

Fatahi and Hoseini (2013) reported that dietary supplementation of 0.25% L-TRP reduced the Cu toxicity and mortality in Caspian roach. Tejpal *et al.* (2014) reported improved in thermal tolerance level and reduced the oxygen consumption rate in *C. mrigala* fingerlings fed with 1.36% L-TRP. Akhtar *et al.* (2014) reported to mitigate temperature and salinity stress induced stress through dietary supplementation L-TRP in *L. rohita* juveniles by reducing AST, ALT and LDH enzyme activity and increase in AchE enzyme activity. Kumar *et al.* (2014) reported that dietary supplementation of L-TRP at either 0.72 or 1.4% in the diet can reduce thermal stress even up to 38^0C in *L. rohita* fingerlings by reducing blood glucose and serum cortisol level, aminotransferase, lactate dehydrogenase, malate dehydrogenase, catalase and superoxide dismutase activities.

Material and Methods

3. Material and Methods

Four separate experiments were conducted to evaluate the stress responses of *Labeo gonius* fingerlings exposed to multiple stressors (temperature and pH) its mitigation through dietary L-tryptophan (L-TRP). All the four experiments were conducted for a period of 60 days each and they are interrelated. Different methodologies have been adapted during the present study, which are discussed below.

3.1 Site of the Experiment

The experimental setup was maintained in the wet laboratory of Aquaculture Division of the Central Institute of Fisheries Education (CIFE), Mumbai, India. Subsequent laboratory analysis was carried out in Aquaculture Division and Fish Nutrition, Biochemistry and Physiology Division of CIFE. Some of the parameters were estimated in laboratories of other divisions of the institute as well as outside of the institute.

3.2 Experimental Animal

Animals used for experimental purpose were fingerlings of *L. gonius*. The fish fingerlings (6.0-7.0 g) were procured from Jungalbalahu fish farm, Nagaon District, Assam (India). The fishes were transported in 10 L polythene packing with sufficient oxygen (10 fish in each pack) by air. On reaching the wet laboratory, they were carefully transferred to a circular tank (1000 L capacity) of flat bottom and were left undisturbed the whole night. In order to ameliorate the handling stress, the fish were given a mild salt treatment the next day followed by antibiotic treatment using oxytetracycline at the rate of 15 mg/L (APHA, 1998) for first three days in the same tank. Water exchange (40-50%) was carried out at every alternate day to maintain conducive water quality in the rearing tank. The stock was acclimatized to the laboratory condition with proper aeration facility before commencing the experiments. During the period, fishes were fed with a control diet containing 35% crude protein.

3.3 Experimental Designs

3.3.1 Experiment I: Studies on the growth and physio-biochemical responses of *L. gonius* fingerlings exposed to four different temperatures.

L. gonius fingerlings of uniform size were randomly distributed into 4 treatments in 3 triplicates. During the experimental period, fishes were exposed to four different temperatures *viz*., T_1 (27^0C), T_2 (30^0C), T3 (33^0C) and T_4 (36^0C) for a period of 60 days. The experimental tubs were arranged following a Completely Randomized Design (CRD). Thermal tolerance study was also conducted under same acclimatization temperature.

3.3.2 Experiment II: Studies on the growth, biochemical and haemato-immunological responses of *L. gonius* fingerlings exposed to multiple stressors.

L. gonius fingerlings of uniform size were randomly distributed into 6 treatments in 3 triplicates. Fingerlings were exposed to combined effect of temperature and pH *viz*., T_1 (27^0C x pH 7.5), T_2 (27^0C x pH 5.5), T3 (27^0C x pH 9.0), T_4 (33^0C x pH 7.5), T_5 (33^0C x pH 5.5) and T_6 (33^0C x pH 9.0) for a period of 60 days. The experimental tubs were arranged following a Completely Randomized Design. The pH range (5.5 and 9.0) was selected based on the findings of Acharya *et al*. (2005) and Das *et al*. (2006) on fingerlings of carp species. They have reported that the chronic exposure to acidic pH (5.5) and alkaline pH (9.0) imparted stress to the fingerlings. Chronic thermal stress (33^0C) was decided based on the findings of the 1st experiment. Das *et al*. (2005) also reported that the chronic exposure to 33^0C has imparted thermal stress to the *L. rohita* fingerlings.

3.3.3 Experiment III: Studies on the growth and immunomodulatory responses of *L. gonius* fingerlings to dietary L-tryptophan supplementation.

The experiments were set up in 5 distinct experimental groups, each group having 3 replicates. Five iso-nitrogenous purified diets were prepared with graded levels of L-tryptophan *viz*., Control T_1 (Basal diet + 0% Tryptophan), T_2 (Basal diet + 0.35% Tryptophan), T_3 (Basal diet + 0.70% Tryptophan), T_4 (Basal diet + 1.4% Tryptophan) and T_5 (Basal diet + 2.1% Tryptophan). The experimental tubs were arranged following a Completely Randomized Design (CRD).

3.3.4 Experiment IV: Studies on the possible role of dietary L-tryptophan on stress mitigation of *L. gonius* fingerlings exposed to multiple stressors.

L. gonius fingerlings of uniform size were randomly distributed into 9 treatments in 3 triplicates. Four iso-nitrogenous purified diets were prepared with graded levels of L-tryptophan (TRP). Nine treatment groups were T_1 (No stress x 0% L-Tryptophan), T_2 (Temperature 33^0C and pH 9.0 x 0% L-Tryptophan), T_3 (Temperature 33^0C and pH 9.0 x 0.7% L-Tryptophan), T_4 (Temperature 33^0C and pH 9.0 x 1.4% L-Tryptophan), T_5 (Temperature 33^0C and pH 9.0 x 2.1% L-Tryptophan), T_6 (Temperature 33^0C and pH 5.5 x 0% L-Tryptophan), T_7 (Temperature 33^0C and pH 5.5 x 0.7% L-Tryptophan), T_8 (Temperature 33^0C and pH 5.5 x 1.4% L-Tryptophan), T_9 (Temperature 33^0C and pH 5.5 x 2.1% L-Tryptophan). The experimental tubs were arranged following a Completely Randomized Design (CRD).

Temperature and pH stress (both acidic and alkaline) were selected based on the observations of the second experiment. Based on the findings of 3rd experiment L-TRP level of 0.35% level was ignored because there was less significance difference between the control and this treatment group on immune parameters.

3.4 Experimental Unit

Rectangular plastic tubs (100 L capacity) were used as experimental units throughout the experiment for all the trials. The tubs were covered with perforated lids. The tubs were initially washed and filled with potassium permanganate solution (4 mg L^{-1}) and were left overnight. They were cleaned on the very next day with chlorine free bore well water. The total volume of the water in each tub was maintained at 85 L throughout the experimental period. Round the clock aeration was provided. The aeration pipe in each tub was provided with an air stone and a regulator to control the air pressure uniformly in all the tubs.

3.5 Chemicals and Glass-wares

The glasswares used throughout the experiment were of neutral glass of Borosil makes. Chemicals of various companies *viz.*, Sigma, SRL, Hi-media, Qualichem, Merck, Stressgen, etc.

3.6 Maintenance of Temperature and pH

The thermostatic water heaters (range up to 50^0C from normal) with specification DTC - PID – 50L X 51B X 52H, General Trading Corporation, Mumbai, India were used to maintain the water temperature in the experimental tanks. Stock solution of sodium bicarbonate ($NaHCO_3$) and hydrochloric acid (HCl) were used to create the different alkaline and acidic conditions, respectively, in water of the experimental tanks.

3.7 Experimental Diets

No reports were available on the protein requirement of *L. gonius* fingerlings. However, related species of Indian major carps attain good growth fed with 35% crude protein as reported by Renukardhyay and Varghese (1986). Thereby, in the present study also, purified diets were prepared so as to keep the crude protein and lipid levels at 35% and 8%, respectively. L-tryptophan was added in different levels to the experimental diets. Fishes were fed with control diet without L-tryptophan (diet-1) in experiment 1 and 2, where as they were fed with diet containing different levels of L-tryptophan in 3rd (Diet 1, 2, 3, 4 and 5) and 4th (Diet 1, 3, 4 and 5) experiment.

3.7.1 Formulation and preparation of experimental diets

Purified ingredients such as casein (fat free, Hi-media Laboratories Ltd., India), gelatin (Himedia Laboratories Ltd., Mumbai, India), cellulose (Himedia Laboratories Ltd., Mumbai, India), betaine chloride (Himedia laboratories Ltd., Mumbai, India), vitamin and mineral mixture (Himedia laboratories Ltd. Mumbai), vitamin C (SD Fines Chemicals Ltd., India), Carboxymethylcellulose (Himedia laboratories Ltd., Mumbai, India.), sunflower oil and cod liver oil (procured locally) were taken for feed formulation. Five isonitrogenous (35%) diets with graded levels of L-tryptophan (0% in diet-1, 0.35% in diet-2, 0.7% in diet-3, 1.4% in diet-4 and 2.1%in diet-5) were prepared. L-tryptophan was procured from Himedia laboratories Ltd., Mumbai, India. Ingredients compositions (% dry matter basis) of the experimental diets are given in Table 1.

Table 1: Composition (% dry matter basis) of the experimental diets fed to *L. gonius* fingerlings in different experiments

Ingredient (% inclusion)	**Diet-1**	**Diet-2**	**Diet-3**	**Diet-4**	**Diet-5**
Casein(vitamin free)[a]	30.0	30.0	30.0	30.0	30.0
Gelatin[a]	10.0	10.0	10.0	10.0	10.0
Dextrin[a]	10.0	10.0	10.0	10.0	10.0
Starch soluble[a]	30.0	30.0	30.0	30.0	30.0
Cellulose powder[a]	7.92	7.57	7.22	6.52	5.82
Cod liver oil[b]	4.0	4.0	4.0	4.0	4.0
Sunflower oil[b]	4.0	4.0	4.0	4.0	4.0
Vitamin C[c]	0.10	0.10	0.10	0.10	0.10
Vit. Min mix[d]	1.95	1.95	1.95	1.95	1.95
Carboxymethyl cellulose(CMC)[a]	2.0	2.0	2.0	2.0	2.0
Betaine Hydrochloride[a]	0.02	0.02	0.02	0.02	0.02
Butylated Hydroxy Toluene(BHT)[a]	0.01	0.01	0.01	0.01	0.01
L-tryptophan (%)[a]	**0.0**	**0.35**	**0.7**	**1.40**	**2.10**
Total	100	100	100	100	100

[a] Himedia Ltd. India; [b] Procured from local market; [c] Sd Fine Chemicals Ltd., India; **[d]** Composition of Vit-min mixture (EMIX PLUS) (quantity/2.5 kg): vitamin A 5,500,000 IU; vitamin D_3 1,100,000 IU; vitamin B_2 2000 mg; vitamin E 750 mg; vitamin K 1000 mg; vitamin B6 1000 mg; vitamin B_{12} 6 mg; calcium pantothenate 2500 mg; nicotinamide 10 g; choline chloride 150 g; Mn 27,000 mg; I 1000 mg; Fe 7500 mg; Zn 5000 mg; Cu 2000 mg; Co 450 mg; Ca 500 g; P 300 g; L-lysine 10 g; DL-methionine 10 g; selenium 50 ppm; Satwari 250 ppm; (*Lactobacillus* 120 million units and Yeast Culture 3000 crore units).

All the ingredients except gelatin, vitamin mineral mixture, L-tryptophan and betaine chloride were mixed in a big plastic bowl. Gelatine crystals were mixed in luke warm water so as to form a jelly like substance. The ingredients were then mixed with gelatin jelly to form dough with the addition of necessary quantity of water. BHT was dissolved in oil and mixed thoroughly in the dough. The dough was then kept for 1 hr for proper conditioning followed by steaming for 5 minutes in a pressure cooker. The vitamin mineral mixture, vitamin C, L-tryptophan and betaine chloride were mixed after cooling. Pellets were prepared by using hand pelletizer having 2 mm diameter size. Finally the pellets were air dried for some time and kept in oven for 3-4 hrs at 60^0C till complete drying. Having dried, the pellets were packed in airtight polythene bags and labelled properly and kept in dry moist free place.

3.7.2 Proximate composition of experimental diets

Proximate compositions (% dry matter basis) of the experimental diets are given in Table 2. In the present study, nutritionally balanced formulated diet containing all the required nutrients in the optimum level was used.

Table 2: Proximate composition of the experimental diets (% Dry matter basis) fed to the *L. gonius* fingerlings

Diet	Moisture	CP	EE	Ash	DM	OM	TC*	**DE
Diet- 1	8.46	35.44	8.57	9.01	91.57	90.98	46.96	406.78
	±0.20	±0.19	±0.32	±0.13	±0.20	±0.13	±0.44	±2.03
Diet- 2	8.37	35.34	8.54	9.09	91.62	90.91	47.03	406.30
	±0.22	±0.16	±0.47	±0.17	±0.22	±0.17	±0.68	±2.28
Diet- 3	8.49	35.37	8.53	9.05	91.50	90.94	47.04	406.46
	±0.15	±0.20	±0.21	±0.23	±0.16	±0.23	±0.44	±1.26
Diet- 4	8.64	35.33	8.59	9.07	91.36	90.93	47.01	406.66
	±0.20	±0.18	±0.09	±0.18	±0.20	±0.18	±0.19	±1.16
Diet- 5	8.37	35.42	8.68	9.16	91.63	90.83	46.95	406.62
	±0.14	±0.20	±0.11	±0.17	±0.14	±0.17	±0.30	±0.29

Data expressed as Mean ± SE, n=6;

*Total carbohydrate % = 100 - (CP% + EE% + Ash %)

CP-crude protein; EE-ether extract;

DM-dry matter; OM-organic matter;

TC-total carbohydrate; DE- digestible energy;

**Digestible energy (Kcal/100g) = protein% x 4 + lipid% x 9 + carbohydrate% x 4

The crude protein content of the experimental diets varied from 35.33 to 35.44%, which is supported by Renukardhyay and Varghese (1986), who suggested that optimum protein requirement of Indian major carp ranging between 30-45%. The carbohydrate content of experimental diets was found to be within the range of 46.95 to 47.04%. Carbohydrate requirement of carps has been studied by Sen *et al.* (1978), who reported 26% carbohydrate level to be present in the diets of carp spawn, fry and fingerlings for optimum growth. In another study by Mohapatra *et al.* (2003), a diet containing 45% gelatinized carbohydrate and 30% crude protein was efficiently utilized by *L. rohita* fry. In the present study, lipid level was found to be 8.53 to 8.68%, which is supported by Mukhopadhyay (1993) who suggested that 7-9% lipid is required for carp grow out stage. In the diets of the present study, isocaloric (406.30 to 406.78 Kcal/100g feed) and iso-nitrogenous diets were given to the fingerlings.

3.8 Experimental Set-up and Rearing

L. gonius fingerlings of uniform size were randomly distributed in different experimental groups. Each group was having three replicates following a completely randomized design (CRD). Fingerlings of uniform size were stocked in each 85-L plastic tub. Each tub was covered with a perforated lid to prevent the animals from jumping out. The experimental conditions were kept same throughout the experiment. Temperature in each treatment was maintained using thermostatic controller fitted with sensors (General Trading Corporation, Mumbai, India). Gradually temperature got increased over ambient water temperature (1^0C per day) to reach the test temperatures and maintained for 60 days. Similarly, gradual decrease or increase of pH was done from the ambient pH of 7.5 to reach the test pH (5.5 and 9.0). Stock solutions of sodium bicarbonate ($NaHCO_3$) and hydrochloric acid (HCl) were used to create the alkaline and acidic conditions, respectively, in water of the experimental tanks.

Exchange of water from the experimental tank was done on daily basis to remove the excreta and uneaten feed, and to maintain the pH. Prior to water exchange, required stock solutions of acid and alkali were added to stored water kept in separate tanks to create the desired pH of 5.5, 7.5 and 9.0. These waters with different pH were used to exchange the water in the corresponding experimental tank with similar pH. Water exchange was done every day during morning hours (8.0-9.0 h) and was followed by final adjustment of pH with addition of stock solutions of acid or alkali to the tank. The exchange was made slowly to avoid any stress to animals. The tanks were continuously aerated throughout the experiments. Water temperature and pH were not adjusted in the tubs of experiment 3. The bore well water was used to exchange the water regularly. However, they were properly aerated before using for exchanging water. Fish were fed twice daily with the purified diet. The fish were deprived of feed for one day before performing the sampling, which was also followed during thermal tolerance study.

3.9 Feeding

Feeding was done @ 3% of their body weight initially and rate was adjusted accordingly. Daily ration was divided into two parts; about 2/3rd of total ration was given at 09:00 hours and the rest 1/3rd at 18:00 hours.

3.10 Physico-chemical Parameters of Water

Water quality parameters *viz.*, temperature, dissolved oxygen, pH, free carbon di-oxide, ammonia, nitrite and nitrate were recorded throughout the experimental period.

3.10.1. Dissolved oxygen and temperature

The dissolved oxygen and water temperature were measured using the digital oxy-meter 330 (sensitivity 0.01mg O_2/L, E-Merck, Germany), for all the treatment and were expressed as mg/L and °C respectively.

3.10.2. pH

Water pH (hydrogen ion concentration) was measured by using a digital pH meter (pH 325-A, MERCK, Germany) for all the experimental tubs.

3.10.3 Free carbon dioxide

The dissolved free Carbon dioxide was measured by titrimetric method (APHA, 1998) and calculated using the following formula.

$$\mathrm{CO_2\ (mg/L)} = \frac{\mathrm{A \times N \times 44 \times 1000}}{\text{Volume of sample (ml)}}$$

Where, A = Volume of titrant (NaOH) and
N = Normality of titrant (N/44)

3.10.4 Ammonia-N

Unionized ammonia nitrogen concentration was measured spectrophotometrically at 640 nm using UV-VIS spectrophotometer (E-Merck, Germany) following Phenate method (APHA, 1998) and compared with standard graph. The concentration was expressed as $\mathrm{mgL^{-1.}}$

3.10.5 Nitrite-N

Nitrite nitrogen of water was estimated using colorimetric method (APHA, 1998), the optical density was measured at 543 nm using UV-VIS spectrophotometer (E-Merck, Germany) and compared with standard graph. The concentration was expressed as $\mathrm{mgL^{-1}}$.

3.10.6 Nitrate-N

Nitrate concentration was estimated spectrophotometrically at 543 nm wave length using UV-VIS spectrophotometer (E-Merck, Germany) following the methodology of APHA (1998) and compared with standard graph. The concentration was expressed as $\mathrm{mgL^{-1}}$.

3.11 Growth Parameters

The growth parameters of the *L. gonius* fingerlings were assessed by taking their body weight at 15 days interval. The animals were kept starved overnight before the body weight measurement. Digital weighing balance was used to measure the weight of the fishes. The growth performance of *L. gonius* fingerlings was assessed using the following formula:

3.11.1 Percentage weight gain

$$\text{Percentage weight gain} = \frac{\text{Final weight} - \text{initial weight}}{\text{Initial weight}} \times 100$$

3.11.2 Specific growth rate (SGR)

$$\text{Specific growth rate} = \frac{\text{Ln (Final weight)} - \text{Ln (Initial weight)}}{\text{Experimental periods in days}} \times 100$$

3.11.3 Food conversion ratio (FCR)

$$\text{Food conversion ratio} = \frac{\text{Feed given (dry weight)}}{\text{Body weight gain (wet weight)}}$$

3.11.4 Feed efficiency ratio (FER)

$$\text{Feed efficiency ratio} = \frac{\text{Net weight gain (wet weight)}}{\text{Feed given (dry weight)}}$$

3.11.5 Protein efficiency ratio (PER)

$$\text{Protein efficiency ratio} = \frac{\text{Net weight gain (wet weight)}}{\text{Crude protein fed}}$$

3.11.6 Survival percentage

Survival percentage was calculated at the end of the experiment by counting the number of fishes in each tub and was calculated as follows:

$$\text{Survival (\%)} = \frac{\text{Total number of animal harvested}}{\text{Total number stocked}} \times 100$$

3.12 Biochemical Analysis of Diets

The proximate compositions of the diets and whole fish tissue were analyzed following the methodologies of AOAC (1995).

3.12.1 Moisture

A known weight of the sample was taken and dried in a hot air oven at 105^0C to constant weight and the moisture content was calculated by using the following formula:

$$\text{Moisture (\%)} = \frac{\text{Wet weight of sample} - \text{Dried weight of sample}}{\text{Wet weight of sample}} \times 100$$

3.12.2 Crude protein (CP)

Nitrogen content of experimental diets and carcass was estimated quantitatively by Kjeltec semi-automatic system (Tecador Sweden) and crude protein was estimated by multiplying nitrogen percentage by a constant factor (6.25).

$$\text{CP (\%)} = \text{Nitrogen (\%)} \times 6.25$$

3.12.3 Ether Extract (EE)

Ether Extract of experimental diets and carcass was estimated by Soxtec (Tecator, Sweden) using diethyl ether (boiling point, 40-60^{0}C) as a solvent.

$$\text{Ether Extract (\%)} = \frac{\text{Weight of ether extract}}{\text{Weight of the sample}} \text{x100}$$

3.12.4 Ash

Ash content of diets and carcass was estimated by taking the sample in vetrosil crucible and placing it in a muffle furnace at 600^{o}C for 6 hrs.

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

3.12.5 Total carbohydrates

Total carbohydrate of experimental diets and carcass was calculated by difference method given by the formula:

$$\text{Total carbohydrate \%} = 100 - (\text{CP\%} + \text{EE\%} + \text{Ash\%})$$

3.12.6 Digestible energy Value

The digestible energy value of experimental diets and carcass was calculated on the basis of standard physiological fuel value of 4 Kcal/g proteins, 4 Kcal/g carbohydrates and 9 Kcal/g lipids (Halver, 1957). It was calculated as per the following formula:

$$\text{Digestible energy (Kcal/100g)} = \text{Protein\% x 4} + \text{Lipid\% x9} + \text{Carbohydrate\% x 4}$$

3.13 Body Indices

3.13.1 Hepatosomatic index (HSI)

The hepatosomatic index was calculated by the following formula:

$$\text{HSI} = \frac{\text{Weight of the liver}}{\text{Weight of the fish}} \times 100$$

3.13.2 Gastrosomatic index (GSI)

The Gastrosomatic index was calculated by the following formula:

$$\text{GSI} = \frac{\text{Weight of the digestive tract}}{\text{Weight of the fish}} \times 100$$

3.14 Sampling Procedure

At the end of the experiment, fishes were collected from each tank and anaesthetized with clove oil (50 µL L^{-1}). Fishes were then dissected out immediately for different tissues, weighed and kept at -20 ^{0}C for enzyme assays and metabolites. For collection of blood and serum, fish were anaesthetized with clove oil (50 µL L^{-1}). The blood was withdrawn from the caudal vein using a medical syringe rinsed with ethylene diamine tetra-acetic acid (EDTA) disodium salt and immediately transferred to an eppendorf tube containing dried EDTA to prevent clotting. For collection of serum, blood was withdrawn without the use of anticoagulant and allowed to clot for some time till the straw colour serum separated out. This serum was then stored at -20^{0}C for further analysis.

3.15 Analysis of Tissue Enzymes

All the colorimetric assays were carried out using UV-VIS spectrophotometer (E-Merck, Germany).

3.15.1 Sample preparation

The muscle, gill, liver, brain and intestine of the fish were removed carefully and weighed. It was homogenized with chilled sucrose solution (0.25 M) in a glass tube using tissue homogenizer (MICCRA D-9, Digitronic, Germany). The tube was continuously kept in ice bath while homogenizing. The homogenate was centrifuged at 5000 rpm for 20 minutes at 4^0C in a cooling centrifuge. The supernatant was kept frozen at -20^0C till further analysis. A 5% homogenate was prepared for all the tissues.

3.15.2 Enzymes of Protein Metabolism

3.15.2.1 Aspartate amino transferase (AST)

(L-aspartate: 2 oxaloglutarate aminotransferase, E.C. 2.6.1.1)

The AST activity was assayed in the tissue homogenate as described by Wooten (1964). The substrate comprised of 0.2M D, L-aspartic acid and 2 mM α- ketoglutarate in 0.05 M phosphate buffer (pH 7.4). To 0.5 ml of substrate, 0.1ml of tissue homogenate was added and incubated at 37°C for 1 hr. The reaction was terminated by the addition of 0.5 ml of 1mM 2,4-dinitrophenylhydrazine (DNPH). In the control tubes, the enzyme source was added after DNPH solution. The tubes were held at room temperature for 20 min. Then 5 ml of 0.4 N NaOH solution was added and the contents were thoroughly mixed. After 10 minutes the OD was recorded at 540 nm against blank. Enzyme activity was expressed as n mol of oxaloacetate released/ min/ mg protein at 37°C.

3.15.2.2 Alanine amino transferase (ALT)

(L –alanine: 2-oxaloglutarate aminotransferase, E.C.2.6.1.2)

The ALT activity was assayed in the tissue homogenate as described by Wooten (1964). The substrate was comprised of 0.2M D, L-alanine and 2 mM α-ketoglutarate in 0.05 M phosphate buffer (pH 7.4). To 0.5 ml of substrate 0.1ml of tissue homogenate was added and incubated at 37°C for 1 hr. The reaction was terminated by the addition of 0.5 ml of 1 mM 2,4-dinitrophenylhydrazine (DNPH). In the control tubes, the enzyme source was added after DNPH solution. The tubes were held at room temperature for 20 min. Then 5 ml of 0.4 N NaOH solution was added and the contents were thoroughly mixed. After 10 minutes the OD was recorded at 540 nm against blank. Enzyme activity was expressed as n mol of sodium pyruvate released/ min/ mg protein at 37°C.

3.15.3 Enzymes of Carbohydrate Metabolism

3.15.3.1 Lactate dehydrogenase (LDH)

LDH (E.C. 1.1.1.27) activity was determined following the methodology of Wroblewski and Ladue (1955). The reaction mixture was comprised of 0.1 M phosphate buffer (pH 7.5), NADH solution, tissue homogenate and sodium pyruvate (0.02 M). The reaction was started by adding substrate sodium pyruvate and OD was recorded at 340 nm at 30 sec interval for 3 min. The enzyme activity was expressed as units per mg protein per min at room temperature where 1 unit was equal to ∆0.01 OD/ min.

3.15.3.2 Malate dehydrogenase (MDH)

The MDH (E.C. 1.1.1.37) activity was estimated following the methodology of Ochoa (1955). The reaction mixture was comprised of 0.1 M phosphate buffer (pH 7.5), NADH solution, tissue homogenate and freshly prepared oxaloacetate solution. The reaction was started by adding oxaloacetate solution as substrate and OD was recorded at 340 nm at 30 sec interval for 3 min. The enzymatic activity was expressed as units per mg protein per min at room temperature where 1 unit was equal to ∆0.01 OD/ min.

3.15.4 Enzymes of Oxidative Stress

3.15.4.1 Superoxide Dismutase (SOD)

The SOD (E.C. 1.15.1.1) activity was estimated following the methodology of Misra and Fridovich (1972). The assay is based on the oxidation of epinephrine-adrenochrome transition by the enzyme. The reaction mixture consisted of 50 µl of sample; 1.5 ml phosphate buffer and 0.5 ml epinephrine. The solution was mixed well and immediately read the change in optical density at 480 nm for 2 min in a UV-VIS spectrophotometer (E-Merck, Germany). One unit of SOD activity was the amount of protein required to give 50% inhibition of epinephrine auto oxidation.

3.15.4.2 Catalase

Catalase (E.C. 1.11.1.7) activity was estimated following the methodology of Takahara *et al*. (1960). To 2.45 ml of phosphate buffer (50mM, pH

7.0) , 50 µl of the tissue homogenate was added and the reaction was started by the addition of 1.0 ml of H_2O_2 solution, the decrease in absorbance was measured at 240 nm at 30 sec intervals for 2 min. The enzyme blank was run simultaneously with 1.0 ml of distilled water instead of hydrogen peroxide. The enzyme activity was expressed as n moles of H_2O_2 decomposed per min per mg protein.

3.15.4.3 Glutathione-s-transferace (GST)

Glutathione-s-transferase (GST) catalyses the conjugation of glutathione to numerous potentially toxic compounds, include aliphatic, aromatic, heterocyclic radicals, epoxides and arene oxides. GST activity was determined by following the methodology of Habig *et al.* (1974).

3.15.5 Enzyme of Neurotransmission

3.15.5.1 Acetylcholine esterase (AChE)

The enzyme AchE (E.C.3.1.1.7) activity was assayed by following the methodology of Hestrin (1949) modified by Augstinasso (1957). Acetylcholine esterase assay system was comprised of 1.0 ml of M/15 phosphate buffer (pH 7.2), 1 ml acetylcholine (0.004 M, pH 4.0) substrate buffer mixture (1:9 dilution) and 0.2 ml of homogenate and incubated for 30 min at 37^0C. Alkaline hydroxylamine (2.0 ml) was added to terminate the reaction. The solution was mixed thoroughly and 1 ml of HCl (2:1) was added followed by thorough mixing. Enzyme solution was then added to the control tubes. The colour was developed by addition of 1 ml of $FeCl_3$ (10%) and OD was recorded at 540 nm after thorough mixing. In this assay, mixing the solution at every step is very essential to avoid trapping of air bubbles.

3.15.6 Enzyme of Phospho Monoesterase

3.15.6.1 Alkaline Phosphatase (ALP)

The ALP (E.C. 3.1.3.1) activity was determined by following the method of Garen and Levinthal (1960). The assay mixture was comprised of 0.2 ml bicarbonate buffer (0.2 M), 0.1 ml of 0.1 M $MgCl_2$, 0.1 ml tissue homogenate, 0.5 ml of distilled water and 0.1 ml of freshly prepared 0.1M para-nitrophenyl phosphate. The reaction mixture was incubated in water bath at 37^0C for 15 minutes and the reaction was stopped by 1.0 ml of 0.1 N NaOH. Optical density was measured at 410 nm.

3.15.7 Enzyme of Gluconeogenic Pathways

3.15.7.1 Fructose-1, 6-diphosphatase (FDPase)

(D FDP-1-Phosphohydrolase; E.C. 3.1.3.11)

FDPase activity from different tissues was assayed by following the methodology outlined by Freeland and Harper (1959). The reaction mixture was comprised of 0.1 ml of 0.05 M FDP (pH 7-7.3), 0.1 ml of 0.5 M $MgSO_4$, 0.2 ml of tissue homogenate and 0.6ml of borate buffer (pH 9.5). The mixture was incubated at 37^0C for 30 minutes and the reaction was terminated by addition of 1 ml of 10% TCA solution. 1ml of the aliquot of the supernatant was used for P estimation by following the methodology of Fiske and Subbarow (1925). Activities of FDPase were expressed as microgram phosphorus released / mg protein / minute at 37°C.

3.15.8 Enzyme of Pentose Phosphate Pathway

3.14.8.1 Glucose-6-phosphate dehydrogenase

(α-D-glucose-6-phosphate: NADP” oxidoreductase; E.C.1.1.1.49).

The activity of glucose-6-phosphate dehydrogenase in the liver tissue was assayed by following the methodology of De Moss (1955). The reaction mixture consisted of 1.5 ml of 0.1 M Tris buffer (pH 7.8), 0.2 ml of 2.7 mM NADP, 0.1ml of tissue homogenate, 1.05 ml of distilled water and 50 µl of 0.2 M G-6-P. The reaction was started by adding G-6-P. The OD was recorded at 340 nm at 15 second interval for 3 minutes. The enzyme activity was expressed as units/mg protein/min. One unit was equal to Δ0.01 OD/min/ml at 25^oC.

3.15.9 Enzyme of Energy Metabolism

3.14.9.1 Adenosine triphosphatase (Total)

(Adenosine triphosphate phosphohydrolase, E.C. 3.6.1.3)

The ATpase enzyme was assayed by following the modified method of Post and Sen (1967). The reaction mixture was comprised of 1.0 ml of 0.1 M Tris-HCl buffer (pH 7.8), 0.1 ml of 100 mM NaCl, 0.1 ml of 20 mM KCl, 0.1 ml of 3 mM of $MgCl_2$, 0.5 ml of 5 mM ATP and 0.1 ml homogenate. The mixture was incubated for 15 min and the reaction was terminated by the addition of 1 ml 10% TCA. After centrifugation, 2.0 ml of supernatant was processed for estimation of inorganic P by following the methodology of Fiske and Subbarow (1925). The ATPase activity was expressed as microgram phosphorus released/ mg protein/ minute at 37°C

3.15.10 Digestive Enzymes

3.15.10.1 Amylase

The reducing sugars produced due to the action of gluco-amylase and alpha amylase on carbohydrates were estimated using Dinitro salicylic acid (DNS) method (Rick and Stegbauer, 1974). The reaction mixture consisted of 1% (w/v) starch solution, phosphate buffer and the tissue homogenate. The reaction mixtures were incubated at 37°C for 30 min. DNS was added after incubation and kept in boiling water bath for 5 min. After cooling, the reaction mixture was diluted with distilled water and absorbance was measured at 540 nm. Maltose was used as the standard. Amylase activity was expressed as mmole of maltose released from starch per min at that temperature.

3.15.10.2 Protease

Protease activity was determined by the casein digestion method as Drapeau (1974). The enzyme reaction mixture consisted of 1% (w/v) casein in 0.05M Tris phosphate buffer (pH 7.8) and incubated for 5 min at 37°C. Then tissue homogenate was added. Ten minutes later, reaction was stopped by adding 10% trichloroacetic acid (TCA) and the whole content was filtered. The reagent blank was made by adding tissue homogenate just before stopping the reaction with TCA and with no incubation. One unit of enzyme activity was defined as the amount of enzyme needed to release acid soluble fragments equivalent to $\Delta 0.001 A_{280}$ per minute at 37°C and pH 7.8.

3.15.10.3 Lipase

The lipase activity was assayed by the method of Cherry and Crandell (1932). The lipase activity was estimated by keeping test and control for each sample. 1 ml of homogenate and 3 ml of distilled water was added to each tube.

The control tubes were kept in boiling water bath for 5 min at 100ºC, this serves to inactivate the lipase enzyme in the control tubes. Then 0.5 ml of buffer and 2 ml of olive oil emulsion were added to the tubes, shaken well and kept for incubation at 37ºC in water bath for 24 hrs. When incubation period got over, 3 ml of 95% alcohol and one drop phenolphthalein indicator were added to each tube and shaken well. This was titrated against 0.05 N NaOH till the appearance of pale pink colour. The titre volume was noted and calculated to obtain the activity of lipase in the sample. The milli equivalent of alkali consumed was taken as a measure of the activity of the enzyme.

3.16 Determination of Metabolite

3.16.1 Glycogen

Muscle and liver glycogen content was estimated colorimetrically following the method described by Hassid and Abraham (1957). The tissue was digested with 30% KOH and 95% ethanol was added. Glycogen was precipitated upon centrifugation at 5000 x g for 5 min, which was dissolved in distilled water. Anthrone reagent was added to known quantity of aliquot and mixed by swirling the tube. The tubes were covered with glass marble and heated for 10 min in boiling water followed by cooling. The absorbance was recorded at 590 nm. The reading was compared with standard glycogen and expressed as mg/g wet tissue.

3.17 Ascorbic Acid

Ascorbic acid (vitamin C) in tissue was estimated following the method of Roe and Keuther (1943). Wet tissue was placed in a pre-weighed test tube containing 10 ml of chilled 6% Trichloro Acetic acid (TCA). The difference in the weight indicates weight of the tissue. The sample was then homogenized and activated charcoal (200-300 mg) was added to the tubes and mixed thoroughly. The solution was filtered using Whatman filter paper (No. 42). 5 ml of aliquot was taken in a test tube to which, one drop of 10% thiourea solution and 1 ml of 2,4 dinitrophenyl hydrazine were added. The test tubes were placed in a water bath at 37°C for 3 hours. The tubes were then transferred to an ice bath and 5 ml of 85% H_2SO_4 was added drop by drop. The test tubes were allowed to stand for 30 minutes in ice bath. At the end of 30 minutes, absorbance was measured at OD 540nm against blank along with standard ascorbic acid.

3.18. Total Protein

Quantification of protein in the liver, muscle, gill, brain and intestinal tissue was carried out by the method described by Lowry *et al*. (1951). To 0.1ml of tissue homogenate, 1 ml of 10% TCA was added to precipitate it. The protein residue was obtained by discarding the supernatant produced after centrifugation at 5000 rpm for 20 min. This residue was dissolved in 0.5ml of 0.1 N NaOH and 0.1ml of the dissolved protein residue was used for further analysis. Alkaline $CuSO_4$ (5 ml) was added to it and left for 10 minutes. To this 1 N folin's reagent was added and incubated for 30 min in dark. Reading was taken at 660nm against blank. Bovine serum albumin was used as standard.

3.19 DNA and RNA

Quantitative determination of nucleic acid in tissue was done by Pentose analysis (Schneider, 1945) and was calculated as follows:

The DNA content of the nucleic acid extract is given by the following equation:

$$\mu\text{g DNA/ml} = \frac{\text{OD at 600 nm}}{0.019}$$

The RNA content of the nucleic acid extract is given by the following equation:

$$\mu\text{g RNA/ml} = \frac{(\text{OD at 600 nm} + 0.008) - (\mu\text{g DNA/ml} \times 0.013)}{0.116}$$

3.20 Blood Parameters

3.20.1 Collection of blood

The body surface of the fingerlings was cleaned with blotting paper. Blood was collected from caudal vein with a glass syringe using anticoagulant (1%) ethylene diamine tetraacetic acid (EDTA) for estimation of blood parameters like blood glucose, haemoglobin percentage, total erythrocyte count, total leucocyte count, haematocrit and respiratory burst activity.

3.20.2. Total erythrocyte count (RBC)

Twenty microlitre of blood was mixed with 3980 µl of RBC diluting fluid in a clean glass vial. The mixture was shaken well to suspend the cells uniformly in the solution. Then the cells were counted using a haemocytometer. The following formula was used to calculate the number of RBC per mm^3 of the blood sample:

$$\text{Number of RBC/mm}^3 = \frac{\text{N x dilution}}{\text{Area counted x Depth of fluid}}$$

$$= \frac{\text{N x 200}}{\text{0.2 x 0.1}} \text{ or N x 10000}$$

Where N is the total number of red blood cells counted in 5 squares of the haemocytometer.

3.20.3. Total leucocytes count (WBC)

Twenty microlitre of blood was mixed with 3980 µl of WBC diluting fluid in a clean glass vial. The mixture was shaken well to suspend the cells uniformly in the solution. Then the cells were counted using a haemocytometer. The number of WBC per mm^3 of the blood sample was calculated using the following formula:

$$\text{Number of WBC/mm}^3 = \frac{\text{N x dilution}}{\text{Area counted x Depth of fluid}}$$

$$= \frac{\text{N x 200}}{\text{4 x 0.1}}$$

$$= \text{N x 500}$$

Where, N denotes the total number of white blood cells counted in 4 squares of the haemocytometer.

3.20.4 Haemoglobin content

The haemoglobin level of blood was analyzed following the Cyanmethemoglobin method using Darbkins Fluid (Qualigens). Twenty microlitre of blood was mixed with 5 ml of Darbkin’s working solution. The absorbance was measured using a spectrophotometer at wavelength of 540 nm. The final concentration was calculated by comparing with the standard cyanmethemoglobin (Qualigens Diagnostics). The haemoglobin concentration was then calculated by using the following formula:

$$\text{Haemoglobin content (g \%)} = \frac{\text{OD (T)}}{\text{OD (S)}} \times \frac{251}{1000} \times 60$$

Where, OD (T) = Absorbance of test; OD (S) = Absorbance of standard

3.20.5 Haematocrit (Hct)

Heamatocrit (Hct-%) was determined following the Wintrobe and Westergreen method as described by Blaxhall and Daisley (1973).

3.20.6 Nitroblue tetrazolium (NBT)

Nitroblue tetrazolium assay was done by the method of Secombes (1992) as modified by Stasiack and Baumann (1996). Fifty microlitre of blood was placed into the wells of 'U' bottom microtitre plates and incubated at 37^0C for 1 hr to facilitate adhesion of cells. Then the supernatant was removed and the loaded wells were washed three times in PBS. Having washed, 50 microlitre of 0.2% NBT was added and plate was incubated for further 1 hr. The cells were then fixed with 100% methanol for 2-3 minutes and again washed thrice with 30% methanol. The plates were then air dried. Sixty microlitres 2N potassium hydroxide and 70 microlitres dimethyl sulphoxide were added into each well to dissolve the formazon blue precipitate formed. The OD of the turquoise blue colored solution was then read in ELISA reader at 540 nm.

3.20.7 Blood glucose

Blood glucose was estimated by the method of Nelson (1944) and Nelson and Somogyi (1975). Blood was deproteinized with zinc sulphate and barium hydroxide, filtered and the supernatant was used for the estimation of glucose. The supernatant was taken in a test tube, alkaline copper sulphate was added and placed in a boiling water bath for 20 min. The test tubes were then cooled to room temperature, arsenomolybdate reagent was added and absorbance was recorded at 540 nm against a blank.

3.21 Blood Serum Parameters

3.21.1 Collection of Serum

Each fish was anesthetized with clove oil (at the rate 50 µl per liter of water) before taking blood from fish. Blood was withdrawn from *vena caudalis* using a medical syringe without using 2.7% EDTA solution. Collected blood was then transferred immediately to a dried eppendorf tube. The tubes were allowed to stand in tilting position at the room temperature for an hour which allowed the blood to clot. After blood clotting, the yellow straw color serum was carefully collected and transferred to another eppendorf tube and stored at -20^0C.

3.21.2 Total serum protein

Serum protein was estimated following biuret method (Reinhold, 1953) using the total protein and albumin kit (MERCK, Mumbai, India). Proteins present in the plasma bind with copper ions in an alkaline medium of the biuret reagent and produce a purple coloured complex, whose absorbance is proportional to the protein concentration. Three test tubes labeled as Blank (B), Standard (S) and Test (T) were taken. Into all the tubes, 1ml of biuret reagent and 2 ml of distilled water were added. A volume of 0.05 ml, protein standard was taken in the test tube labeled as standard and 0.05 ml of plasma was added in to the test tube labeled as test. It was then mixed well and incubated at 37^0C for 10 minutes. The absorbance of Standard (S) and Test (T) were measured against blank (B) in a spectrophotometer at 630 nm. Total serum protein calculation was done as follows:

$$\text{Total proteins in gm \%} = \frac{\text{Absorbance of Test (T) x 6}}{\text{Absorbance of Standard (S)}}$$

3.21.3. Albumin

Albumin was estimated following Bromocresol green binding method (Doumas *et al.*, 1971). Albumin in a buffered medium binds with Bromocresol green (BCG) and produces a green colour whose absorbance is proportional to the albumin concentration. Three test tubes labeled as Blank (B), Standard (S) and Test (T) were taken. Into all the tubes, 1ml of buffered dye reagent and 2 ml of distilled water were added. 0.01ml of albumin standard was taken in the test tube labeled as standard and 0.01 ml of plasma was added in to the test tube labeled as test. It was then mixed well and incubated at 37^0C for 10 minutes. The absorbance of Standard (S) and Test (T) were measured immediately against blank (B) in a spectrophotometer at 630nm. The calculation was done as follows:

$$\text{Albumin in gm \%} = \frac{\text{Absorbance of Test (T) X 4}}{\text{Absorbance of Standard (S)}}$$

3.21.4. Globulin

Globulin was calculated by subtracting albumin values from total plasma protein.

$$\text{Globulins (gm \%)} = \text{Total protein (gm \%)} - \text{Albumin (gm \%)}$$

3.21.5. Albumin- Globulin ratio

Albumin- globulin ratio **(**A/G ratio) was calculated by dividing albumin values by globulin values.

$$\text{A/G ratio} = \frac{\text{Albumin (gm \%)}}{\text{Globulins (gm \%)}}$$

3.21.6 Serum lysozyme activity

For assay of serum lysozyme activity, serum samples were diluted with phosphate buffer (pH 7.4) to a concentration of 0.33 mg per ml. In a suitable cuvette, 3 ml of *Micrococcus luteus* suspension in phosphate buffer (A_{450} = 0.5 - 0.7) was taken, to which 50 microlitre of diluted serum sample was added. The content of the cuvette was mixed well for 15 seconds and reading was taken in a spectrophotometer at 450 nm exactly after 60 seconds of addition of serum sample. This absorbance was compared with standard lysozyme of known activity following the same procedure as above. The activity was expressed as Unit/min/mg protein at 37^0C.

3.21.7 Biochemical parameters of serum

Serum biochemical parameters like GOT (glutamic oxaloacetate transaminase), GPT (glutamic pyruvic transaminase), ALP (alkaline phosphatase), ACP (acid phosphatase), LDH (Lactate Dehydrogenase) were quantified using respective calorimetric assay kits procured from Merck, Germany and analysis was done in auto blood analyser, Spectra Junior (Merck, Germany) following the standard enzymatic procedures.

3.21.8 Serum cortisol

The DSL-10-2000 ACTIVE$^{@}$ Enzyme Immunoassay (EIA) kit (Diagnostic Systems Laboratories, Inc., USA) was used for the quantitative measurement of cortisol in serum. The assay was performed according to the protocol provided along with the kit. The absorbance was read in the ELISA plate reader (Biotek Pvt. Ltd., India). Serum cortisol was expressed as ng/mL.

3.21.9 Heat shock protein (Hsp-70)

The assay Designs ^{TM} Anti-Human Hsp70 (total) ELISA (enzyme-linked immunosorbent assay) kit (Assay Design, Inc., USA) was used for the quantitative measurement of Hsp-70 in serum. The assay was performed according to the protocol provided along with the kit and expressed as ng/ml. Hsp-70 was also analyzed in tissue samples (muscle, gill and liver) with the same test kit. However, the tissue samples were processed before the measurement of Hsp-70.

Tissue homogenate was prepared in chilled phosphate buffer (50 mM, pH 7.2) containing 0.1 mM phenyl methane sulfonyl fluoride (PMSF) as protease inhibitor. The homogenates were centrifuged at 6000 rpm for 20 minutes, at -4°C. The supernatants were collected in glass vials and stored at -20°C until further analysis.

3.21.10 Caspase-3 activity

The activity of Caspase-3 enzyme in the serum samples was determined calorimetrically using the Caspase-3 calorimetric detection kit (Catalog no. 907-013 procured from assay designs, Stressgen). The assay was performed according to the protocol provided along with the kit. The amount of chromophore, p-nitroaniline (pNA), released by caspase-3 activity was quantitated by measuring the optical density at 405 nm. The absorbance was read in the plate reader (Biotek Pvt. Ltd., India). Caspase-3 activity was expressed as µM p-NA released/ hr/ mg cellular protein.

3.21.11 Thyroid hormones

Thyroid hormones (T3 and T4) were analyzed with an EIA Total T3 Kit and EIA Total T4 Kit (Adaltis Italia S.P.A., Italy). The assay was performed according to the protocol provided along with the kit and the concentration was expressed as ng/mL.

3.21.12 Electrolytes

Electrolytes (Na^+, K^+ and Cl^-) in serum samples were analyzed using an Electrolyte analyzer, Roche (version 9180.0) and expressed as mEq/L.

3.21.13 Osmolality

Serum osmolality was measured using a freezing point depression Osmomat-030 osmometer (Gonotec, Berlin, Germany) and expressed as Osmol/L.

3.22 Thermal Tolerance Study

3.22.1 Thermal tolerance limit and polygon

Thermal tolerance limit of *L. gonius* fingerlings was assessed after acclimated to four different temperatures (27, 30, 33 and 36°C) following the critical thermal methodology (CTM) as described previously by Beitinger and Bennetta (2000) and Das *et al*. (2004, 2005). Twelve fish (six for CT_{min} & LT_{min} and six for CT_{max} & LT_{max}, separately) were randomly selected from each acclimated temperature and were shifted to separate thermostatic aquaria (Suan Scientific Instruments & Equipments, Kolkata, India, 52L water capacity, sensitivity ± 0.2^0C) for thermal tolerance study. The temperatures of the water in the thermostatic aquaria were maintained similar to the experimental groups. Dissolved oxygen concentration was maintained at 5.8 ± 0.5 mg/L throughout the thermal tolerance study by continuous aeration using a 2-HP air blower.

Water temperature in the aquarium was increased/ decreased at a constant rate of 0.3°C/ min, until loss of equilibrium (LOE) was reached, which was designated as the CT_{max} / CT_{min} (Paladino *et al*., 1980). The lethal thermal maxima (LT_{max}) / lethal thermal minima (LT_{min}) were determined by further increasing/ decreasing the temperature until the opercula movements were ceased (Tsuchida, 1995 and Kita *et al*., 1996). This technique has been critically evaluated by numerous workers (Das *et al.*, 2004, 2005) and is well established as a powerful tool for studying the thermal tolerance in fishes (Paladino *et al.*, 1980). A partial thermal tolerance polygon was generated from the CT_{max} and CT_{min} data by plotting acclimation temperatures (℃) on the X-axis and thermal tolerance limit (℃) on the Y-axis. The area of thermal tolerance was calculated from the polygon and expressed as $^0C^2$. The fish were deprived of feed for one day before performing the thermal tolerance study.

3.22.2 Oxygen consumption rates and temperature quotients (Q_{10})

Oxygen consumption rates were measured in a static respirometer chamber, using a separate group of fish acclimated to different temperatures (27, 30, 33, and 36℃), following the method adopted by earlier investigations Das *et al.* (2004, 2005). Briefly, six fish per treatment, acclimated to a particular temperature were placed individually into a sealed glass chamber (5L) with 6.4 mm thick glass lid, cut to cover the top portion completely. An opening in the lid fitted with a gasket to ensure an air-tight seal permitted the insertion of a DO probe. The chamber was placed inside the thermostatic aquarium set at the respective test temperatures. All four sides of the aquarium were covered with opaque screens to minimize visual disturbances of the experimental fish. The oxygen consumption experiment was carried out for an hour. The initial and final oxygen contents in the static respirometer were measured using a digital oxy-meter 330 (sensitivity 0.01mg O_2/L, E-Merck, Germany) and the oxygen consumption rates for individual fish were expressed as mg O_2 /kg/ hr.

Similarly another set of 24 fish, maintained at an ambient temperature, was subjected to an increase in water temperature to reach the test temperatures (27, 30, 33 and 36^{0}C) in thermostatic aquaria separately, so as to delaminate the effect of an acute increase of temperature vs. acclimation procedure on oxygen consumption rate. The temperature quotients (Q_{10}) were calculated to assess the effect of acclimation on oxygen consumption rate by using the formula (Schmidt-Nielsen, 1997):

$$Q_{10} = (\text{Rate2} / \text{Rate1})^{(10/\text{Temp2}-\text{Temp1})}$$

3.23 Challenge Study

After the feeding trial, intraperitoneal injection over the fish in various experimental groups were challenged with the pathogenic isolates of *Aeromonas hydrophila*. For this, the pathogenic isolates of *A. hydrophila* were grown on nutrient agar for 24hr at 30^0C in a BOD incubator. The cells were harvested and washed thrice in sterile PBS and then re-suspended in PBS at concentration of 10^6 cells ml^{-1}. The fishes in each experimental group were injected with 0.2 ml of this suspension intraperitoneally. The mortality (%) was observed in the challenged fishes up to a week along with the changes in behaviour and morphology. At the end of week of post challenge, haemato-immunological parameters were studied.

3.24 Histological Study

The fish liver, gill and intestinal tissues were immediately fixed in neutral-buffered formalin, embedded in paraffin wax, cut at 5 µm and stained with haematoxylin and eosin (H&E) as described by Roberts (1989). Prepared slides were examined under a light microscope (40X and 160X). The tissues were fixed in buffered formalin and were dehydrated in 90% alcohol for an hour and three times in absolute alcohol for 45 min each separately. The samples were then cleared two times in xylene for 30 min each and embedded in paraffin for 45 min. The samples were then blocked, allowed to cool, cut on a rotatory microtome at 5µm and mounted sections were dewaxed in xylene and dehydrated serially in alcohol and then the slides were washed in tap water for 1 min, stained in haematoxylene for 12 min, washed with tap water, dipped in 2% acid alcohol and again washed in tap water. The sections were dehydrated through 50%, 70% and 90% alcohol for 2 min each and stained in eosin for 4 min and dipped in absolute alcohol for 1 min. Finally the stained sections were cleared in xylene for 5 min and mounted with DPX.

3.25 Scanning Electron Microscopy (SEM) study

Gills tissue was sampled for SEM observation. The second gill arch from each group of fishes was dissected out and kept in normal saline solution. All the isolated arches were fixed in primary fixative 2.5% glutaraldehyde solution, after that washed with 0.1M sodium cacodylate buffer at 4^0C overnight. The buffer was changed for three times at the interval of 15 minutes each at same cool environment. And then post fixation was done with osmium tetroxide for 2 hours. Later they were washed with distilled water for 5-10 times. After fixation, the sample was dehydrated with increasing concentration of ethanol (20%, 30%, 40%, 50%, 60%, 70%, 80 % and absolute ethanol. Having dried, the tissues were mounted on specimen stubs with double-sided adhesive carbon tabs. These samples are very sensitive to electron beam. The charging of the specimen causes artifacts and also focusing problem in the SEM. To avoid the charging, the specimen was coated with a thin layer (250-300°A) of conducting material *viz.*, gold/palladium using sputter coater and examined in Philips XL 30 SEM at 10KV with tilt angle of 45°. Using suitable magnification, micrographs of the tissue outer surface were recorded.

3.26 Statistical Analysis

Data were analyzed using one-way and two-way analysis of variance (ANOVA) and the significant difference between the treatments was determined by Duncan's Multiple Range Test (DMRT) using SPSS 16.0 (SPSS, Chicago, IL, USA). The mean values of pre-challenge parameters were compared with post-challenge value by Student's paired t-test. The level of significance employed was 0.05. Regression analysis was also carried out on some selected parameters. Oxygen consumption values were mass-adjusted considering mass exponent of 0.80 and reported at standard temperature and pressure (Cech, 1990).

Results

4. Results

4.1 Experiment 1

4.1.1 Physico-chemical Properties of Water

The physico-chemical parameters of water such as temperature, pH, dissolved oxygen, free carbon dioxide, ammonia, Nitrite-N and Nitrate-N recorded in all the experimental groups are given in Table 3. Water temperatures of experimental groups were maintained at 27, 30, 33 and 36^{0}C during the entire experimental period. The pH values were recorded within the range of 6.87 to 7.61 during the experimental period. Dissolved oxygen content was recorded with in the range of 5.89 to 7.29 mg/L and free carbon dioxide (mg/L) was found to be negligible. The total ammonia, nitrite-N and nitrate-N content was recorded before water exchange and found to be in the range of 0.15 to 0.27, 0.001 to 0.009 and 0.03 to 0.14 mg/L, respectively.

Table 3: Water quality parameters in rearing tanks exposed to four different temperatures for a period of 60 days

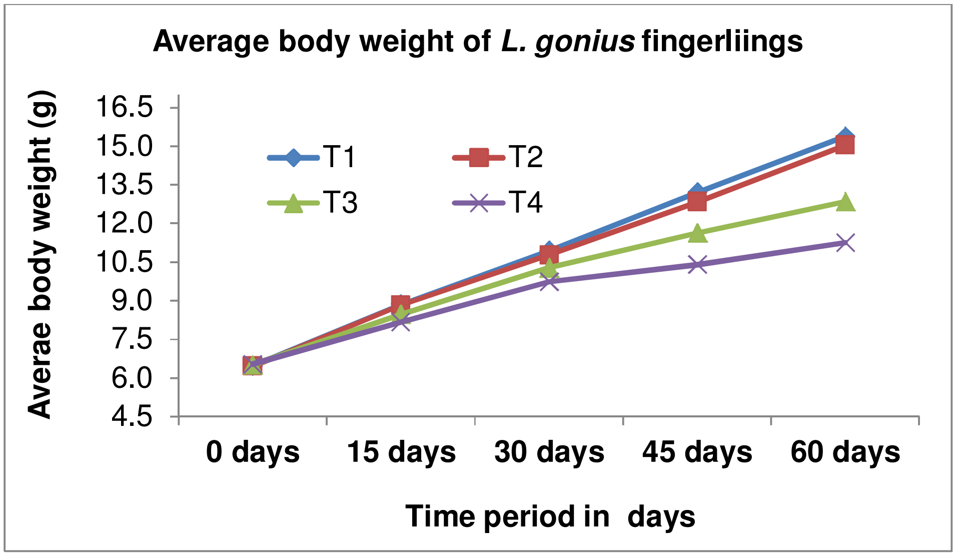
Treatments	pH	Dissolved oxygen	Ammonia - N	Nitrite - N	Nitrate - N
T_1 (27^{0}C)	7.43- 7.61	6.29- 7.29	0.15- 0.19	0.001- 0.003	0.03- 0.06
T_2 (30^{0}C)	7.39- 7.55	6.37- 7.17	0.17- 0.22	0.002- 0.004	0.04- 0.08
T_3 (33^{0}C)	7.29- 7.44	6.21- 6.49	0.20- 0.24	0.005- 0.006	0.07- 0.12
T_4 (36^{0}C)	6.87- 7.11	5.89- 6.23	0.21- 0.27	0.006- 0.009	0.10- 0.14

Values expressed in range; Dissolved oxygen, Ammonia-N, Nitrite-N and Nitrate-N are expressed as mg/L

4.1.2 Growth and Survival

The body weight gain of *L. gonius* fingerlings exposed to four different temperatures was recorded at 15 days intervals as shown in Table 4 and Figure 1. The final body weight gain of the fingerlings decreased significantly ($p<0.05$) on exposure to higher temperatures. The highest final body weight was recorded in T_1, which is not significantly different and T_2 group and the lowest was in T_4 group followed by T_3 group. After 15 days of exposure, there was no significant difference in average body weight gain of the fingerlings in different treatment groups, which varied afterwards.

Figure 1: Body weight (g) gain (at 15 days interval) of *L. gonius* fingerlings exposed to four different temperatures for a period of 60 days



The body weight gain %, SGR, FCR, FER, PER and survival rate of *L. gonius* fingerlings exposed to four different temperatures are presented in Table 5. Body weight gain (%), SGR, FER, PER and survival rate decreased significantly ($p<0.05$) on exposure to higher temperature. The higher values were recorded in T_1 and T_2 group and the lowest was in T_4 group followed by T_3 group. On the contrary, FCR followed an opposite trend.

4.1.3 DNA, RNA and RNA/DNA Ratio

DNA, RNA and RNA/DNA ratio in liver and muscle tissue of of *L. gonius* fingerlings exposed to four different temperatures are presented in Table 6. DNA content in both the tissues did not vary significantly ($p>0.05$) on exposure to higher temperature. RNA and RNA/DNA ratio in both the tissues decreased significantly ($p<0.05$) on exposure to higher temperature. The higher values were recorded in T_1 and T_2 group. The lowest value was recorded in T_4 group followed by T_3 group. A linear relationship was observed with weight gain % and RNA, RNA/DNA ratio of *L. gonius* fingerlings (Figure 2 & 3).

Figure 2: Relationship between weight gain % and RNA content in muscle and liver tissue of *L. gonius* fingerlings exposed to four different temperatures for a period of 60 days

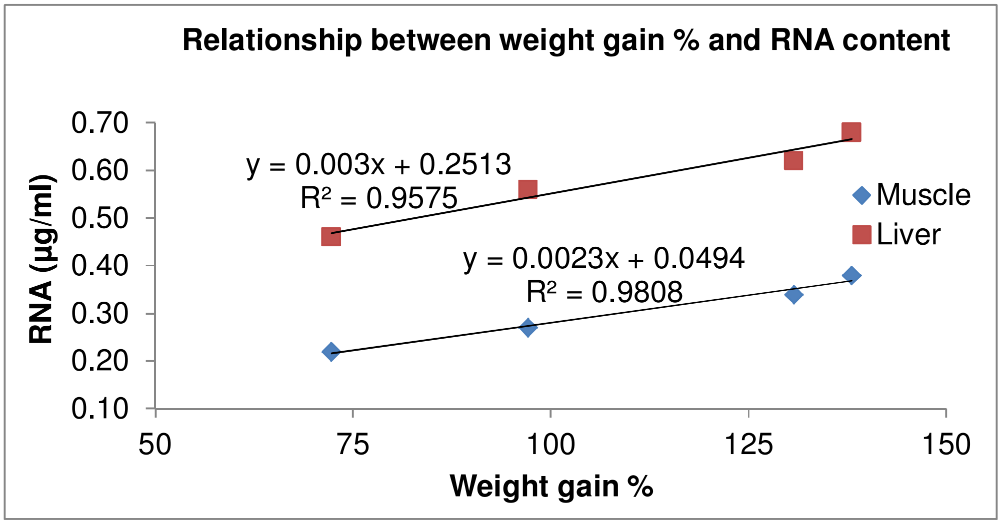


Figure 3: Relationship between weight gain % and RNA/DNA ratio in muscle and liver tissue of *L. gonius* fingerlings exposed to four different temperatures for a period of 60 days

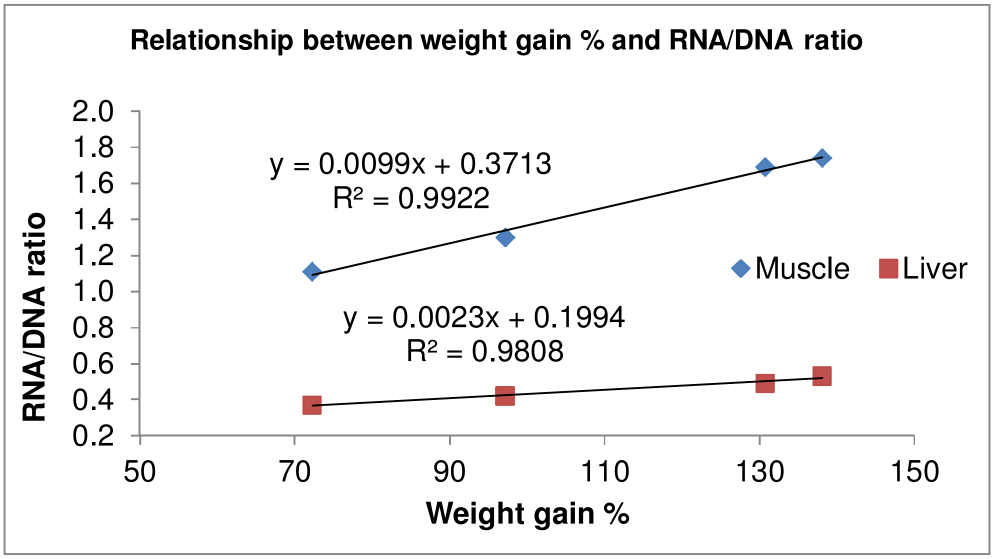


Table 4: Body weight (g) gain (at 15 days interval) of *L. gonius* fingerlings exposed to four different temperatures for a period of 60 days

Treatment	Initial	0-15 days	0-30 days	0-45 days	0-60 days
T_1 (27^0C)	$6.46^a \pm 0.08$	$8.86^b \pm 0.25$	$10.94^b \pm 0.19$	$13.21^c \pm 0.43$	$15.37^c \pm 0.26$
T_2 (30^0C)	$6.45^a \pm 0.09$	$8.73^{ab} \pm 0.16$	$10.66^{bc} \pm 0.16$	$12.70^c \pm 0.36$	$14.88^c \pm 0.07$
T_3 (33^0C)	$6.51^a \pm 0.09$	$8.43^{ab} \pm 0.11$	$10.28^{ab} \pm 0.12$	$11.56^b \pm 0.35$	$12.84^b \pm 0.17$
T_4 (36^0C)	$6.54^a \pm 0.10$	$8.13^a \pm 0.10$	$9.74^a \pm 0.15$	$10.40^a \pm 0.14$	$11.26^a \pm 0.09$
P-Value	0.545	0.061	0.003	0.002	0.001

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=3 (each replicate is having 12 fish)

Table 5: Growth and survival of *L. gonius* fingerlings exposed to four different temperatures for a period of 60 days

Treatments	Weight gain %	FCR	FER	PER	SGR	% survival
T_1 (27^{0}C)	138.09^c ±1.17	1.84^c ±0.02	0.54^c ±0.01	1.54^c ±0.01	1.45^c ±0.01	100.0^c ±0.00
T_2 (30^{0}C)	130.74^c ±2.26	1.90^c ±0.03	0.52^c ±0.01	1.49^c ±0.03	1.39^c ±0.02	100.0^c±0.00
T_3 (33^{0}C)	97.16^b ±3.78	2.42^b ±0.08	0.42^b ±0.02	1.17^b ±0.03	1.13^b ±0.03	94.45^{bc} ±2.77
T_4 (36^{0}C)	72.26^a ±1.23	3.07^a ±0.05	0.33^a ±0.01	0.92^a ±0.01	0.91^a ±0.01	77.78^a ±2.76
P-Value	0.001	0.001	0.001	0.001	0.001	0.001

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=3 (each replicate is having 12 fish for growth parameters)

FCR: Feed conversion ratio; FER: Feed efficiency ratio;

PER: Protein efficiency ratio; SGR: Specific growth rate

Table 6: DNA, RNA and RNA/DNA ratio of *L. gonius* fingerlings exposed to four different temperatures for a period of 60 days

Treatment	Muscle			Liver		
	µg DNA/ml	µg RNA/ml	RNA/DNA ratio	µg DNA/ml	µg RNA/ml	RNA/DNA ratio
T_1 (27^0C)	0.22±0.02	0.38^b±0.02	1.74^c±0.04	1.28±0.01	0.68^b±0.04	0.53^b±0.03
T_2 (30^0C)	0.20±0.01	0.34^b±0.03	1.69^c±0.02	1.27±0.02	0.62^b±0.02	0.49^b±0.02
T_3 (33^0C)	0.21±0.01	0.27^a±0.01	1.30^b±0.02	1.28±0.02	0.56^a±0.01	0.42^a±0.01
T_4 (36^0C)	0.21±0.01	0.22^a±0.01	1.11^a±0.02	1.27±0.01	0.46^a±0.02	0.37^a±0.02
P-Value	0.421	0.001	0.001	0.520	0.001	0.002

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6

Table 7: Glycogen, ascorbic acid and body indices (HSI and GSI) of *L. gonius* fingerlings exposed to four different temperatures

Treatments	Glycogen		Ascorbic acid	Body indices	
	Muscle	Liver	Muscle	HSI	GSI
T_1 (27^0C)	1.25^c ±0.14	12.93^c±0.84	131.48^c±2.26	1.45^b±0.21	2.64^a±0.41
T_2 (30^0C)	1.18^{bc}±0.07	11.58^b±0.70	126.23^b±3.47	1.36^b±0.11	2.52^a±0.28
T_3 (33^0C)	0.89^{ab}±0.09	8.43^a±0.59	87.05^a±2.76	1.18^{ab}±0.18	2.27^a±0.51
T_4 (36^0C)	0.74^a±0.04	5.77^a±0.41	68.51^a±1.70	0.81^a±0.09	2.13^a±0.23
P-Value	0.015	0.001	0.001	0.030	0.379

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6 (n=9 for body indices);

Glycogen: mg glycogen/g tissue;

Ascorbic acid: microgram/g wet tissue;

Hepatosomatic Index (HSI) and Gastrosomatic Index (GSI) are expressed as %

4.1.4 Body Indices

Body indices (HSI and GSI) of *L. gonius* fingerlings exposed to four different temperatures are presented in Table 7. HSI decreased significantly ($p<0.05$) on exposure to higher temperature. The higher values were recorded in T_1 and T_2 group. The lowest value was recorded in T_4 group followed by T_3 group. GSI values of different experimental groups did not vary significantly ($p>0.05$).

4.1.5 Tissue Glycogen

Glycogen content in liver and muscle tissue of *L. gonius* fingerlings exposed to four different temperatures is presented in Table 7. Glycogen content in both the tissue decreased significantly ($p<0.05$) on exposure to higher temperature. The highest tissue glycogen was recorded in T_1, which is not significantly different and T_2 group. The lowest value was recorded in T_4 group followed by T_3 group.

4.1.6 Ascorbic Acid

Ascorbic acid in muscle tissue of *L. gonius* fingerlings exposed to four different temperatures is presented in Table 7. Ascorbic acid in muscle tissue decreased significantly ($p<0.05$) on exposure to higher temperature. The highest ascorbic content was recorded in T_1, which is not significantly different from T_2 group. The lowest value was recorded in T_4 group followed by T_3 group.

4.1.7 Enzymatic Activity in Tissues

4.1.7.1 Enzymes of protein metabolism (AST and ALT)

AST and ALT activity in liver and muscle tissue of *L. gonius* fingerlings exposed to four different temperatures are presented in Table 8. AST and ALT activity in liver and muscle tissue increased significantly ($p<0.05$) on exposure to higher temperature. The lowest AST and ALT activity was recorded in T_1 group and the highest value was in T_4 group followed by T_3 group. However, there was no significant difference ($p>0.05$) between T_1 and T_2 group. AST and ALT activity was higher in muscle tissue than the liver tissue.

Table 8: AST, ALT, LDH and MDH enzyme activity in tissues of *L. gonius* fingerlings exposed to four different temperatures for a period of 60 days

Treatments	GOT/AST		GPT/ALT		LDH		MDH	
	Liver	Muscle	Liver	Muscle	Liver	Muscle	Liver	Muscle
T_1 (27^{0}C)	$15.04^{c} \pm 0.59$	$19.94^{c} \pm 0.51$	$5.28^{c} \pm 0.38$	$7.41^{c} \pm 0.35$	$3.32^{c} \pm 0.37$	$4.77^{c} \pm 0.38$	$2.08^{c} \pm 0.14$	$3.19^{c} \pm 0.38$
T_2 (30^{0}C)	$17.78^{c} \pm 0.63$	$22.57^{c} \pm 0.60$	$7.29^{c} \pm 0.56$	$9.25^{c} \pm 0.48$	$3.78^{c} \pm 0.34$	$5.21^{c} \pm 0.33$	$2.22^{c} \pm 0.18$	$3.58^{c} \pm 0.45$
T_3 (33^{0}C)	$31.17^{b} \pm 1.14$	$38.56^{b} \pm 2.57$	$12.37^{b} \pm 1.01$	$14.97^{b} \pm 0.74$	$9.02^{b} \pm 0.41$	$10.18^{b} \pm 0.63$	$7.16^{b} \pm 0.40$	$8.59^{b} \pm 0.69$
T_4 (36^{0}C)	$47.59^{a} \pm 1.59$	$52.30^{a} \pm 1.47$	$17.76^{a} \pm 0.76$	$24.44^{a} \pm 0.90$	$13.66^{a} \pm 0.66$	$15.63^{a} \pm 0.69$	$11.11^{a} \pm 0.84$	$14.15^{a} \pm 0.61$
P-Value	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6;

AST activity was expressed as n mol of oxaloacetate released/ min/ mg protein at 37^{o}C;

ALT activity was expressed as n mol of sodium pyruvate released/ min/ mg protein at 37^{o}C;

LDH and MDH activity was expressed as Unit/min/mg protein at 37^{0}C

Table 9: AchE, catalase, SOD and GST enzyme activity in tissues of *L. gonius* fingerlings exposed to four different temperatures for a period of 60 days

Treatments	AchE	Catalase		SOD		GST	
	Brain	Liver	Gill	Liver	Gill	Liver	Gill
T_1 (27^{0}C)	6.12^{c} ±0.06	3.53^{c}±0.26	6.69^{c}±0.46	11.84^{c} ±0.49	25.04^{c} ±0.67	0.367^{c} ±0.01	0.465^{c} ±0.01
T_2 (30^{0}C)	5.95^{c}±0.12	3.87^{c}±0.35	7.46^{c}±0.58	12.61^{c} ±0.28	25.44^{c}±0.27	0.382^{c} ±0.01	0.476^{c} ±0.01
T_3 (33^{0}C)	4.71^{b}±0.22	6.76^{b}±0.40	12.02^{b}±0.60	19.74^{b} ±0.45	34.061^{b}±0.73	0.586^{b} ±0.01	0.704^{b} ±0.01
T_4 (36^{0}C)	3.93^{a} ±0.26	10.80^{a}±0.86	17.21^{a}±0.66	26.75^{a} ±0.54	43.68^{a} ±0.44	0.799^{a} ±0.02	1.08^{a} ±0.02
P-Value	0.001	0.001	0.001	0.001	0.001	0.002	0.002

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6;

Catalase: Nano moles H_2O_2 decomposed / min / mg protein at 37^{0}C;

SOD: micromole /mg protein/min at 37^{0}C;

GST: units /mg protein;

AchE: micromoles acetylcholine hydrolyzed / mg protein / min at 37^{0}C

Table 10: FDPase, G6PDH and ATPase enzyme activity in tissues of *L. gonius* fingerlings exposed to four different temperatures for a period of 60 days

Treatments	FDPase		G6PDH		ATPase	
	Liver	Gill	Liver	Muscle	Liver	Gill
T_1 (27^0C)	8.72^c ±0.43	28.94^c ±1.02	11.41^c ±0.35	6.45^c ±0.42	2.89^b±0.11	5.31^b±0.25
T_2 (30^0C)	12.97^c ±0.75	34.88^c ±1.61	11.59^c ±0.31	5.70^{bc} ±0.52	3.04^b±0.14	5.54^b±0.13
T_3 (33^0C)	27.46^b ±1.17	59.11^b ±2.23	8.52^b ±0.60	4.41^{ab} ±0.63	3.27^b±0.10	5.96^b±0.15
T_4 (36^0C)	41.44^a ±2.64	91.96^a ±2.32	6.51^a ±0.71	3.16^a ±0.42	3.98^a±0.15	6.95^a±0.21
P-Value	0.001	0.001	0.001	0.009	0.002	0.001

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6;

FDPase: microgram phosphorous released / mg protein / minute at 37^0C;

G6PDH: Δ 0.01 optical density / mg protein / minute at 37^0C;

ATPase: microgram phosphorous released / mg protein / minute at 37^0C

4.1.7.2 Enzymes of carbohydrate metabolism (LDH and MDH)

LDH and MDH activity in liver and muscle tissue of *L. gonius* fingerlings exposed to four different temperatures are presented in Table 8. LDH and MDH activity in liver and muscle tissue increased significantly ($p<0.05$) on exposure to higher temperature. The lowest value was recorded in T_1 group and the highest value was in T_4 group followed by T_3 group. However, there was no significant difference ($p>0.05$) between T_1 and T_2 group.

4.1.7.3 Enzyme of neurotransmission (AchE)

AchE activity in brain tissue of *L. gonius* fingerlings exposed to four different temperatures is presented in Table 9. AchE activity in brain decreased significantly ($p<0.05$) on exposure to higher temperature. The highest value was recorded in T_1 group and the lowest activity was in T_4 group followed by T_3 group. However, there was no significant difference ($p>0.05$) between T_1 and T_2 groups.

4.1.7.4 Enzymes of oxidative stress (Catalase, SOD and GST)

Catalase, SOD and GST activity in liver and gill tissue of *L. gonius* fingerlings exposed to four different temperatures are presented in Table 9. Catalase activity in liver and gill tissue increased significantly ($p<0.05$) on exposure to higher temperature. The lowest activity was recorded in T_1 group and the highest activity was in T_4 group followed by T_3 group. However, there was no significant difference ($p>0.05$) between T_1 and T_2 group.

4.1.7.5 Enzyme of gluconeogenic pathways (FDPase)

FDPase activity in liver and gill tissue of *L. gonius* fingerlings exposed to four different temperatures is presented in Table 10. FDPase activity in liver and gill tissue increased significantly ($p<0.05$) on exposure to higher temperature. The lowest value was recorded in T_1 group and the highest activity was in T_4 group followed by T_3 group.

4.1.7.6 Enzyme of energy metabolism (ATPase)

ATPase activity in liver and gill tissue of *L. gonius* fingerlings exposed to four different temperatures is presented in Table 10. ATPase activity in liver and gill tissue increases significantly ($p<0.05$) on exposure to extreme higher temperature. The lowest value was recorded in T_1 group and the highest activity was in T_4 group. There was no significance difference between T_1, T_2 and T_3 groups.

4.1.7.7 Enzyme of pentose phosphate pathway (G6PDH)

G6PDH activity in liver and muscle tissue of *L. gonius* fingerlings exposed to four different temperatures is presented in Table 10. G6PDH activity in liver and muscle tissue decreased significantly ($p<0.05$) on exposure to higher temperature. The highest activity was recorded in T_1 and T_2 group and the lowest activity was recorded in T_4 group followed by T_3 group.

4.1.7.8 Enzyme of phospho monoesterase (ALP)

ALP activity in muscle and liver tissues of *L. gonius* fingerlings exposed to four different temperatures is presented in Table 11. ALP activity in all the tissue increased significantly ($p<0.05$) on exposure to higher temperature. The lowest activity was recorded in T_1 and T_2 group and the highest activity was in T_4 group followed by T_3 group.

Table 11: ALP and protease enzyme activity in tissues of *L. gonius* fingerlings exposed to four different temperatures for a period of 60 days

Treatments	ALP		Protease
	Muscle	Liver	Intestine
T_1 (27^0C)	12.01^c ±1.04	32.38^c±1.38	17.91^c ±0.53
T_2 (30^0C)	14.61^c ±1.15	36.40^c±1.77	16.69^c ±0.63
T_3 (33^0C)	28.47^b ±1.18	69.80^b±1.57	14.72^b ±0.81
T_4 (36^0C)	48.58^a ±1.75	95.33^a±2.43	13.82^a ±0.65
P-Value	0.001	0.001	0.009

Values in the same column with different superscript differ significantly ($p<0.05$);
Data expressed as Mean ± SE, n=6;
Protease activity as mmole of tryosine released/min/g protein at 37^0C;
ALP: nanomoles P-nitrophenol released / mg protein / minute at 37^0C

4.1.7.9 Digestive enzyme (Protease)

Protease enzyme activity in intestinal tissue of *L. gonius* fingerlings exposed to four different temperatures is presented in Table 11. Protease activity in intestinal tissue decreased significantly ($p<0.05$) on exposure to higher temperature. However, amylase activity did not vary significantly ($p>0.05$). The highest activity was recorded in T_1 followed by T_2 group. The lowest activity was recorded in T_4 group.

4.1.8 Haemato-immunological Parameters

4.1.8.1 Erythrocyte (RBC), haemoglobin (Hb) and haematocrit (Hct)

RBC, Hb and Hct count of *L. gonius* fingerlings exposed to four different temperatures are presented in Table 12. WBC count in serum decreased significantly ($p<0.05$) on exposure to higher temperature. The highest count was recorded in T_1 followed by T_2 group. The lowest count was recorded in T_4 followed by T_3 group.

4.1.8.2 Leucocyte (WBC) count

WBC count of *L. gonius* fingerlings exposed to four different temperatures is presented in Table 12. WBC count in serum decreased significantly ($p<0.05$) on exposure to higher temperature. The highest count was recorded in T_1 followed by T_2 group. The lowest count was recorded in T_4 followed by T_3 group.

4.1.8.3 Respiratory burst activity (NBT)

Respiratory burst activity of *L. gonius* fingerlings exposed to four different temperatures is presented in Table 12. NBT values decreased significantly ($p<0.05$) on exposure to higher temperature. The highest activities were recorded in T_1 group. However, there was no significant difference ($p>0.05$) between T_1 and T_2 group. The lowest activity was recorded in T_4 group followed by T_3 group.

4.1.8.4 Blood glucose

Blood glucose level of *L. gonius* fingerlings exposed to four different temperatures is presented in Table 12. Blood glucose level increased significantly

($p<0.05$) on exposure to higher temperature. The lowest value was recorded in T_1 followed by T_2 group. The highest value was recorded in T_4 followed by T_3 group.

4.1.8.5 Total serum protein, albumin (A), globulin (G) and A-G ratio

Total serum protein, albumin, globulin and A-G ratio of *L. gonius* fingerlings exposed to four different temperatures are presented in Table 13. Total serum protein, albumin and globulin values decreased significantly ($p<0.05$) on exposure to higher temperature. The highest value was recorded in T_1 followed by T_2 group. The lowest value was recorded in T_4 group followed by T_3 group. On the contrary, A-G ratio shows an opposite trend.

4.1.8.6 Serum lysozyme activity

Serum lysozyme activity of *L. gonius* fingerlings exposed to four different temperatures is presented in Table 13. Lysozyme activities decreased significantly ($p<0.05$) on exposure to higher temperature. The highest value was recorded in T_1 group. However, there was no significance difference between T_1 and T_2. The lowest value was recorded in T_4 followed by T_3 group.

4.1.9 Serum Enzyme Activity

Serum GOT, GPT, ALP, ACP and LDH activity in serum of *L. gonius* fingerlings exposed to four different temperatures are presented in Table 14. The enzyme activities increased significantly ($p<0.05$) on exposure to higher temperature. The lowest activity was recorded in T_1. However, there was no significance difference between T_1 and T_2. The highest activity was recorded in T_4 group followed by T_3 group.

4.1.10 Serum Cortisol

Cortisol levels of *L. gonius* fingerlings exposed to four different temperatures is presented in Table 15. The cortisol level in serum increases significantly ($p<0.05$) on exposure to higher temperature. The lowest cortisol value was recorded in T_1 group. However, there was no significant difference ($p>0.05$) between T1 and T2 group. The highest cortisol was recorded in T_4 group followed by T_3 group.

Table 12: Total leucocytes (WBC), total erythrocyte (RBC), hemoglobin (Hb), Haematocrit (Hct), NBT and blood glucose of *L. gonius* fingerlings exposed to four different temperatures for a period of 60 days

Treatments	**WBC (10^{3}cells/mm^{3})**	**RBC (10^{6}cells/mm^{3})**	**Hb (g/dL)**	**Hct (%)**	**NBT (A_{540})**	**Blood glucose (mg/100 mL)**
T_1 (27^{0}C)	$218.50^{c}\pm1.8$	$1.98^{c}\pm0.04$	$9.63^{c}\pm0.30$	$29.10^{c}\pm0.46$	$0.324^{c}\pm0.01$	$38.22^{c}\pm0.20$
T_2 (30^{0}C)	$211.17^{c}\pm2.09$	$1.88^{c}\pm0.02$	$9.46^{c}\pm0.34$	$28.37^{c}\pm0.24$	$0.310^{c}\pm0.01$	$40.06^{c}\pm0.48$
T_3 (33^{0}C)	$184.31^{b}\pm5.20$	$1.76^{b}\pm0.03$	$6.92^{b}\pm0.11$	$18.78^{b}\pm0.34$	$0.224^{b}\pm0.01$	$53.07^{b}\pm0.90$
T_4 (36^{0}C)	$163.13^{a}\pm3.45$	$1.54^{a}\pm0.04$	$6.12^{a}\pm0.05$	$16.42^{a}\pm0.42$	$0.192^{a}\pm0.01$	$61.71^{a}\pm0.75$
P-Value	0.001	0.001	0.001	0.001	0.001	0.001

Values in the same column with different superscript differ significantly $p<0.05$);

Data expressed as Mean ± SE, n=6;

NBT (Respiratory burst activity) expressed in optimal density at 540 nm

Table 13: Total protein, albumin, globulin, A/G ratio and serum lysozyme activity in serum of *L. gonius* fingerling exposed to four different temperatures for a period of 60 days

Treatments	Total protein (g/dL)	Albumin (A) (g/dL)	Globulin (G) (g/dL)	A/G Ratio	Serum lysozyme
T_1 (27^0C)	3.92^c±0.03	0.87^c±0.02	3.05^c±0.05	0.28^c±0.01	375.95^c±9.48
T_2 (30^0C)	3.79^c±0.11	0.84^c±0.03	2.95^c±0.08	0.29^c±0.01	375.16^c±6.61
T_3 (33^0C)	2.81^b±0.04	0.70^b±0.02	2.11^b±0.03	0.33^b±0.01	254.16^b±4.91
T_4 (36^0C)	2.06^a±0.03	0.59^a±0.02	1.48^a±0.03	0.40^a±0.02	213.44^a±7.46
P-Value	0.001	0.001	0.001	0.001	0.001

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6;

Serum lysozyme activity is expressed as unit/ min/ mg serum protein at 37^0C

Table 14: Serum enzyme activity of *L. gonius* fingerlings exposed to four different temperatures for a period of 60 days

Treatments	sGOT (IU/L)	sGPT (IU/L)	sALP (IU/L)	sACP (IU/L)	sLDH (IU/L)
T_1 (27^0C)	$25.57^c \pm 0.59$	$9.83^c \pm 0.39$	$95.80^c \pm 1.35$	$19.97^c \pm 1.33$	$6.26^c \pm 0.37$
T_2 (30^0C)	$28.31^c \pm 0.66$	$11.81^c \pm 0.53$	$100.41^c \pm 2.37$	$22.31^c \pm 1.06$	$6.69^c \pm 0.36$
T_3 (33^0C)	$41.89^b \pm 1.13$	$17.61^b \pm 0.96$	$133.27^b \pm 1.64$	$29.07^b \pm 1.19$	$11.58^b \pm 0.77$
T_4 (36^0C)	$58.07^a \pm 1.52$	$26.82^a \pm 0.91$	$159.38^a \pm 3.01$	$35.89^a \pm 1.07$	$17.20^a \pm 0.54$
P-value	0.001	0.001	0.001	0.001	0.001

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6;

Table 15: Hsp-70 expression, cortisol, caspase-3 activity and thyroid hormones (T3 and T4) of *L. gonius* fingerlings exposed to four different temperatures for a period of 60 days

Treatments	Hsp-70 expression (ng/mL)				Cortisol (ng/mL)	Caspase-3	Thyroid hormones (ng/mL)	
	Muscle	**Gill**	**Liver**	**Serum**	**Serum**	**Serum**	**T3**	**T4**
T$_1$ (27^{0}C)	$1.18^a \pm 0.03$	$2.62^c \pm 0.15$	$3.10^c \pm 0.11$	$5.35^c \pm 0.21$	$97.25^c \pm 2.65$	$17.87^c \pm 0.71$	$1.35^a \pm 0.04$	$21.36^c \pm 0.37$
T$_2$ (30^{0}C)	$1.23^a \pm 0.05$	$2.93^c \pm 0.28$	$3.54^c \pm 0.31$	$6.19^c \pm 0.26$	$103.55^c \pm 1.52$	$19.07^c \pm 0.78$	$1.26^a \pm 0.07$	$20.30^c \pm 0.93$
T$_3$ (33^{0}C)	$1.25^a \pm 0.05$	$4.85^b \pm 0.45$	$6.24^b \pm 0.32$	$15.29^b \pm 0.63$	$156.20^b \pm 2.20$	$24.97^b \pm 0.23$	$0.77^b \pm 0.03$	$15.87^b \pm 0.57$
T$_4$ (36^{0}C)	$1.28^a \pm 0.06$	$5.94^a \pm 0.34$	$8.74^a \pm 0.19$	$22.95^a \pm 0.87$	$189.50^a \pm 2.50$	$32.63^a \pm 0.63$	$0.63^c \pm 0.04$	$13.21^a \pm 0.55$
P-Value	0.519	0.001	0.001	0.001	0.001	0.001	0.002	0.001

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6;

Activity of caspase-3 is expressed as p-NA/ hr / mg of protein

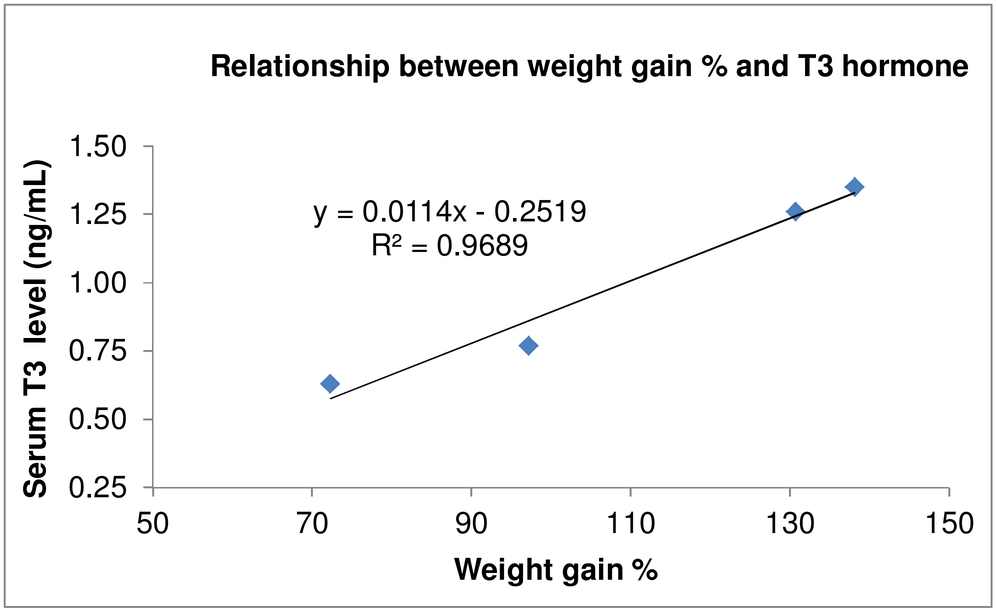
4.1.11 Hsp-70 Expression

The quantitative expression of Hsp-70 in serum, liver, gill and muscle *L. gonius* fingerlings exposed to four different temperatures is presented in Table 15. The Hsp-70 expression was significantly higher ($p<0.05$) in T_3 and T_4 treatment group. The lowest value was recorded in T_1 group. However, there was no significant difference ($p>0.05$) between T_1 and T_2 group. The expression of Hsp-70 in muscle was not significantly varied.

4.1.12 Caspase-3 Activity

The caspase-3 activity of *L. gonius* fingerlings exposed to four different temperatures is presented in Table 15. Caspase-3 activity in serum increases significantly ($p<0.05$) with higher water temperatures in T3 and T4. The lowest caspase-3 activity was recorded in T_1 group. However, there was no significant difference ($p>0.05$) between T_1 and T_2 group. The highest caspase-3 activity was recorded in T_4 group followed by T_3 group.

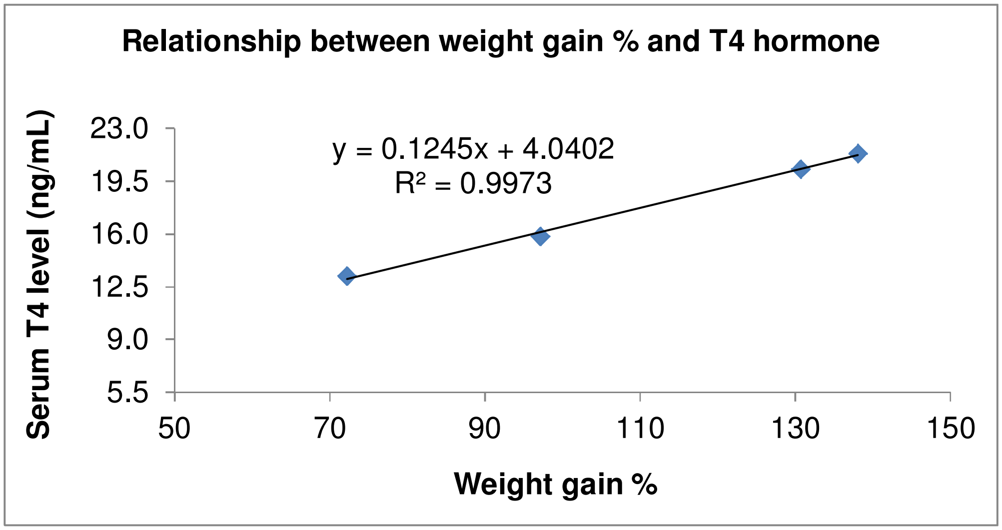
Figure 4: Relationship between weight gain % and T3 hormone in serum of *L. gonius* fingerlings exposed to four different temperatures for a period of 60 days



4.1.13 Thyroid Hormones (T3 and T4)

Thyroid hormones thyroxine (T4) and tri-iodothyronine (T3) levels in serum of *L. gonius* fingerlings exposed to four different temperatures are presented in Table 15.The serum thyroid hormone (T3 and T4) levels decreased significantly ($p<0.05$) on exposure to higher temperature. The highest T3 and T4 hormone was recorded in T_1 and T_2 group. The lowest was recorded in T_4 group followed by T_3 group. A linear relationship was observed between weight gain % and thyroid hormone levels of *L. gonius* fingerlings (Figure 4 & 5).

Figure 5: Relationship between weight gain % and T4 hormone in serum of *L. gonius* fingerlings exposed to four different temperatures for a period of 60 days



4.1.14 Thermal Tolerance Study

4.1.14.1 Thermal tolerance limit

Thermal tolerance limit of *L. gonius* fingerlings acclimated to four different temperatures is given in Table 16. Thermal tolerance limit of the fingerlings is widely influenced by the acclimated temperatures. Critical thermal maxima, CT_{max} and critical thermal minima, CT_{min} increased significantly ($p<0.05$) with increasing acclimation temperatures. Similarly, Lethal thermal maxima, LT_{max} and Lethal thermal minima, LT_{min} increased significantly ($p<0.05$) with increasing acclimation temperatures. A thermal tolerance polygon over the range of 27 to 36 °C had a calculated area of 256.24 $°C^2$ (Figure 6).

4.1.14.2 Oxygen consumption rate

The oxygen consumption rates of *L. gonius* fingerlings with and without acclimated (mgO_2/kg/hr) are presented in Table 17. Oxygen consumption rate increased significantly ($p<0.05$) with increasing acclimation temperatures. Similarly, oxygen consumption rates also significantly ($p<0.05$) differ without acclimating at different temperatures.

Table 16: Thermal tolerance limit of *L. gonius* fingerlings acclimated to four different temperatures

Acclimated temperature	Parameters			
	CT_{Max} (^0C)	LT_{Max} (^0C)	CT_{Min} (^0C)	LT_{Min} (^0C)
27^0C	$40.97^d\pm0.049$	$41.63^d\pm0.040$	$12.72^d\pm0.042$	$12.24^d\pm0.025$
30^0C	$41.89^c\pm0.045$	$42.41^c\pm0.049$	$13.62^c\pm0.038$	$13.25^c\pm0.034$
33^0C	$42.69^b\pm0.035$	$42.99^b\pm0.021$	$13.99^b\pm0.049$	$13.68^b\pm0.045$
36^0C	$43.05^a\pm0.053$	$43.42^a\pm0.041$	$14.39^a\pm0.021$	$13.97^a\pm0.039$
Mean ± SE	42.15±0.167	42.61±0.141	13.67±0.130	13.29±0.137

Values in the same column with different superscript differ significantly ($p<0.05$); Data expressed as Mean ± SE, n=6

Table 17: Oxygen consumption rates in acclimated and non-acclimated condition of *L. gonius* fingerlings at four different temperatures

Temperatures	Oxygen consumption rates (mgO_2 /kg/ hr)	
	Acclimated group	Non-acclimated group
27^0C	$72.78^d \pm1.420$	$73.67^d \pm2.850$
30^0C	$87.75^c \pm2.966$	$93.84^c \pm2.351$
33^0C	$111.31^b \pm2.392$	$120.30^b \pm3.472$
36^0C	$119.37^a \pm2.457$	$157.23^a \pm3.202$
Mean ± SE	97.80±4.021	111.26±6.691

Values in the same column with different superscript differ significantly ($p<0.05$); Data expressed as Mean ± SE, n=6

Figure 6: Thermal tolerance polygon of *L. gonius* fingerlings over four acclimation temperatures (27, 30, 33 and 36°C) using CT_{max} and CT_{min} values.

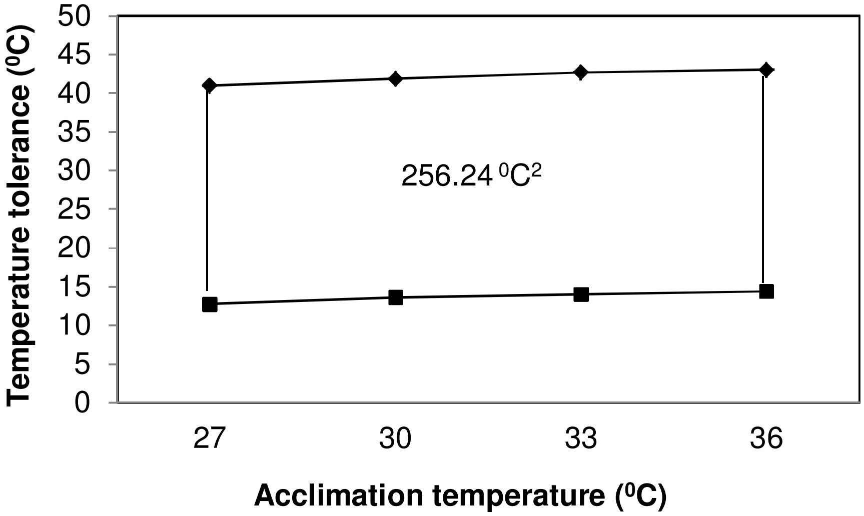


Table 18: Temperature quotients (Q_{10} values) of *L. gonius* fingerlings acclimated to four different temperatures

Temperatures	Temperature differences	Temperature quotients (Q_{10} values)
27-30^{0}C	3^{0}C	1.865^b
30-33^{0}C	3^{0}C	2.209^c
33-36^{0}C	3^{0}C	1.262^a
27-36^{0}C (Acclimated)	9^{0}C	1.722^A
27-36^{0}C (Non-acclimated	9^{0}C	2.322^B

Different superscript letters in the same column indicate significance difference

4.1.14.3 Temperature quotient (Q_{10})

Temperature quotients (Q_{10} values) of *L. gonius* fingerlings acclimated to four different temperatures is presented in 18. Temperature quotient (Q_{10}) was found to be 1.72 for acclimated fish and 2.23 for non-acclimated fish over the range of acclimation temperatures (27-36℃). Final preferred temperature estimated from the Q_{10} value (1.865) was between 27-30℃ and (2.209) 30-33℃.

4.1.15 Histological Study

4.1.15.1 Gill tissue

Histoarchitecture (H&E 40X) of gill tissue of *L. gonius* fingerlings was found to be normal at 27^0C (Plate 1) and 30^0C (H&E 40X) (Plate 4). However, gill tissue showed loss of secondary lamellae in the lower half of the filament at 33^0C (H&E 160X) (Plate 7). In another slide, gill tissue showed marked loss of primary and secondary lamellae at 33^0C (H&E 160X) (Plate 8). Similarly, gill tissue showed extensive loss of secondary gill lamellae, atrophy of primary lamellae and congestion at the branchial arch at 36^0C (H&E 40X) (Plate 11). In another slide, gill tissue showed complete loss of gill lamellae at 36^0C (H&E 160X) (Plate 12).

4.1.15.2 Intestinal tissue

Histoarchitecture of intestinal tissue of *L. gonius* fingerlings was found to be normal at 27^0C (H&E 160X) (Plate 2) and 30^0C (H&E 160X) (Plate 5). However, Intestinal tissue showed mild loss of brush boarder at the mucosa at 33^0C (H&E 160X) (Plate 9). Similarly, intestinal tissue showed degeneration of mucosa at some places and vacuolation in the lamina propria at 36^0C (H&E 160X) (Plate 13).

4.1.15.3 Liver tissue

Histoarchitecture of the liver tissue of *L. gonius* fingerlings was found to be normal at 27^0C (H&E 40X) (Plate 3) and 30^0C (H&E 160X) (Plate 6). However, liver tissue showed diffuse vacuolation of the hepatocytes at 33^0C (H&E 160X) (Plate 10). Similarly, liver tissue showed extensive vacuolation at 36^0C (H&E 160X) (Plate 14).

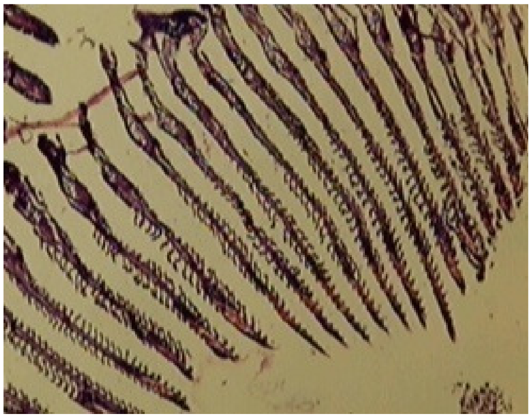


Plate 1: Histoarchitecture of normal gill tissue of *L. gonius* fingerlings at 27^0C (H&E 40X)

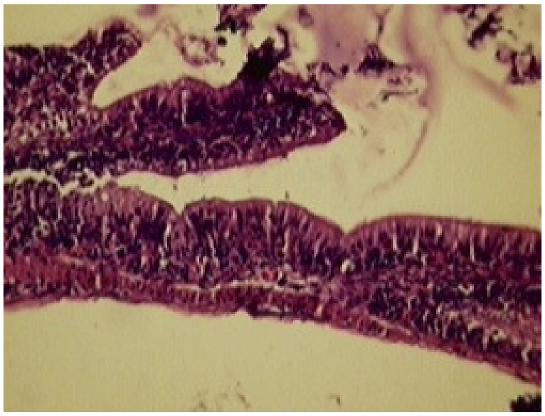


Plate 2: Histoarchitecture of normal intestinal tissue of *L. gonius* fingerlings at 27^0C (H&E 160X)

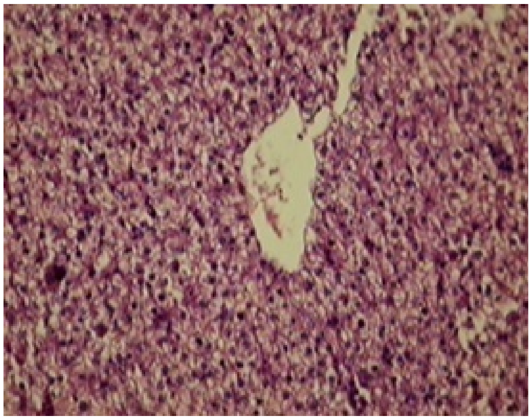


Plate 3: Histology of normal liver tissue of *L. gonius* fingerlings at 27^0C (H&E 40X)

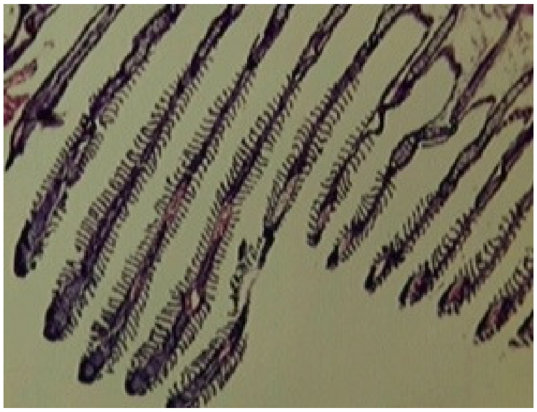


Plate 4: Gill tissues of *L. gonius* fingerlings at 30^0C showing normal architecture (H&E 40X)

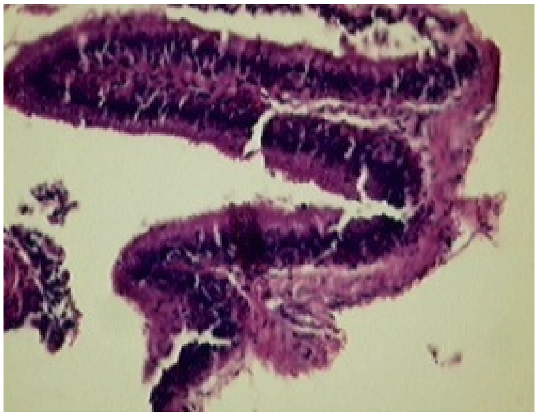


Plate 5: Histology of intestinal tissue of *L. gonius* fingerlings at 30^0C showing normal structure (H&E 160X)

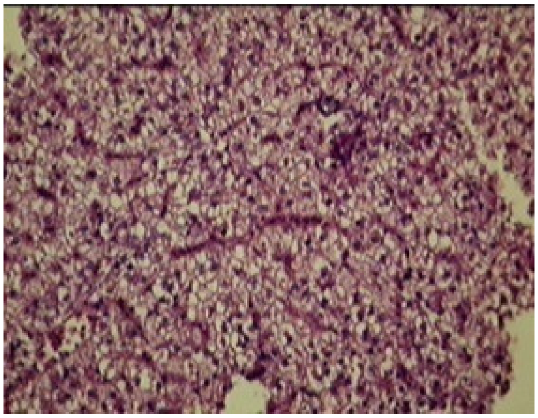


Plate 6: Histoarchitecture of normal liver tissue of *L. gonius* fingerlings at 30^0C (H&E 160X)

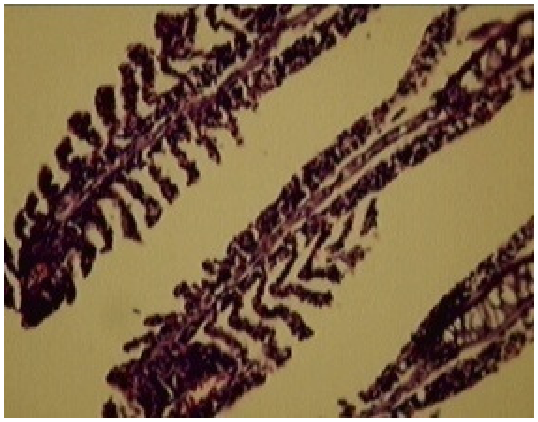


Plate 7: Gill tissue of *L. gonius* fingerlings showed loss of secondary lamellae in the lower half of the filament at 33^0C (H&E 160X)

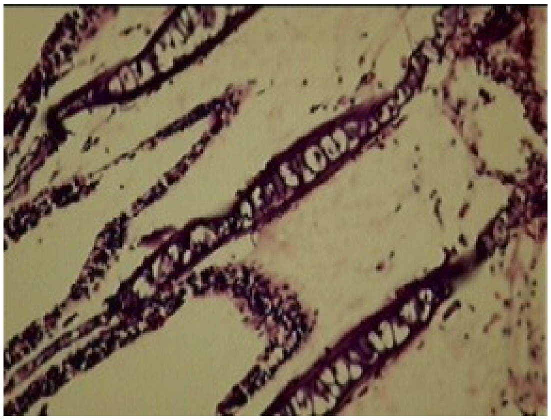


Plate 8: Gill tissue of *L. gonius* fingerlings showed marked loss of primary and secondary lamellae at 33^0C (H&E 160X)

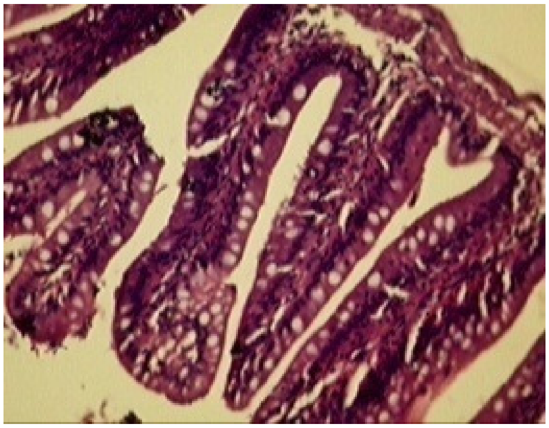


Plate 9: Intestinal tissue of *L. gonius* fingerlings showed mild loss of brush boarder at the mucosa at 33^0C (H&E 160X)

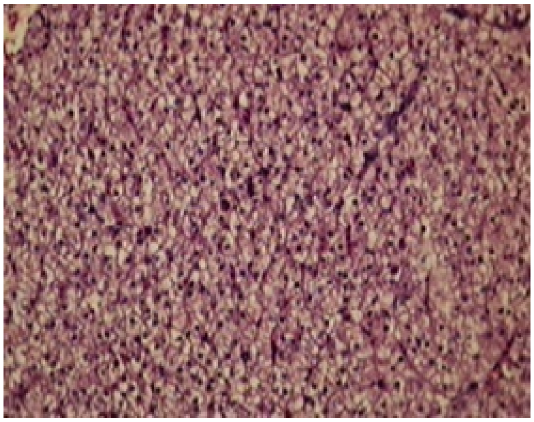


Plate 10: Liver tissue of *L. gonius* fingerlings showed diffuse vacuolation of the hepatocytes at 33^0C (H&E 160X)

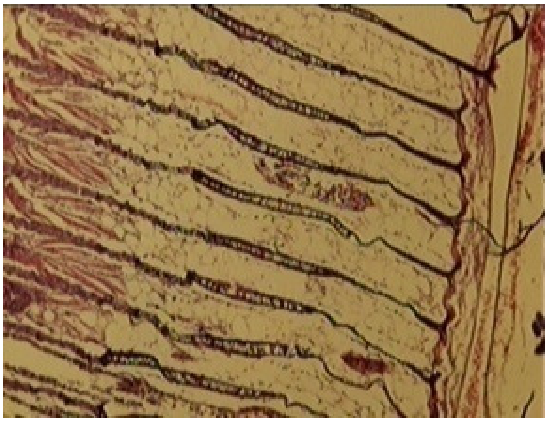


Plate 11: Gill tissue of *L. gonius* fingerlings showed extensive loss of secondary gill lamellae, atrophy of primary lamellae and congestion at the branchial arch at 36^0C (H&E 40X)

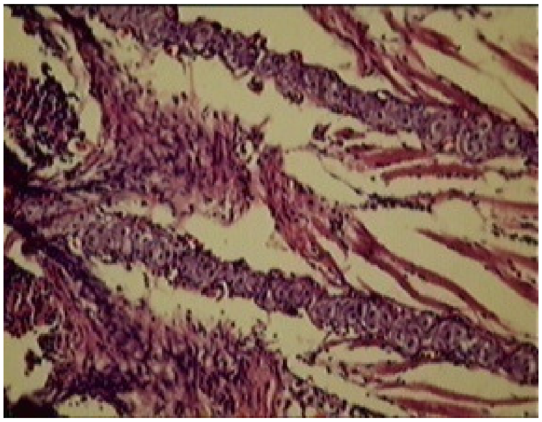


Plate 12: Gill tissue of *L. gonius* fingerlings showing severe damage of gill lamellae at 36^0C (H&E 160X)

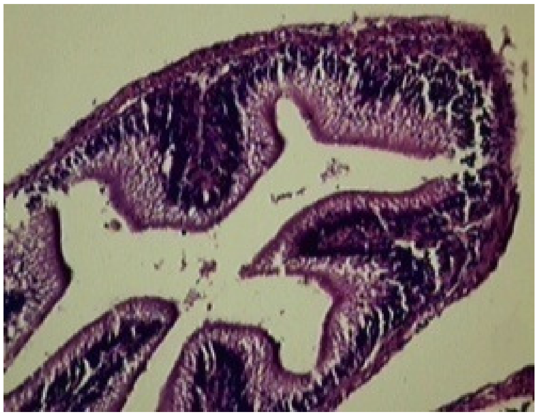


Plate 13: Intestinal tissue of *L. gonius* fingerlings showed degeneration of mucosa at some places and vacuolation in the lamina propria at 36^{0}C (H&E 160X)

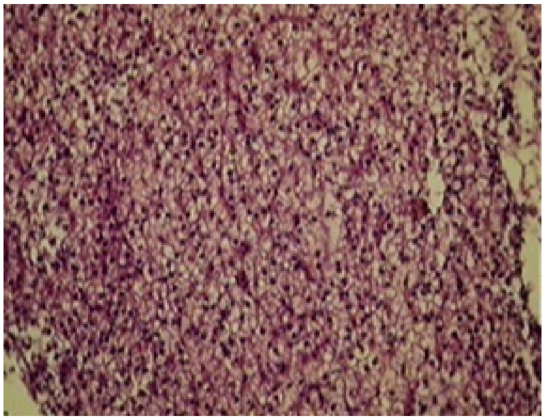


Plate 14: Liver tissue of *L. gonius* fingerlings showed extensive vacuolation at 36^{0}C (H&E 160X)

4.1.16 Scanning Electron Microscopy (SEM) study

4.1.16.1 Gill tissue

At 27^{0}C, SEM photograph (Magnification 400X) of the gill tissue of *L. gonius* fingerlings showed normal structure of primary and secondary gill lamella (Plate 15). Similarly 30^{0}C, SEM photograph (Magnification 500X) showed normal structure of primary and secondary gill lamella (Plate 16). At 33^{0}C, SEM photograph showed damaged of the primary lamella and loss of secondary lamellae alongside the damaged primary cells (Plate 17). In another SEM photograph at 33^{0}C (Magnification 350X), showed necrotic secondary lamellae appearing as shreds of tissue (Plate 18). Thin layers of netlike structure at the dark background are probably mucus was also noted. At 36^{0}C, SEM photograph (Magnification 200X) showed extensive loss of secondary lamellae and damaged of the primary lamellae at some places (Plate 19). In another SEM photograph (Magnification 200X) at 36^{0}C, severe damaged of the lamellar tissues were observed (Plate 20).

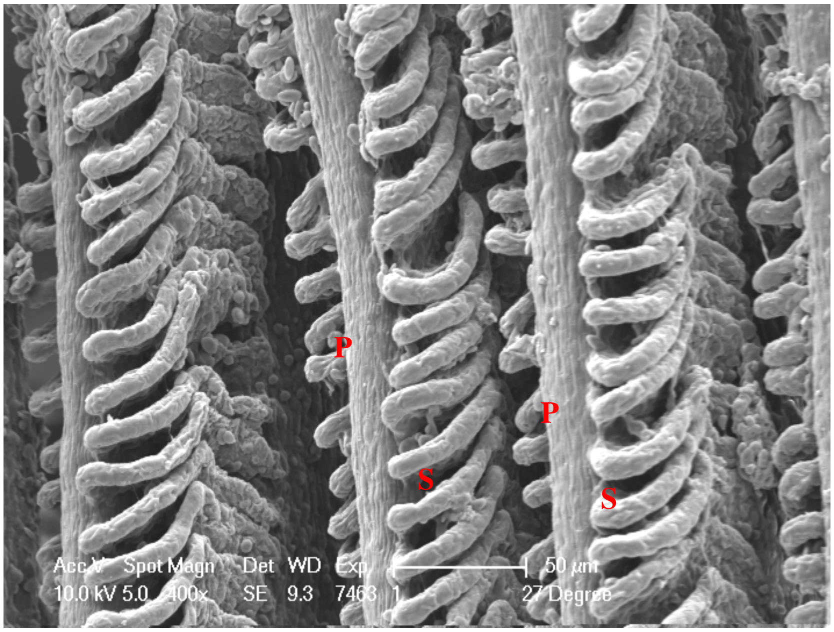


Plate 15: SEM photographs of the gill tissue of *L. gonius* fingerlings at 27^{0}C (Spot Magnification 400X) showing normal structure of gill (P- Primary lamella and S- Secondary lamella)

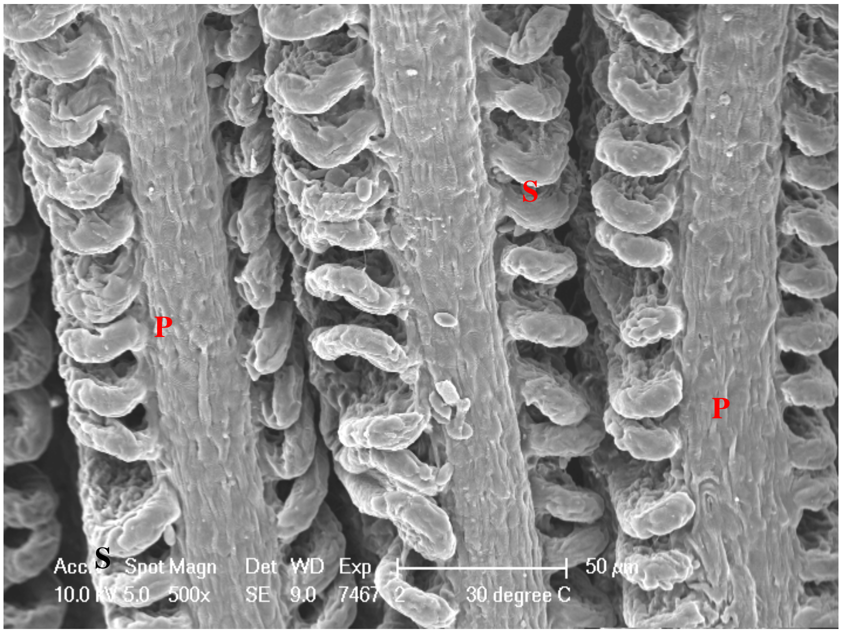


Plate 16: SEM photographs of the gill tissue of *L. gonius* fingerlings at 30^{0}C (Spot Magnification 500X) showing normal structure of gill (P- Primary lamella and S- Secondary lamella)

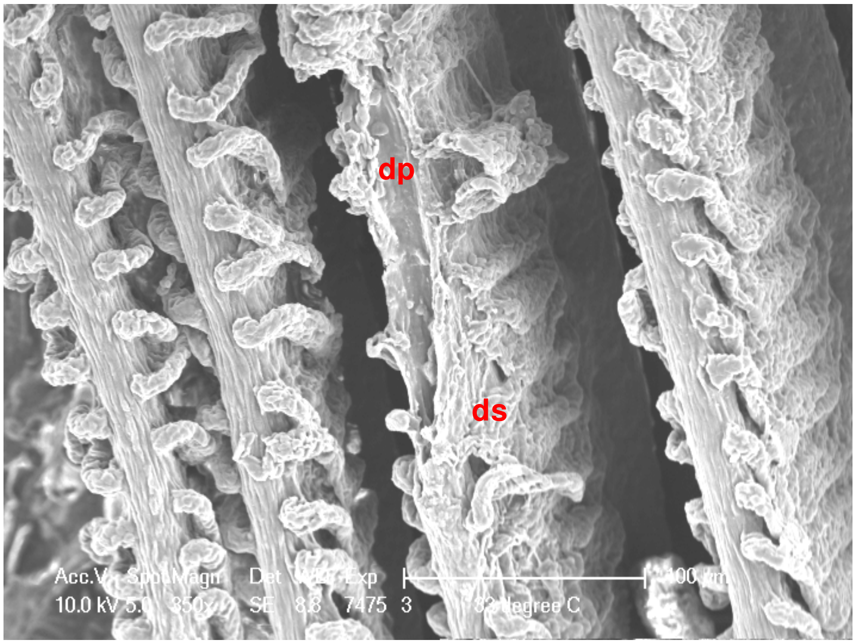


Plate 17: SEM photographs (Spot Magnification 350X) showed the gill structure of *L. gonius* fingerlings at 33^{0}C and ambient pH 7.5. Damaged of the primary lamella (dp). Observe the loss of secondary lamellae alongside the damaged primary cells (ds)

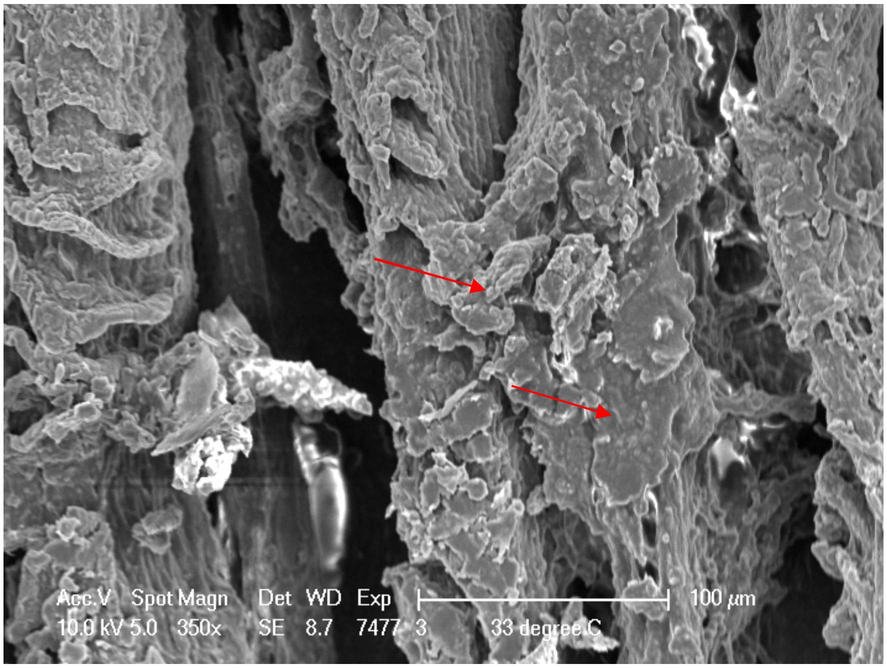


Plate 18: SEM photographs showed the gill structure of *L. gonius* fingerlings at 33^{0}C (350X). Observe the necrotic secondary lamellae appearing as shreds of tissue. Thin layers of netlike structure at the dark background are probably mucus

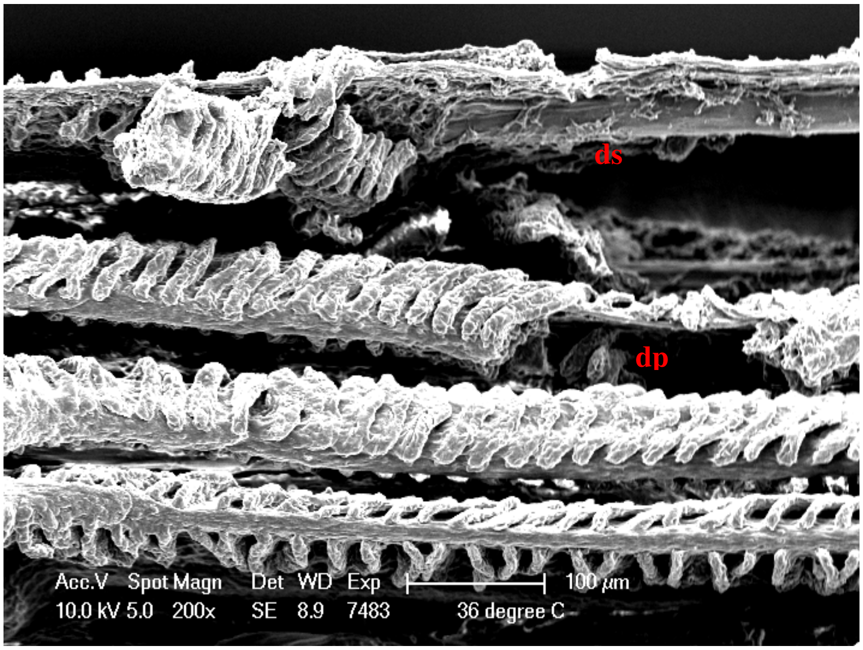


Plate 19: SEM photographs showed the gill structure of *L. gonius* fingerlings at 36^{0}C (Spot Magnification 200X). Observe the extensive loss of secondary gill lamellae (ds) and damaged of the primary lamellae at some places (dp)

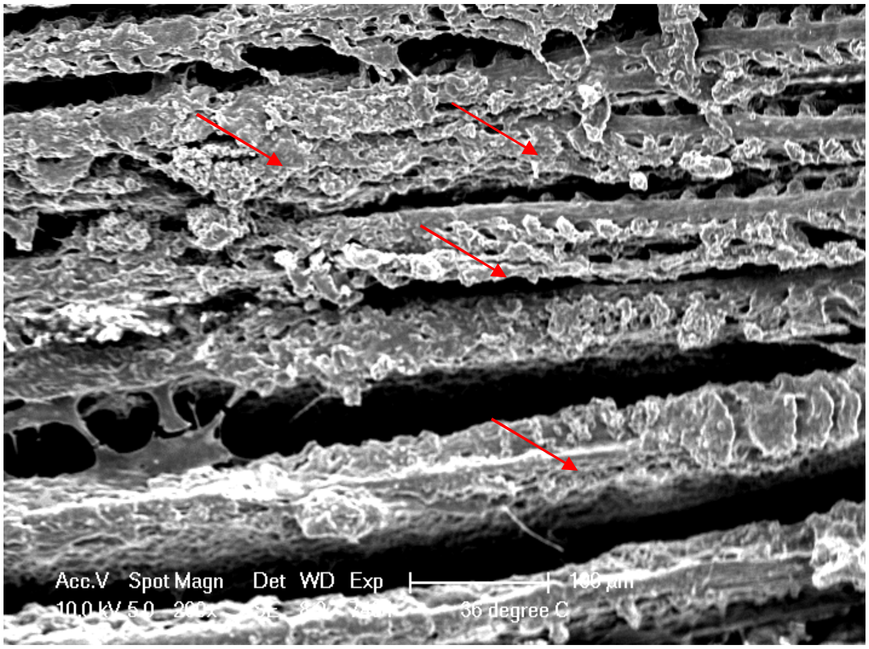


Plate 20: SEM photographs showed the gill structure of *L. gonius* fingerlings 36^{0}C (Spot Magnification 200X). Severe damaged of the lamellar tissues were observed

4.2 Experiment 2

4.2.1 Physico-Chemical Properties of Water

The physico- chemical parameters of water such as temperature (^{0}C), pH, dissolved oxygen (mg/L), free carbon dioxide (mg/L), ammonia (mg/L), Nitrite-N (mg/L), Nitrate-N (mg/L) were recorded and the average value of all the experimental groups are given in Table 19. The water temperature of the experimental groups was maintained at 27^{0}C in T_1, T_2 and T_3, whereas it was maintained at 33^{0}C in T_4, T_5 and T_6 during the experimental period of 60 days. The pH values of experimental groups were maintained at 7.5 in T_1, 5.5 in T_2 and 9.0 in T_3, whereas pH was maintained at 7.5 in T_4, 5.5 in T_5 and 9.0 in T_6 during the experimental period of 60 days.

The dissolved oxygen content of all the experimental tubs was recorded within the range of 5.85 to 7.21 mg/L. Free carbon dioxide (mg/L) was found to be negligible during the entire period. The total ammonia, nitrite- N and nitrate-N content was recorded before water exchange and found to be in the range of 0.15 to 0.29, 0.002 to 0.012 and 0.04 to 0.15 mg/L respectively.

Table 19: Water quality parameters in rearing tank during the 60 days of experimental period

Treatments	Dissolved oxygen	Ammonia- N	Nitrite-N	Nitrate-N
T_1	6.49-7.21	0.15- 0.18	0.002- 0.004	0.04-0.06
T_2	6.13-6.89	0.20- 0.26	0.005 - 0.009	0.07-0.13
T_3	6.25 -7.05	0.19- 0.23	0.005 - 0.007	0.05-0.10
T_4	6.23 -6.57	0.19- 0.24	0.004 - 0.007	0.06-0.09
T_5	5.85 -6.33	0.23- 0.29	0.008 - 0.012	0.13-0.15
T_6	5.92 -6.39	0.22- 0.28	0.008 - 0.011	0.12-0.14

Values expressed in range

Dissolved oxygen, Ammonia-N, Nitrite-N and Nitrate-N are expressed as mg/L

4.2.2 Growth Parameters

The body weight gain of *L. gonius* fingerlings exposed to multiple stressors was recorded at 15 days intervals is shown in Table 20 and Figure 7. The final body weight gain of the fingerlings differed significantly ($p<0.05$) in all the treatments. The highest final body weight was recorded in T_1 group and the lowest was in T_5 group followed by T_6 group. After 15 days of exposure, effects of stressors are more prominent in all the treatment groups.

The body weight gain % SGR, FCR, FER, PER and survival rate of *L. gonius* fingerlings exposed to multiple stressors are presented in Table 21. Body weight gain %, SGR, PER, FER and survival rate decreased significantly ($p<0.05$) in T_5 and T_6 treatment groups followed by T_2, T_4 and T_3 group. However, there was no significant difference ($p>0.05$) between T_3 and T_4 group. The highest body weight gain (%) was recorded in T_1 group and the lowest was in T_5 group followed by T_6 group. On the contrary, FCR shows an opposite trend. The effect of temperature and/or pH stress was significantly higher after 15 days of exposure.

Figure 7: Body weight (g) gain (at 15 days interval) of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

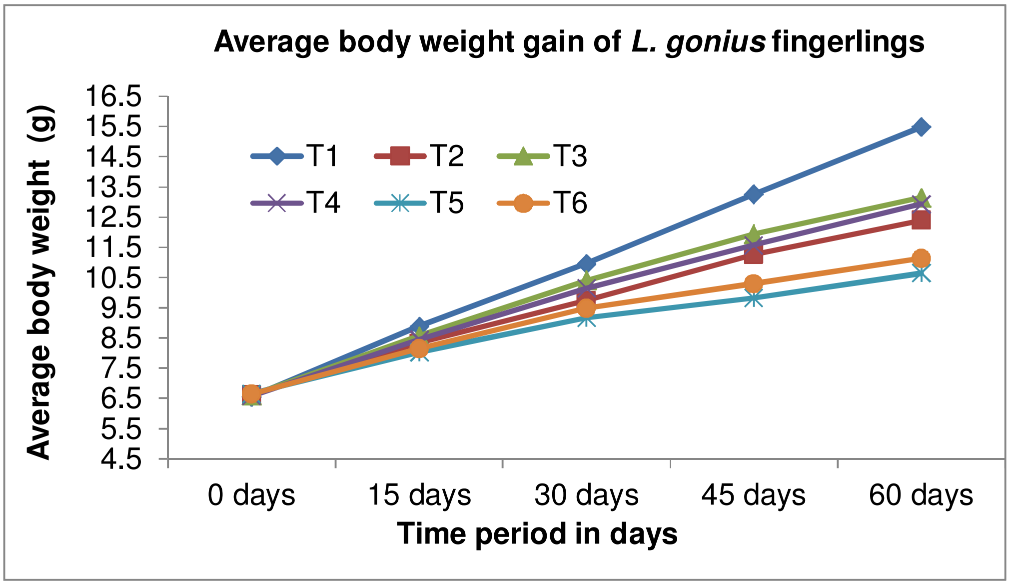


Table 20: Body weight (g) gain (at 15 days interval) of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

Treatment	Initial	0-15 days	0-30 days	0-45 days	0-60 days
T_1 (27^0C × pH 7.5)	$6.56^a \pm 0.09$	$8.89^c \pm 0.25$	$10.96^d \pm 0.19$	$13.26^d \pm 0.40$	$15.49^d \pm 0.28$
T_2 (27^0C × pH 5.5)	$6.62^a \pm 0.11$	$8.33^{ab} \pm 0.13$	$9.75^{bc} \pm 0.08$	$11.27^b \pm 0.10$	$12.40^b \pm 0.20$
T_3 (27^0C × pH 9.0)	$6.61^a \pm 0.10$	$8.57^{bc} \pm 0.08$	$10.41^c \pm 0.11$	$11.95^c \pm 0.18$	$13.15^c \pm 0.26$
T_4 (33^0C × pH 7.5)	$6.59^a \pm 0.08$	$8.45^{abc} \pm 0.11$	$10.15^c \pm 0.26$	$11.58^{bc} \pm 0.15$	$12.93^{bc} \pm 0.23$
T_5 (33^0C × pH 5.5)	$6.66^a \pm 0.11$	$8.04^a \pm 0.14$	$9.18^a \pm 0.14$	$9.84^a \pm 0.12$	$10.65^a \pm 0.19$
T_6 (33^0C × pH 9.0)	$6.64^a \pm 0.08$	$8.15^{ab} \pm 0.07$	$9.49^{ab} \pm 0.18$	$10.30^a \pm 0.10$	$11.14^a \pm 0.21$
P-Value	0.494	0.015	0.002	0.001	0.001

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=3 (each replicate is having 12 fish)

Table 21: Growth and survival of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

Treatments	Wt. Gain	FCR	FER	PER	SGR	Survival
Temperature						
27^{0}C	107.60^b±7.39	2.28^b±0.11	0.45^b±0.02	1.27^b±0.07	1.21^b±0.06	92.59^b±2.57
33^{0}C	74.65^a±5.55	3.05^a±0.17	0.34^a±0.02	0.95^a±0.06	0.92^a±0.05	78.70^a±3.14
pH						
7.5	116.26^b±9.01	2.13^b±0.13	0.48^b±0.03	1.35^b±0.08	1.27^b±0.07	94.44^c±2.78
5.5	73.72^a±6.24	3.07^a±0.21	0.34^a±0.02	0.94^a±0.06	0.92^a±0.06	79.17^a±3.57
9.0	83.40^a±7.02	2.80^a±0.19	0.37^a±0.02	1.03^a±0.07	1.01^a±0.06	83.33^{bc}±4.81
Temperature x pH						
T_1 (27^{0}C × pH 7.5)	136.32^e±1.08	1.84^e±0.01	0.54^e±0.01	1.53^e±0.01	1.43^e±0.01	100.0^c± 0.00
T_2 (27^{0}C × pH 5.5)	88.19^c±1.42	2.60^c±0.03	0.39^c±0.01	1.08^c±0.02	1.05^c±1.01	86.11^b ± 2.78
T_3 (27^{0}C × pH 9.0)	99.01^d±0.99	2.38^d±0.03	0.42^d±0.01	1.18^d±0.02	1.15^d±0.01	91.66^{bc} ± 4.81
T_4 (33^{0}C × pH 7.5)	96.71^d±1.22	2.41^{cd}±0.01	0.41^d±0.01	1.17^d±0.01	1.12^d±0.01	88.90^b ± 2.78
T_5 (33^{0}C × pH 5.5)	59.97^a±1.85	3.53^a±0.10	0.28^a±0.01	0.80^a±0.02	0.78^a±0.02	72.22^a ± 2.77
T_6 (33^{0}C × pH 9.0)	67.78^b±1.20	3.21^b±0.06	0.31^b±0.01	0.88^b±0.02	0.86^b±0.01	75.00^a ± 4.80

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=3 (each replicate is having 12 fish for growth parameters)

FCR: Feed conversion ratio; FER: Feed efficiency ratio;

PER: Protein efficiency ratio; SGR: Specific growth rate

Figure 8: Relationship between weight gain % and RNA content in muscle and liver tissue of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

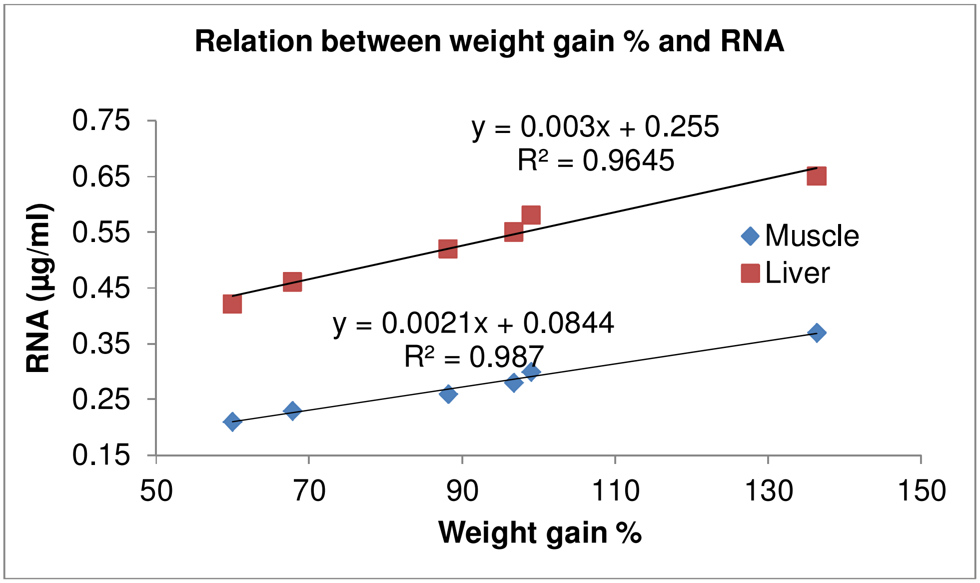
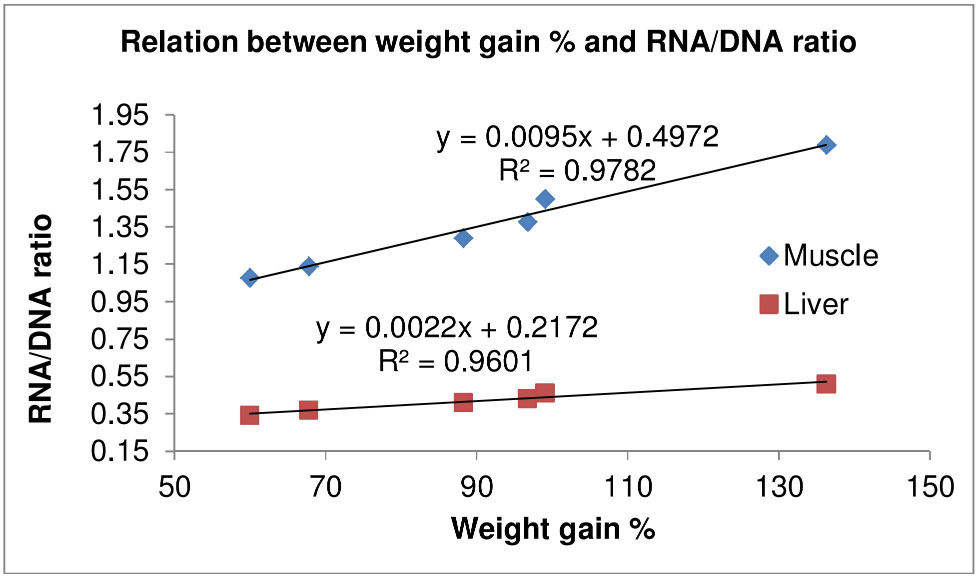


Figure 9: Relationship between weight gain % and RNA/DNA ratio in muscle and liver tissue of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days



4.2.3 DNA, RNA and RNA/DNA Ratio

DNA, RNA and RNA/DNA ratio in liver and muscle tissue of of *L. gonius* fingerlings exposed multiple stressors are presented in Table 22. DNA content in both the tissue did not vary significantly ($p>0.05$) among different treatments. RNA and RNA/DNA ratio in both the tissue decreased significantly ($p<0.05$) in T_5 and T_6 treatment groups followed by T_2, T_3 and T_4 group. However, there was no significant difference ($p>0.05$) between T_3 and T_4 group. The highest value was recorded in T_1 group and the lowest was in T_5 group followed by T_6 group. A linear relationship was observed between weight gain % and RNA, RNA/DNA ratio of *L. gonius* fingerlings (Figure 8 & 9).

4.2.4 Body Indices

Body indices (HSI and GSI) of *L. gonius* fingerlings exposed to multiple stressors are presented in Table 23. HSI value decreases decreased significantly ($p<0.05$) in T_5 and T_6 treatment groups followed by T_2, T_3 and T_4 group. However, GSI value did not vary significantly ($p>0.05$) among different treatments.

4.2.5 Tissue Glycogen

Glycogen content in liver and muscle tissues of *L. gonius* fingerlings exposed to multiple stressors is presented in Table 23. Glycogen content in liver and muscle tissue decreased significantly ($p<0.05$) in T_5 and T_6 treatment groups followed by T_2, T_3 and T_4 group. The highest value was recorded in T_1 group and the lowest was in T_5 group followed by T_6 group.

4.2.6 Ascorbic Acid

Ascorbic acid content in muscle tissue of *L. gonius* fingerlings exposed to multiple stressors is presented in Table 23. Ascorbic acid content in muscle tissue decreased significantly ($p<0.05$) in T_5 and T_6 treatment groups followed by T_2, T_3 and T_4 group. However, there was no significant difference ($p>0.05$) between T_3 and T_4 group. The highest value was recorded in T_1 group and the lowest was in T_5 group followed by T_6 group.

Table 22: DNA, RNA and RNA/DNA ratio of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

Treatments	Muscle			Liver		
	µg DNA/ml	µg RNA/ml	RNA/ DNA ratio	µg DNA/ml	µg RNA/ml	RNA/DNA ratio
Temperature						
27^{0}C	0.20^{a}±0.01	0.31^{b}±0.02	1.53^{b}±0.08	1.28^{a}±0.01	0.58^{b}±0.02	0.46^{b}±0.02
33^{0}C	0.20^{a}±0.01	0.24^{a}±0.01	1.20^{a}±0.05	1.27^{a}±0.01	0.48^{a}±0.02	0.38^{a}±0.02
pH						
7.5	0.21^{a}±0.01	0.33^{b}±0.02	1.58^{b}±0.09	1.28^{a}±0.01	0.60^{b}±0.02	0.47^{b}±0.02
5.5	0.20^{a}±0.01	0.24^{a}±0.02	1.18^{a}±0.05	1.27^{a}±0.01	0.47^{a}±0.02	0.31^{a}±0.02
9.0	0.20^{a}±0.01	0.27^{a}±0.02	1.31^{a}±0.08	1.27^{a}±0.01	0.52^{a}±0.03	0.41^{a}±0.02
Temperature x pH						
T$_1$ (27^{0}C × pH 7.5)	0.21^{a}±0.01	0.37^{e}±0.02	1.79^{d}±0.03	1.29^{a}±0.01	0.65^{e}±0.01	0.51^{e}±0.01
T$_2$ (27^{0}C × pH 5.5)	0.20^{a}±0.01	0.26^{bc}±0.01	1.29^{b}±0.01	1.28^{a}±0.01	0.52^{c}±0.01	0.41^{c}±0.01
T$_3$ (27^{0}C × pH 9.0)	0.19^{a}±0.01	0.30^{cd}±0.01	1.50^{c}±0.07	1.28^{a}±0.02	0.58^{d}±0.01	0.46^{d}±0.02
T$_4$ (33^{0}C × pH 7.5)	0.21^{a}±0.01	0.28^{d}±0.02	1.38^{b}±0.03	1.27^{a}±0.01	0.55^{d}±0.01	0.43^{cd}±0.02
T$_5$ (33^{0}C × pH 5.5)	0.20^{a}±0.01	0.21^{a}±0.01	1.08^{a}±0.01	1.26^{a}±0.01	0.42^{a}±0.01	0.34^{a}±0.01
T$_6$ (33^{0}C × pH 9.0)	0.21^{a}±0.01	0.23^{ab}±0.01	1.14^{a}±0.03	1.27^{a}±0.01	0.46^{b}±0.02	0.37^{b}±0.02

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6

Table 23: Glycogen, ascorbic acid content and body indices (HSI and GSI) of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

Treatments	Glycogen		Ascorbic acid	Body indices	
	Muscle	Liver	Muscle	HSI	GSI
Temperature					
27^{0}C	$1.01^{b}\pm0.06$	$9.8^{b}\pm0.82$	$101.09^{b}\pm8.06$	$1.26^{b}\pm0.07$	$2.77^{a}\pm0.10$
33^{0}C	$0.81^{a}\pm0.03$	$6.5^{a}\pm0.53$	$67.71^{a}\pm4.81$	$1.01^{a}\pm0.06$	$2.50^{a}\pm0.14$
pH					
7.5	$1.04^{b}\pm0.08$	$10.47^{b}\pm1.09$	$108.94^{b}\pm10.51$	$1.29^{b}\pm0.07$	$2.49^{a}\pm0.20$
5.5	$0.79^{a}\pm0.03$	$7.70^{a}\pm0.63$	$77.79^{a}\pm5.86$	$1.11^{a}\pm0.08$	$2.61^{a}\pm0.12$
9.0	$0.90^{ab}\pm0.05$	$6.30^{a}\pm0.75$	$66.48^{a}\pm6.37$	$1.02^{ab}\pm0.09$	$2.79^{a}\pm0.13$
Temperature x pH					
T$_1$ (27^{0}C × pH 7.5)	$1.20^{c}\pm0.05$	$12.67^{e}\pm0.86$	$132.30^{e}\pm2.38$	$1.38^{b}\pm0.10$	$2.94^{a}\pm0.19$
T$_2$ (27^{0}C × pH 5.5)	$0.84^{ab}\pm0.03$	$7.49^{bc}\pm0.48$	$79.30^{d}\pm2.31$	$1.12^{ab}\pm0.10$	$2.62^{a}\pm0.18$
T$_3$ (27^{0}C × pH 9.0)	$0.98^{b}\pm0.05$	$9.31^{d}\pm0.46$	$91.67^{c}\pm1.32$	$1.28^{b}\pm0.12$	$2.75^{a}\pm0.19$
T$_4$ (33^{0}C × pH 7.5)	$0.88^{ab}\pm0.07$	$8.26^{cd}\pm0.55$	$85.60^{c}\pm1.10$	$1.16^{ab}\pm0.07$	$2.65^{a}\pm0.36$
T$_5$ (33^{0}C × pH 5.5)	$0.75^{a}\pm0.04$	$5.12^{a}\pm0.61$	$53.65^{a}\pm1.53$	$0.91^{ab}\pm0.12$	$2.37^{a}\pm0.18$
T$_6$ (33^{0}C × pH 9.0)	$0.81^{a}\pm0.04$	$6.11^{ab}\pm0.28$	$63.89^{b}\pm2.87$	$0.94^{a}\pm0.10$	$2.36^{a}\pm0.17$

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6 (n=9 for body indices);

Glycogen: mg glycogen/g tissue; Ascorbic acid: microgram/g wet tissue;

Hepatosomatic Index (HSI) and Gastrosomatic Index (GSI) are expressed as %

4.2.7 Enzymatic Activity in Tissues

4.2.7.1 Enzymes of protein metabolism (AST and ALT)

AST and ALT activity in liver and muscle tissue of *L. gonius* fingerlings exposed to multiple stressors are presented in Table 24. AST and ALT activity in liver and muscle tissue increased significantly ($p<0.05$) in T_5 and T_6 treatment groups followed by T_2, T_3 and T_4 group. However, there was no significant difference ($p>0.05$) between T_3 and T_4 group. The lowest activity was recorded in T_1 group and the lowest was in T_5 group followed by T_6 group.

4.2.7.2 Enzymes of carbohydrate metabolism (LDH and MDH)

LDH and MDH activity in liver and muscle tissues of *L. gonius* fingerlings exposed to multiple stressors are presented in Table 25. The activity in liver and muscle tissue increased significantly ($p<0.05$) in T_5 and T_6 treatment groups followed by T_2, T_3 and T_4 group. However, there was no significant difference ($p>0.05$) between T_3 and T_4 group. The highest value was recorded in T_1 group and the lowest was in T_5 followed by T_6 group.

4.2.7.3 Enzyme of neurotransmission (AchE)

AchE activity in brain tissue of *L. gonius* fingerlings exposed to multiple stressors is presented in Table 26. AchE activity in brain tissue decreased significantly ($p<0.05$) in T_5 and T_6 treatment groups followed by T_2, T_3 and T_4 group. The highest activity was recorded in T_1 group and the lowest was in T_5 followed by T_6 group.

4.2.7.4 Enzymes of oxidative stress (Catalase, SOD and GST)

Catalase, SOD and GST activity in liver and gill tissue of *L. gonius* fingerlings exposed to multiple stressors are presented in Table 26. The activities in liver and gill tissue increased significantly ($p<0.05$) in T_5 and T_6 treatment groups followed by T_2, T_3 and T_4 group. However, there was no significant difference ($p>0.05$) between T_3 and T_4 group. The lowest value was recorded in T_1 group the highest was in T_5 group followed by T_6 group.

4.2.7.5 Enzyme of gluconeogenic pathways (FDPase)

FDPase activity in liver and gill tissue of *L. gonius* fingerlings exposed to multiple stressors is presented in Table 27. FDPase activity in liver and gill tissue increased significantly ($p<0.05$) in T_5 and T_6 treatment groups followed by T_2, T_3 and T_4 group. However, there was no significant difference ($p>0.05$) between T_3 and T_4 group. The lowest value was recorded in T_1 group and the highest was in T_5 group followed by T_6 group.

4.2.7.6 Enzyme of pentose phosphate pathway (G6PDH)

G6PDH activity in liver and muscle tissue of *L. gonius* fingerlings exposed to multiple stressors is presented in Table 27. G6PDH activity in liver and muscle tissue decreased significantly ($p<0.05$) in T_5 and T_6 treatment groups followed by T_2, T_3 and T_4 group. However, there was no significant difference ($p>0.05$) between T_2, T_3 and T_4 group. The highest activity was recorded in T_1 group and the lowest was in T_5 group followed by T_6 group.

4.2.7.7 Enzyme of energy metabolism (ATPase)

ATPase activity in gill and liver tissue of *L. gonius* fingerlings exposed to multiple stressors is presented in Table 27. ATPase activity in liver and gill tissue decreased significantly ($p<0.05$) in T_5 and T_6 treatment groups, followed by T_2 and T_3. However, there was no significant difference ($p>0.05$) between T_5 and T_2 group and T_6 and T_4. The highest activity was recorded in T_1 group and there was no significance difference between T_1 and T_4.

4.2.7.8 Enzyme of phospho ponoesterase (ALP)

ALP activity in liver, muscle and intestinal tissue of *L. gonius* fingerlings exposed to multiple stressors is presented in Table 28. ALP activity in muscle, liver and intestinal tissue increased significantly ($p<0.05$) in T_5 and T_6 treatment groups followed by T_2, T_3 and T_4 group. However, there was no significant difference ($p>0.05$) between T_3 and T_4 group. The lowest value was recorded in T_1 group and the highest was in T_5 group followed by T_6 group.

Table 24: AST and ALT enzymes activity in tissues of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

Treatments	AST		ALT	
	Liver	Muscle	Liver	Muscle
Temperature				
27 ^{0}C	25.35^{b}±2.83	30.28^{b}±2.84	8.83^{b}±1.15	11.66^{b}±1.40
33 ^{0}C	44.21^{a}±4.07	48.79^{a}±4.02	15.84^{a}±1.36	24.48^{a}±2.70
pH				
7.5	22.31^{b}±3.23	27.02^{b}±3.24	8.10^{a}±1.51	10.54^{b}±1.62
5.5	45.76^{a}±5.13	50.32^{a}±5.05	16.35^{a}±1.79	24.10^{a}±3.52
9.0	36.54a±4.32	41.28^{a}±4.29	12.57^{ab}±1.63	19.57^{ab}±3.57
Temperature x pH				
T_1 (27^{0}C × pH 7.5)	15.17^{e}±0.69	19.87^{e}±0.76	4.82^{e}±0.29	6.97^{e}±0.31
T_2 (27^{0}C × pH 5.5)	34.42^{c}±0.87	39.15^{c}±0.96	12.49^{c}±0.50	16.33^{c}±1.02
T_3 (27^{0}C × pH 9.0)	27.01^{d}±0.81	31.83d±0.75	9.19^{d}±0.88	11.69^{d}±0.59
T_4 (33^{0}C × pH 7.5)	29.46^{d}±0.89	34.17^{d}±0.93	11.38^{cd}±0.78	14.12^{d}±0.48
T_5 (33^{0}C × pH 5.5)	57.09^{a}±1.56	61.48^{a}±1.38	20.20^{a}±0.91	31.87^{a}±0.74
T_6 (33^{0}C × pH 9.0)	46.07^{b}±1.36	50.73^{b}±1.41	15.90^{b}±1.08	27.47^{b}±1.11

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6;

AST activity was expressed as n mol of oxaloacetate released/ min/ mg protein at 37^{o}C;

ALT activity was expressed as n mol of sodium pyruvate released/ min/ mg protein at 37^{o}C

Table 25: LDH and MDH enzymes activity in tissues of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

Treatments	LDH		MDH	
	Liver	Muscle	Liver	Muscle
Temperature				
27 ^{0}C	8.51^{b}±1.27	9.87^{b}±1.24	6.36^{b}±1.14	8.06^{b}±1.17
33 ^{0}C	14.37^{a}±1.16	15.97^{a}±1.25	11.75^{a}±1.04	13.82^{a}±1.11
pH				
7.5	7.06^{b}±1.39	8.45^{b}±1.37	5.14^{b}±1.31	6.96^{b}±1.39
5.5	15.10^{a}±1.22	16.81^{a}±1.40	12.23^{a}±1.23	14.51^{a}±1.38
9.0	12.15^{a}±1.49	13.49^{a}±1.47	9.80^{a}±1.29	11.36^{a}±1.27
Temperature x pH				
T$_1$ (27^{0}C × pH 7.5)	4.01^{e} ±0.16	5.44^{e} ±0.19	2.25^{e} ±0.11	3.90^{e} ±0.11
T$_2$ (27^{0}C × pH 5.5)	12.51^{c} ±0.71	13.79^{c} ±0.60	9.05^{c} ±0.46	11.58^{c}±0.79
T$_3$ (27^{0}C × pH 9.0)	9.01^{d} ±0.67	10.37^{d} ±0.82	7.13^{d} ±0.84	8.72^{d}±0.83
T$_4$ (33^{0}C × pH 7.5)	10.12^{d} ±0.50	11.47^{d} ±0.57	8.04^{d} ±0.37	10.02^{d}±0.57
T$_5$ (33^{0}C × pH 5.5)	17.69^{a} ±0.51	19.84^{a} ±0.52	14.75^{a}±0.80	17.44^{a}±0.60
T$_6$ (33^{0}C × pH 9.0)	15.29^{b} ±0.87	16.60^{b} ±0.66	12.46^{b}±0.72	14.01^{b}±0.64

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6;

LDH and MDH specifies activity expressed as Unit/min/mg protein at 37^{0}C

Table 26: AchE, catalase, SOD and GST enzyme activity in tissues of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

Treatments	AchE	Catalase		SOD		GST	
	Brain	Liver	Gill	Liver	Gill	Liver	Gill
Temperature							
27 ^{0}C	$5.18^{b}\pm0.27$	$5.91^{b}\pm0.65$	$10.04^{b}\pm0.93$	$16.79^{b}\pm1.67$	$31.89^{b}\pm1.88$	$0.509^{b}\pm0.04$	$0.618^{b}\pm0.04$
33 ^{0}C	$3.90^{a}\pm0.26$	$11.10^{a}\pm1.12$	$17.37^{a}\pm1.45$	$26.34^{a}\pm1.91$	$45.19^{a}\pm3.38$	$0.755^{a}\pm0.05$	$0.931^{a}\pm0.06$
pH							
7.5	$5.43^{b}\pm0.33$	$5.33^{b}\pm0.83$	$9.31^{b}\pm1.16$	$15.12^{b}\pm1.89$	$28.96^{b}\pm1.83$	$0.469^{b}\pm0.05$	$0.581^{b}\pm0.05$
5.5	$3.76^{a}\pm0.29$	$11.22^{a}\pm1.51$	$17.11^{a}\pm1.94$	$27.12^{a}\pm2.21$	$46.04^{a}\pm3.78$	$0.743^{a}\pm0.06$	$0.901^{a}\pm0.08$
9.0	$4.44^{a}\pm0.33$	$8.97^{ab}\pm1.24$	$14.71^{a}\pm1.93$	$22.39^{a}\pm2.46$	$40.63^{a}\pm3.77$	$0.685^{a}\pm0.05$	$0.843^{a}\pm0.08$
Temperature x pH							
T_1 (27^{0}C × pH 7.5)	$6.11^{d}\pm0.10$	$3.50^{e}\pm0.20$	$6.76^{e}\pm0.23$	$10.95^{e}\pm0.55$	$25.13^{e}\pm0.67$	$0.462^{f}\pm0.01$	$0.366^{f}\pm0.01$
T_2 (27^{0}C × pH 5.5)	$4.35^{b}\pm0.19$	$7.89^{c}\pm0.22$	$12.86^{c}\pm0.51$	$22.31^{c}\pm0.72$	$37.90^{c}\pm0.55$	$0.625^{c}\pm0.01$	$0.609^{c}\pm0.02$
T_3 (27^{0}C × pH 9.0)	$5.09^{c}\pm0.23$	$6.36^{d}\pm0.21$	$10.50^{d}\pm0.80$	$17.08^{d}\pm0.69$	$32.97^{d}\pm0.87$	$0.567^{e}\pm0.01$	$0.553^{e}\pm0.01$
T_4 (33^{0}C × pH 7.5)	$4.76^{bc}\pm0.29$	$7.15^{cd}\pm0.24$	$11.86^{cd}\pm0.41$	$19.31^{d}\pm0.59$	$33.79^{d}\pm0.43$	$0.599^{d}\pm0.01$	$0.572^{d}\pm0.01$
T_5 (33^{0}C × pH 5.5)	$3.18^{a}\pm0.17$	$14.55^{a}\pm0.46$	$21.38^{a}\pm0.63$	$32.02^{a}\pm0.73$	$50.83^{a}\pm1.14$	$1.077^{a}\pm0.02$	$0.877^{a}\pm0.02$
T_6 (33^{0}C × pH 9.0)	$3.78^{ab}\pm0.23$	$11.89^{b}\pm0.92$	$18.90^{b}\pm0.54$	$28.04^{b}\pm1.05$	$45.28^{b}\pm0.76$	$1.017^{b}\pm0.01$	$0.817^{b}\pm0.01$

Values in the same column with different superscript differ significantly ($p<0.05$); Data expressed as Mean ± SE, n=6;

AchE: micromoles acetylcholine hydrolyzed / mg protein / min at 37^0 C;

Catalase: Nano moles H_2O_2 decomposed / min / mg protein at 37^{0}C;

SOD: micromole /mg protein/min at 37 ^{0}C; GST: units /mg protein

Table 27: FDPase, G6PDH and ATPase enzyme activity in tissues of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

Treatments	FDPase		G6PDH		ATPase	
	Liver	Gill	Liver	Muscle	Liver	Gill
Temperature						
27 ^{0}C	$22.22^{b}\pm3.54$	48.95 ± 5.44	$9.14^{b}\pm0.75$	$5.12^{b}\pm0.48$	$1.96^{a}\pm0.22$	$4.08^{a}\pm0.30$
33 ^{0}C	$39.35^{a}\pm3.25$	82.45 ± 6.19	$7.07^{a}\pm0.41$	$3.56^{a}\pm0.32$	$1.99^{a}\pm0.26$	$4.11^{a}\pm0.44$
pH						
7.5	$18.50^{b}\pm4.21$	$44.04^{b}\pm6.83$	$9.93^{b}\pm0.77$	$5.51^{b}\pm0.57$	$3.07^{b}\pm0.11$	$5.69^{b}\pm0.24$
5.5	$40.42^{a}\pm3.83$	$81.64^{a}\pm8.16$	$6.56^{a}\pm0.52$	$3.46^{a}\pm0.37$	$1.36^{a}\pm0.05$	$3.10^{a}\pm0.16$
9.0	$33.44^{a}\pm4.10$	$71.40^{a}\pm8.19$	$7.72^{a}\pm0.64$	$4.04^{a}\pm0.52$	$1.49^{a}\pm0.05$	$3.49^{a}\pm0.10$
Temperature x pH						
T$_1$ (27^{0}C × pH 7.5)	$9.15^{e}\pm0.54$	$28.97^{e}\pm1.03$	$11.51^{c}\pm0.34$	$6.59^{c}\pm0.41$	$2.86^{c}\pm0.09$	$5.25^{c}\pm0.27$
T$_2$ (27^{0}C × pH 5.5)	$32.40^{c}\pm2.63$	$64.34^{c}\pm4.21$	$7.07^{ab}\pm1.04$	$3.88^{ab}\pm0.58$	$1.47^{ab}\pm0.04$	$3.38^{ab}\pm0.12$
T$_3$ (27^{0}C × pH 9.0)	$25.10^{d}\pm1.32$	$53.53^{d}\pm2.81$	$8.83^{b}\pm0.81$	$4.90^{b}\pm0.65$	$1.58^{b}\pm0.04$	$3.62^{b}\pm0.12$
T$_4$ (33^{0}C × pH 7.5)	$27.84^{cd}\pm1.14$	$59.12^{cd}\pm2.22$	$8.36^{ab}\pm0.62$	$4.44^{ab}\pm0.53$	$3.29^{c}\pm0.09$	$6.13^{c}\pm0.10$
T$_5$ (33^{0}C × pH 5.5)	$48.44^{a}\pm1.40$	$98.96^{a}\pm3.97$	$6.26^{a}\pm0.42$	$3.04^{a}\pm0.41$	$1.28^{a}\pm0.05$	$2.83^{a}\pm0.22$
T$_6$ (33^{0}C × pH 9.0)	$41.77^{b}\pm3.58$	$89.29^{b}\pm2.72$	$6.61^{a}\pm0.38$	$3.17^{a}\pm0.43$	$1.39^{a}\pm0.02$	$3.35^{ab}\pm0.15$

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6;

FDPase: microgram phosphorous released / mg protein / minute at 37^{0}C;

G6PDH: Δ 0.01 optical density / mg protein / minute at 37^{0}C;

ATPase: microgram phosphorous released / mg protein / minute at 37^{0}C

4.4.7.9 Digestive enzyme (Protease)

Protease enzyme activity in intestinal tissue of *L. gonius* fingerlings exposed to multiple stressors is presented in Table 28. Protease and lipase activity in intestinal tissue decreased significantly ($p<0.05$) in T_5 and T_6 treatment groups followed by T_2, T_3 and T_4 group. However, there was no significant difference ($p>0.05$) between T_3 and T_4 group. The highest activity was recorded in T_1 group and the lowest was in T_5 group followed by T_6 group. Amylase does not show any significance difference.

Table 28: ALP and Protease enzymes activity in tissues of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

Treatments	ALP		Protease
	Muscle	Liver	Intestine
Temperature			
27 ^{0}C	$25.63^{b}\pm3.75$	$58.70^{b}\pm7.52$	$15.72^{b}\pm0.71$
33 ^{0}C	$46.60^{a}\pm5.38$	$97.34^{a}\pm8.14$	$13.39^{a}\pm0.32$
pH			
7.5	$18.81^{b}\pm3.42$	$47.99^{b}\pm8.55$	$16.21^{b}\pm0.92$
5.5	$48.41^{a}\pm5.72$	$99.17^{a}\pm10.10$	$13.06^{a}\pm0.33$
9.0	$41.12^{a}\pm5.43$	$86.89^{a}\pm7.60$	$14.40^{ab}\pm0.56$
Temperature x pH			
T_1 (27^{0}C × pH 7.5)	$11.41^{e}\pm0.84$	$29.08^{e}\pm2.10$	$18.11^{d}\pm0.70$
T_2 (27^{0}C × pH 5.5)	$36.13^{c}\pm1.83$	$76.88^{c}\pm1.84$	$13.67^{ab}\pm0.27$
T_3 (27^{0}C × pH 9.0)	$29.36^{d}\pm1.10$	$70.14^{d}\pm1.12$	$15.38^{c}\pm0.69$
T_4 (33^{0}C × pH 7.5)	$26.21^{d}\pm1.69$	$66.91^{cd}\pm1.71$	$14.31^{bc}\pm0.35$
T_5 (33^{0}C × pH 5.5)	$60.70^{a}\pm3.09$	$121.48^{a}\pm3.09$	$12.44^{a}\pm0.31$
T_6 (33^{0}C × pH 9.0)	$52.88^{b}\pm2.79$	$107.63^{b}\pm2.74$	$13.42^{ab}\pm0.34$

Values in the same column with different superscript differ significantly ($p<0.05$); Data expressed as Mean ± SE, n=6;

Protease activity as mmole of tryosine released/min/g protein at 37^{0}C;

ALP: nanomoles P-nitrophenol released / mg protein / minute at 37^{0}C

4.2.8 Haemato-immunological Parameters

4.2.8.1 RBC, WBC, haemoglobin (Hb) and haematocrit (Hct)

RBC, WBC, Hb and Hct of *L. gonius* fingerlings exposed to multiple stressors are presented in Table 29. The counts decreased significantly ($p<0.05$) in

T_5 and T_6 treatment groups followed by T_2, T_3 and T_4 group. However, there was no significant difference ($p>0.05$) between T_3 and T_4 group. The higher value was recorded in T_1 group and the lowest was in T_5 followed by T_6 group.

4.2.8.2 Respiratory burst activity (NBT)

NBT activity of *L. gonius* fingerlings exposed to multiple stressors is presented in Table 29. NBT activity decreased significantly ($p<0.05$) in T_5 and T_6 treatment groups followed by T_2, T_3 and T_4 group. However, there was no significant difference ($p>0.05$) between T_3 and T_4 group. The lowest value was recorded in T_1 group and highest was in T_5 group followed by T_6 group.

4.2.8.3 Blood glucose

Blood glucose level of *L. gonius* fingerlings exposed to multiple stressors is presented in Table 29. Blood glucose level increased significantly ($p<0.05$) in T_5 and T_6 treatment groups followed by T_2, T_3 and T_4 group. However, there was no significant difference ($p>0.05$) between T_3 and T_4 group. The lowest value was recorded in T_1 group and highest was in T_5 group followed by T_6 group.

4.2.8.4 Total serum protein, albumin (A), globulin (G) and A-G ratio

Total serum protein, albumin, globulin and A-G ratio of *L. gonius* fingerlings exposed to multiple stressors are presented in Table 30. Total serum protein, albumin and globulin decreased significantly ($p<0.05$) in T_5 and T_6 followed by T_2, T_3 and T_4 group. However, there was no significant difference ($p>0.05$) between T_3 and T_4 group. The higher value was recorded in T_1 group and the lowest was in T_5 followed by T_6 group. On the contrary, A-G ratio shows an opposite trend.

4.2.8.5 Serum lysozyme activity

Serum lysozyme activity of *L. gonius* fingerlings exposed to multiple stressors is presented in Table 30. The activity decreased significantly ($p<0.05$) in T_5 and T_6 treatment groups followed by T_2, T_3 and T_4 group. However, there was no significant difference ($p>0.05$) between T_3 and T_4 group. The higher value was recorded in T_1 group and the lowest was in T_5 group followed by T_6 group.

Table 29: Total leucocytes (WBC), total erythrocyte (RBC), hemoglobin (Hb), haematocrit (Hct), NBT and blood glucose of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

Treatments	WBC (10^{3}cells/mm^{3})	RBC (10^{6}cells/mm^{3})	Hb (g/dL)	Hct (%)	NBT (A$_{540}$)	Blood glucose (mg/100mL)
Temperature						
27 ^{0}C	193.21^{b}±7.22	1.83^{b}±0.05	8.03^{b}±0.53	22.27^{b}±1.77	0.261^{b}±0.02	48.51^{b}±2.66
33 ^{0}C	163.57^{a}±5.10	1.56^{a}±0.05	6.18^{a}±0.24	15.13^{a}±0.88	0.190^{a}±0.01	62.60^{a}±2.42
pH						
7.5	201.07^{b}±8.84	1.87^{b}±0.07	8.58^{b}±0.68	23.82b±2.38	0.279^{b}±0.02	45.55^{b}±3.39
5.5	161.46^{a}±5.65	1.55^{a}±0.06	6.10^{a}±0.27	15.18a±1.09	0.189^{a}±0.01	61.70^{a}±3.10
9.0	172.64^{a}±6.52	1.66^{a}±0.06	6.64^{a}±0.32	17.10a±1.39	0.209^{a}±0.01	59.42^{a}±3.03
Temperature x pH						
T$_1$ (27^{0}C × pH 7.5)	220.17d±3.51	2.01^{d}±0.05	10.10^{d}±0.32	29.11^{d}±0.52	0.325^{d}±0.01	38.01^{e}±0.45
T$_2$ (27^{0}C × pH 5.5)	172.63b±3.83	1.67^{b}±0.04	6.68^{b}±0.11	17.55^{b}±0.44	0.217^{b}±0.01	54.88^{c}±0.89
T$_3$ (27^{0}C × pH 9.0)	186.82c±1.80	1.79c±0.03	7.33^{c}±0.08	20.14^{c}±0.59	0.241^{c}±0.01	52.66^{d}±0.36
T$_4$ (33^{0}C × pH 7.5)	181.97bc±3.75	1.73bc±0.02	7.09^{bc}±0.10	18.53^{b}±0.25	0.232^{bc}±0.01	53.10^{cd}±0.62
T$_5$ (33^{0}C × pH 5.5)	150.29a±4.46	1.43a±0.05	5.51^{a}±0.06	12.81^{a}±0.34	0.161^{a}±0.01	68.53^{a}±0.76
T$_6$ (33^{0}C × pH 9.0)	158.46a±2.84	1.52a±0.04	5.94^{a}±0.08	14.05^{a}±0.08	0.177^{a}±0.01	66.18^{b}±0.41

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6;

NBT (Respiratory burst activity) expressed in optimal density at 540 nm

Table 30: Total protein, albumin, globulin, A/G ratio and serum lysozyme activity of *L. gonius* fingerling exposed to multiple stressors for a period of 60 days

Treatments	Total protein (g/dL)	Albumin (A) (g/dL)	Globulin (G) (g/dL)	A/G Ratio	Serum lysozyme
Temperature					
27 ^{0}C	$3.18^{b}\pm0.21$	$0.76^{b}\pm0.03$	$2.41^{b}\pm0.18$	$0.32^{b}\pm0.01$	$299.45^{b}\pm23.33$
33 ^{0}C	$2.20^{a}\pm0.16$	$0.61^{a}\pm0.03$	$1.59^{a}\pm0.14$	$0.39^{a}\pm0.02$	$210.10^{a}\pm10.99$
pH					
7.5	$3.39^{b}\pm0.26$	$0.79^{b}\pm0.04$	$2.61^{b}\pm0.22$	$0.31^{b}\pm0.01$	$320.89^{b}\pm31.53$
5.5	$2.17^{a}\pm0.20$	$0.61^{a}\pm0.03$	$1.57^{a}\pm0.16$	$0.40^{a}\pm0.02$	$209.75^{a}\pm13.87$
9.0	$2.51^{a}\pm0.21$	$0.67^{a}\pm0.03$	$1.84^{a}\pm0.17$	$0.37^{a}\pm0.02$	$233.68^{a}\pm15.43$
Temperature x pH					
T_1 (27^{0}C × pH 7.5)	$3.96^{e}\pm0.09$	$0.86^{e}\pm0.01$	$3.10^{e}\pm0.09$	$0.28^{d}\pm0.01$	$390.74^{e}\pm7.85$
T_2 (27^{0}C × pH 5.5)	$2.60^{c}\pm0.06$	$0.68^{c}\pm0.01$	$1.92^{c}\pm0.05$	$0.36^{c}\pm0.01$	$240.09^{c}\pm4.72$
T_3 (27^{0}C × pH 9.0)	$2.96^{d}\pm0.08$	$0.75^{d}\pm0.01$	$2.22^{d}\pm0.07$	$0.33^{c}\pm0.01$	$267.52^{d}\pm2.55$
T_4 (33^{0}C × pH 7.5)	$2.82^{d}\pm0.05$	$0.71^{c}\pm0.01$	$2.12^{d}\pm0.03$	$0.34^{c}\pm0.01$	$251.04^{cd}\pm5.48$
T_5 (33^{0}C × pH 5.5)	$1.74^{a}\pm0.07$	$0.53^{a}\pm0.01$	$1.21^{a}\pm0.06$	$0.44^{a}\pm0.02$	$179.40^{a}\pm4.36$
T_6 (33^{0}C × pH 9.0)	$2.05^{b}\pm0.06$	$0.59^{b}\pm0.01$	$1.46^{b}\pm0.05$	$0.41^{b}\pm0.01$	$199.82^{b}\pm6.21$

Values in the same column with different superscript differ significantly ($p<0.05$); Data expressed as Mean ± SE, n=6

Serum lysozyme activity is expressed as unit/ min/ mg serum protein

Table 31: Serum enzyme activity of *L. gonius* fingerling exposed to multiple stressors for a period of 60 days

Treatments	sGOT (IU/L)	sGPT (IU/L)	sALP (IU/L)	sACP (IU/L)	sLDH (IU/L)
Temperature					
27 ^{0}C	36.03^{b}±2.84	13.92^{b}±1.41	122.08^{b}±7.51	29.08^{b}±2.40	11.47^{b}±1.30
33 ^{0}C	54.72^{a}±4.07	26.94^{a}±2.70	160.71^{a}±8.12	38.05^{a}±2.07	19.55^{a}±1.72
pH					
7.5	32.78^{b}±3,24	12.92^{b}±1.63	111.42^{b}±8.54	25.07^{b}±2.47	10.03^{b}±1.39
5.5	56.29^{a}±5.14	26.53^{a}±3.56	162.54^{a}±10.10	39.50^{a}±2.04	20.09^{a}±2.03
9.0	47.05^{a}±4.31	21.81^{a}±3.66	150.23^{a}±7.61	36.13^{a}±1.74	16.42^{a}±2.13
Temperature x pH					
T_1 (27^{0}C × pH 7.5)	25.61±0.76^{e}	09.34±0.48^{e}	92.49±1.69^{e}	19.76±1.01^{e}	6.97±0.11^{e}
T_2 (27^{0}C × pH 5.5)	44.93±0.94^{c}	18.68±0.70^{c}	140.26±3.09^{c}	35.05±0.62^{c}	15.67±0.79^{c}
T_3 (27^{0}C × pH 9.0)	37.53±0.86^{d}	13.74±1.14^{d}	141.40±2.78^{cd}	32.44±0.81^{cd}	11.78±0.83^{d}
T_4 (33^{0}C × pH 7.5)	39.95±0.91^{d}	16.56±0.28^{c}	130.33±2.10^{d}	30.37±1.20^{d}	13.09±0.56^{d}
T_5 (33^{0}C × pH 5.5)	67.65±1.65^{a}	34.38±1.09^{a}	184.83±1.84^{a}	43.94±0.88^{a}	25.15±0.60^{a}
T_6 (33^{0}C × pH 9.0)	56.56±1.32^{b}	29.87±0.84^{b}	166.98±1.10^{b}	39.83±0.89^{b}	21.06±0.64^{b}

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6

Table 32: Hsp-70 expression, cortisol and caspase-3 activity in *L. gonius* fingerling exposed to multiple stressors for a period of 60 days

Treatments	Hsp-70 expression (ng/mL)				Cortisol (ng/mL)	Caspase-3
	Muscle	Gill	Liver	Serum	Serum	Serum
Temperature						
27 ^{0}C	$1.20^{a}\pm0.29$	$4.02^{b}\pm0.44$	$5.01^{b}\pm0.56$	$12.95^{b}\pm1.89$	$140.96^{b}\pm11.22$	$21.92^{b}\pm1.18$
33 ^{0}C	$1.27^{a}\pm0.37$	$6.12^{a}\pm0.38$	$8.08^{a}\pm0.58$	$21.71^{a}\pm1.56$	$181.02^{a}\pm5.58$	$30.71^{a}\pm1.17$
pH						
7.5	$1.18^{a}\pm0.04$	$3.77^{b}\pm0.58$	$4.54^{b}\pm0.66$	$10.96^{b}\pm2.28$	$129.61^{b}\pm14.12$	$20.94^{b}\pm1.54$
5.5	$1.28^{a}\pm0.04$	$6.16^{a}\pm0.40$	$8.13^{a}\pm0.74$	$22.41^{a}\pm1.89$	$184.99^{a}\pm6.15$	$30.27^{a}\pm2.18$
9.0	$1.25^{a}\pm0.05$	$5.28^{a}\pm0.62$	$6.97^{a}\pm0.80$	$18.61^{a}\pm1.89$	$160.89^{a}\pm7.59$	$27.73^{a}\pm2.28$
Temperature x pH						
T_1 (27^{0}C × pH 7.5)	$1.13^{a}\pm0.03$	$2.52^{d}\pm0.05$	$3.08^{d}\pm0.16$	$5.88^{e}\pm0.28$	$98.16^{e}\pm1.93$	$17.55^{e}\pm0.39$
T_2 (27^{0}C × pH 5.5)	$1.25^{a}\pm0.06$	$5.40^{bc}\pm0.26$	$6.63^{b}\pm0.51$	$18.51^{c}\pm1.11$	$172.40^{c}\pm4.01$	$25.33^{c}\pm0.48$
T_3 (27^{0}C × pH 9.0)	$1.22^{a}\pm0.04$	$4.14^{c}\pm0.38$	$5.30^{c}\pm0.45$	$14.45^{d}\pm0.34$	$152.33^{d}\pm4.04$	$22.69^{d}\pm0.24$
T_4 (33^{0}C × pH 7.5)	$1.24^{a}\pm0.05$	$5.02^{c}\pm0.37$	$5.99^{bc}\pm0.22$	$16.06^{d}\pm0.37$	$161.06^{d}\pm1.89$	$24.34^{d}\pm0.31$
T_5 (33^{0}C × pH 5.5)	$1.31^{a}\pm0.06$	$6.91^{a}\pm0.42$	$9.62^{a}\pm0.50$	$26.29^{a}\pm1.25$	$197.57^{a}\pm3.82$	$35.01^{a}\pm1.01$
T_6 (33^{0}C × pH 9.0)	$1.27^{a}\pm0.04$	$6.43^{ab}\pm0.69$	$8.64^{a}\pm0.43$	$22.78^{b}\pm0.69$	$184.44^{b}\pm3.69$	$32.77^{b}\pm0.74$

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6;

Caspase-3 enzyme activity is expressed as p-NA/hr/mg of protein

4.2.9 Serum Enzyme Activity

Serum GOT, GPT, ALP, ACP and LDH enzyme activity of *L. gonius* fingerlings exposed to multiple stressors are presented in Table 31. The values increased significantly ($p<0.05$) in T_5 and T_6 treatment groups followed by T_2, T_3 and T_4 group. However, there was no significant difference ($p>0.05$) between T_3 and T_4 group. The lowest value was recorded in T_1 group and the highest level was in T_5 group followed by T_6 group.

4.2.10 Serum Cortisol

Cortisol level of *L. gonius* fingerlings exposed to multiple stressors is presented in Table 32. The cortisol level in serum was significantly higher ($p<0.05$) in T_5 and T_6 treatment group followed by T_2, T_3 and T_4 group. However, there was no significant difference ($p>0.05$) between T_3, T_4 and T_5 group. The lowest cortisol value was recorded in T_1 group. The highest value was in T_5 followed by T_6 group.

4.2.11 Hsp-70 Expression

The quantitative expression of Hsp-70 in serum, liver and gill tissue of *L. gonius* fingerlings exposed to multiple stressors is presented in Table 32. The Hsp-70 expression was significantly higher ($p<0.05$) in T_5 and T_6 treatment group followed by T_2, T_3 and T_4 group. The lowest expression was recorded in T_1 group.

4.2.12 Caspase-3 Activity

Caspase-3 activity of *L. gonius* fingerlings exposed multiple stressors is presented in Table 32. Caspase-3 activity in serum was significantly higher ($p<0.05$) in T_5 and T_6 treatment group followed by T_2, T_3 and T_4 group. The lowest caspase-3 activity was recorded in T_1 group. The highest caspase-3 activity was recorded in T_5 group followed by T_6 group.

4.2.13 Thyroid Hormones (T3 and T4)

Thyroid hormones (T3 and T4) levels of *L. gonius* fingerlings exposed multiple stressors are presented in Table 33. The T3 and T4 hormone levels in serum decreased significantly ($p<0.05$) in T_5 and T_6 treatment groups followed by T_2,

T_3 and T_4 group. The higher level T3 and T4 hormone was recorded in T_1 group. The lowest level was recorded in T_5 followed by T_6 group. A linear relationship was observed between weight gain % and thyroid hormone levels (Figure 10 & 11).

Figure 10: Relationship between weight gain % and T3 hormone in serum of *L. gonius* fingerlings exposed multiple stressors for a period of 60 days

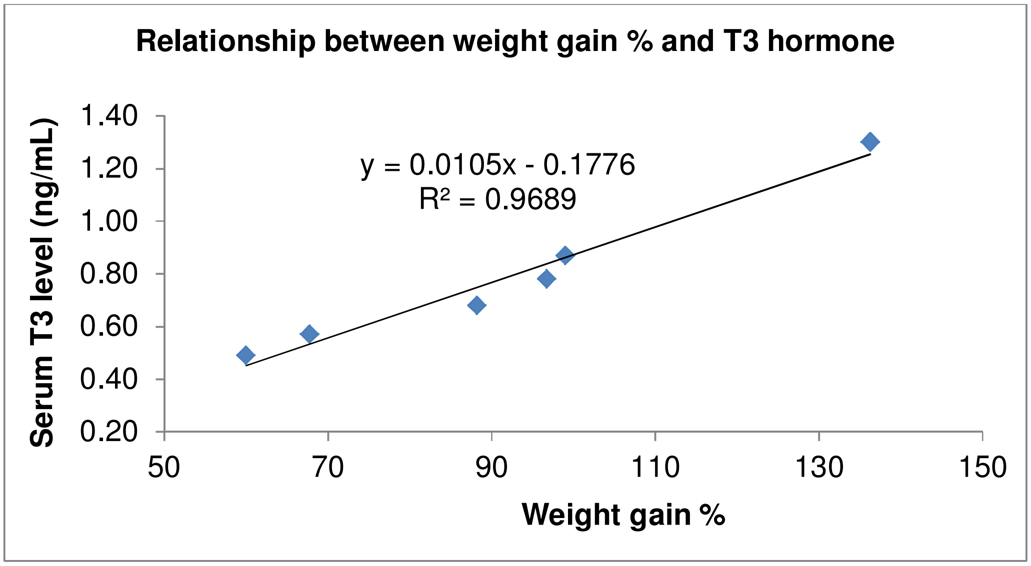


Figure 11: Relationship between weight gain % and T4 hormone in serum of *L. gonius* fingerlings exposed multiple stressors for a period of 60 days

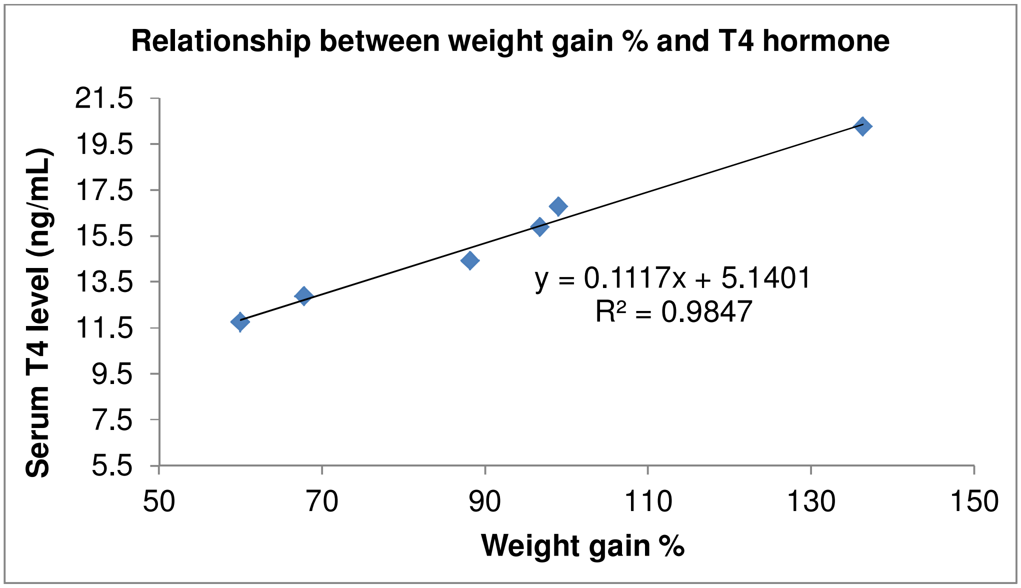


Table 33: Thyroid hormone (T3 and T4), electrolytes and osmolality in serum of *L. gonius* fingerling exposed to multiple stressors for a period of 60 days

Treatments	Thyroid hormones (ng/mL)		Electrolytes (mEq/L)			Osmolality
	T3	**T4**	**Na^{+}**	**K^{+}**	**Cl^{-}**	**(Osmol/L)**
Temperature						
27 ^{0}C	0.95^{b}±0.05	17.16^{b}±0.61	137.11^{a}±2.47	4.31^{a}±0.31	127.01^{a}±2.36	0.266^{a}±0.008
33 ^{0}C	0.61^{a}±0.09	13.52^{a}±0.91	132.78^{a}±3.05	4.77^{a}±0.29	123.22^{a}±2.45	0.254^{a}±0.009
pH						
7.5	1.04b±0.12	18.09^{b}±1.06	143.83^{b}±1.76	3.62^{b}±0.32	132.83^{b}±0.75	0.286^{b}±0.009
5.5	0.58a±0.05	13.09^{a}±0.72	129.83^{a}±2.32	5.12^{a}±0.19	120.33^{a}±2.22	0.245^{a}±0.006
9.0	0.72a±0.07	14.84^{a}±0.95	131.17^{a}±2.73	4.88^{a}±0.25	122.17^{a}±2.50	0.251^{a}±0.007
Temperature x pH						
T_1 (27^{0}C × pH 7.5)	1.30^{e}±0.04	20.26^{e}±0.61	145.33^{c}±3.19	3.46^{c}±0.49	133.67^{c}±0.88	0.291^{c}±0.015
T_2 (27^{0}C × pH 5.5)	0.68^{bc}±0.04	14.43^{bc}±0.64	132.33^{ab}±2.90	4.93^{ab}±0.30	122.67^{a}±3.48	0.251^{ab}±0.008
T_3 (27^{0}C × pH 9.0)	0.87^{d}±0.03	16.79^{d}±0.68	133.67^{ab}±1.86	4.60^{ab}±0.40	124.68^{ab}±4.40	0.256^{abc}±0.007
T_4 (33^{0}C × pH 7.5)	0.78^{cd}±0.03	15.92b^{cd}±0.73	142.33^{bc}±1.76	3.83^{bc}±0.47	132.01^{bc}±1.55	0.281^{bc}±0.013
T_5 (33^{0}C × pH 5.5)	0.49^{a}±0.05	11.76^{a}±0.61	127.33^{a}±3.48	5.30^{a}±0.23	110.01^{a}±2.65	0.239^{a}±0.008
T_6 (33^{0}C × pH 9.0)	0.57^{ab}±0.05	12.80^{ab}±0.51	128.67^{a}±5.24	5.17^{a}±0.24	119.67^{a}±2.33	0.245^{a}±0.011

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6

4.2.14 Serum Electrolytes (Na^+, K^+ and Cl^-)

Serum electrolytes (Na^+, K^+ and Cl^-) *L. gonius* fingerlings exposed to multiple stressors are presented in Table 33. Na^+ and Cl^- levels in serum decreased significantly ($p<0.05$) in T_5 and T_6 treatment groups followed by T_2, T_3 and T_4 group. The higher values were observed in T_1 group. The lowest level was recorded in T_5 followed by T_6 group. However, there was least significant difference among T_1 and T_4 group. On the contrary, K^+ levels followed an opposite trend.

4.2.15 Serum Osmolality

Serum osmolality of *L. gonius* fingerlings exposed to multiple stressors is presented in Table 33. Serum osmolality decreased significantly ($p<0.05$) in T_5 and T_6 treatment groups followed by T_2, T_3 and T_4 group. The higher values were observed in T_1 group. The lowest level was recorded in T_5 followed by T_6 group. However, there was least significance difference among T_1 and T_4 group.

4.2.16 Histological Study

4.2.16.1 Gill tissue

Histoarchitecture of gill tissue (H&E 40X) of *L. gonius* fingerlings found to be normal at T_1 group (Plate 21). At T_2 group, gill tissue (H&E 40X) showed mild congestion at the branchial arch (Plate 24). At T_3 group, gill tissue (H&E 160X) showed mild hyperplasia of secondary lamellae (Plate 27). At T_4 group, gill tissue (H&E 40X) showed loss of secondary lamellae in the lower half of the filament (Plate 30). At T_5 group, gill tissue (H&E 160X) showed haemorrhages in the branchial arch (Plate 33). Another view at T_5 group, gill tissue (H&E 160X) showed accumulation of mononuclear cells in inter-lamellar space and congested branchial arch (Plate 34). At T_6 group, gill tissue (H&E 40X) showed thinning of primary lamellae and marked loss of secondary lamellae (Plate 37).

4.2.16.2 Intestinal tissue

Histoarchitecture of intestinal tissue (H&E 160X) of *L. gonius* fingerlings found to be normal at T_1 group (Plate 22). At T_2 group, intestinal tissue (H&E 40X) showed sloughing of the mucosa (Plate 25). At T_3 group, intestinal tissue (H&E 160X) showed minor damage (Plate 28). At T_4 group, intestinal tissue

(H&E 160X) showed mild loss of brush boarder at the mucosa (Plate 31). At T_5 group, intestinal tissue (H&E 160X) showing marked destruction of sub mucosa and mucosal layer (Plate 35). At T_6 group, intestinal tissue (H&E 40X) showed severe desquamation of the lamina propria (Plate 38). Another view at T_6 group, intestinal tissue (H&E 160X) showed desquamation of the intestinal mucosa (Plate 39).

4.2.16.3 Liver tissue

Histopathology of liver tissue (H&E 40X) of *L. gonius* fingerlings found to be normal at ambient temperature T_1 group (Plate 23). At T_2 group, liver tissue (H&E 160X) showed mild vacuolated haepatocyte (Plate 26). At T_3 group, liver tissue (H&E 160X) showed moderate degeneration of hepatocytes (Plate 29). At T_4 group, liver tissue (H&E 160X) showed diffuse vacuolation of the hepatocytes (Plate 32). At T_5 group, liver tissue (H&E 160X) showing marked necrosis, extensive vacuolation of the hepatocytes and obliteration of the architecture (Plate 36). At T_6 group, liver tissue (H&E 160X) showed moderately vacuolated haepatocyte (Plate 39).

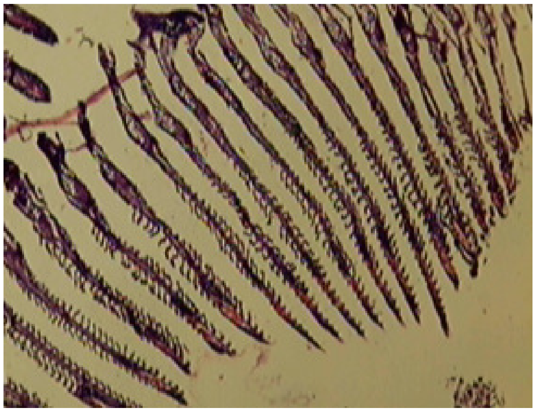


Plate 21: Histoarchitecture of normal gill tissue of *L. gonius* fingerlings at ambient temperature 27^{0}C and ambient pH 7.5 (H&E 40X)

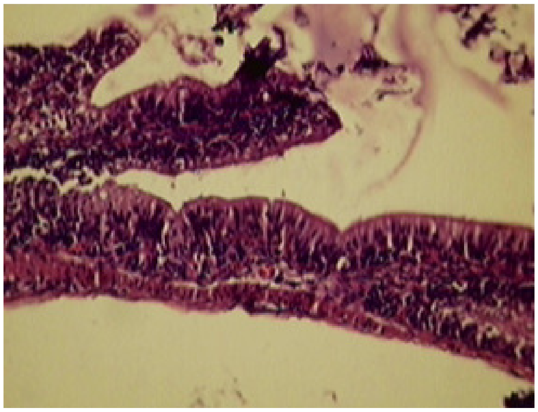


Plate 22: Histoarchitecture of normal intestine tissue of *L. gonius* fingerlings at ambient temperature 27^{0}C and ambient pH 7.5 (H&E 160X)

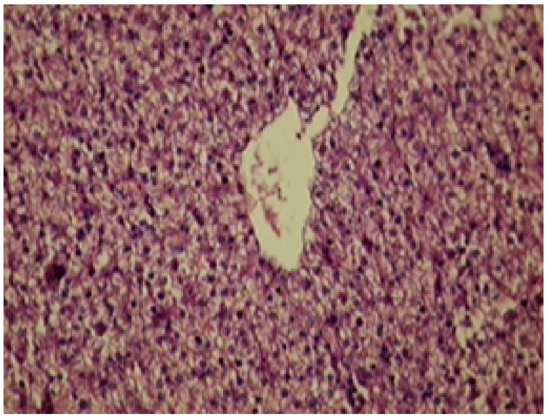


Plate 23: Histology of normal liver tissue of *L. gonius* fingerlings at ambient temperature 27^{0}C and ambient pH 7.5 (H&E 40X)

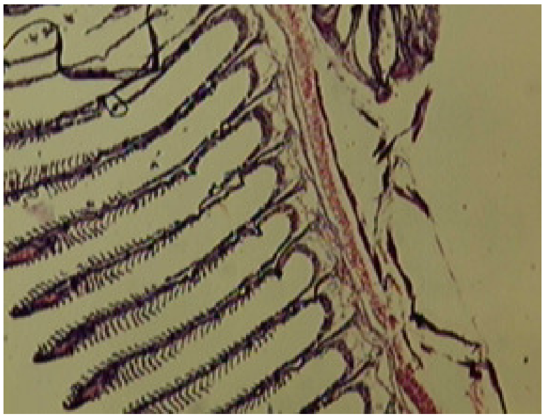


Plate 24: Gill tissue of *L. gonius* fingerlings showed mild congestion at the branchial arch at ambient temperature 27^{0}C and low acidic pH 5.5 (H&E 40X)

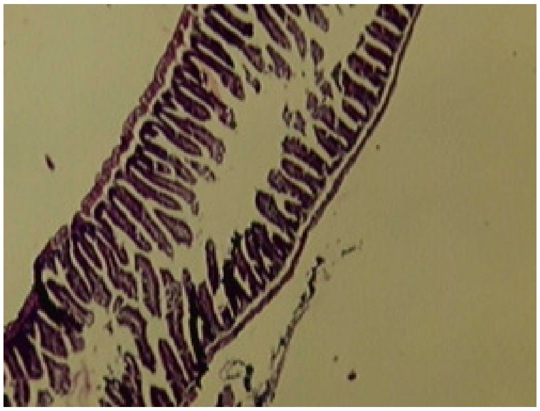


Plate 25: Intestinal tissue of *L. gonius* fingerlings showing sloughing of the mucosa at 27^{0}C and low acidic pH 5.5 (H&E 40X)

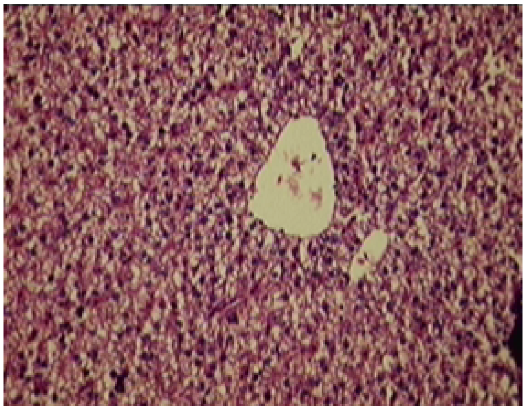


Plate 26: Liver tissue of *L. gonius* fingerlings showed mild vacuolated haepatocyte at 27^{0}C and pH 5.5 (H&E 160X)

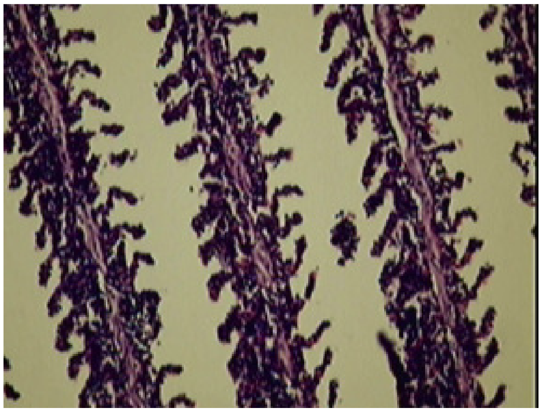


Plate 27: Gill tissue of *L. gonius* fingerlings showing mild hyperplasia of secondary lamellae at 27^{0}C and high alkaline pH 9.0 (H&E 160X)

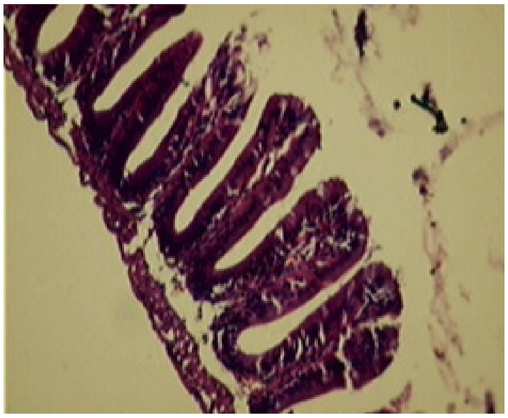


Plate 28: Intestinal tissue of *L. gonius* fingerlings showing minor damage at 27^{0}C and high alkaline pH 9.0 (H&E 160X)

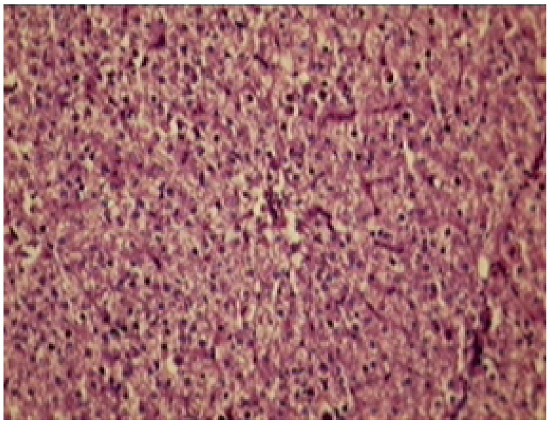


Plate 29: Liver tissue of *L. gonius* fingerlings showing moderate degeneration of hepatocytes at 27^{0}C and high alkaline pH 9.0 (H&E 160X)

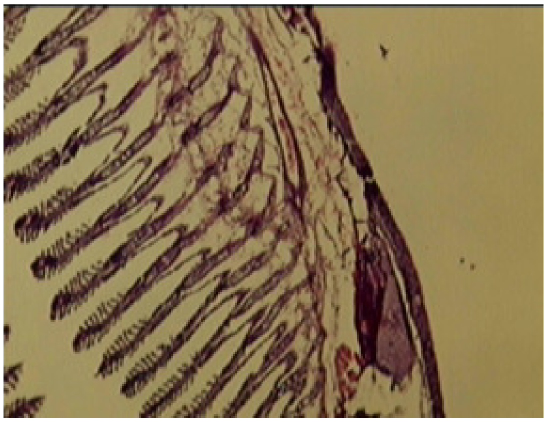


Plate 30: Gill tissue of *L. gonius* fingerlings showed loss of secondary lamellae at high temperature 33^{0}C and ambient pH 7.5 in the lower half of the filament (H&E 40X)

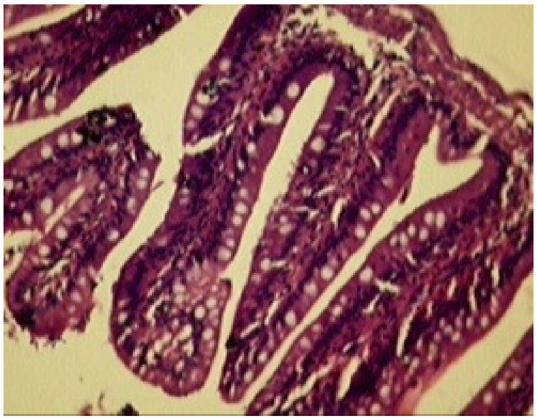


Plate 31: Intestinal tissue of *L. gonius* fingerlings showed mild loss of brush boarder at the mucosa at high temperature 33^{0}C and ambient pH 7.5 (H&E 160X)

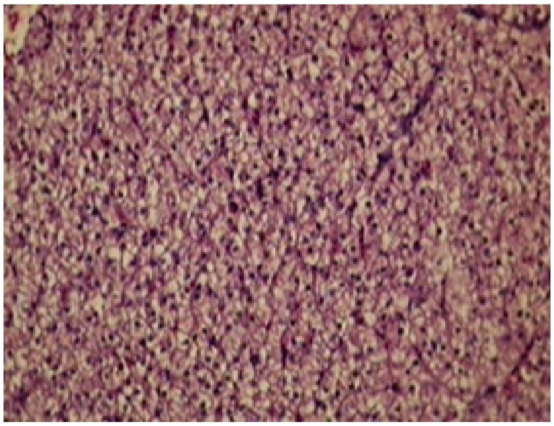


Plate 32: Liver tissue of *L. gonius* fingerlings showed diffuse vacuolation of the hepatocytes at high temperature 33^{0}C and ambient pH 7.5 (H&E 160X)

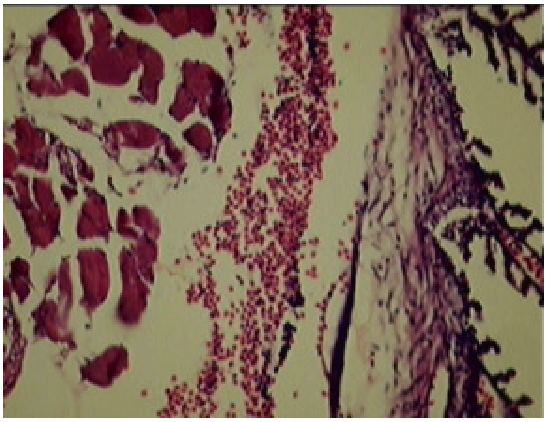


Plate 33: Gill tissues of *L. gonius* fingerlings showed haemorrhages in the branchial arch at high temperature 33^{0}C and low acidic pH 5.5 (H&E 160X)

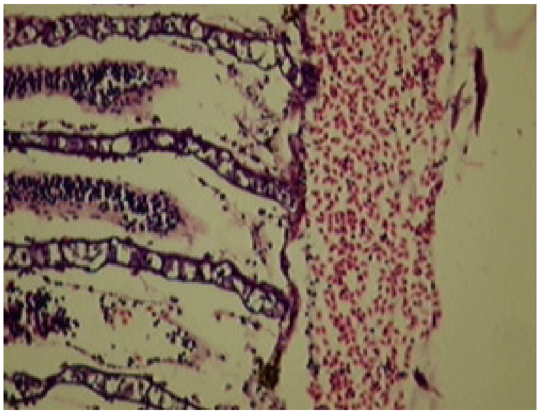


Plate 34: Gill tissue of *L. gonius* fingerlings showing accumulation of mononuclear cells in inter-lamellar space and congested branchial arch at 33^{0}C and low acidic pH 5.5 (H&E 160X)

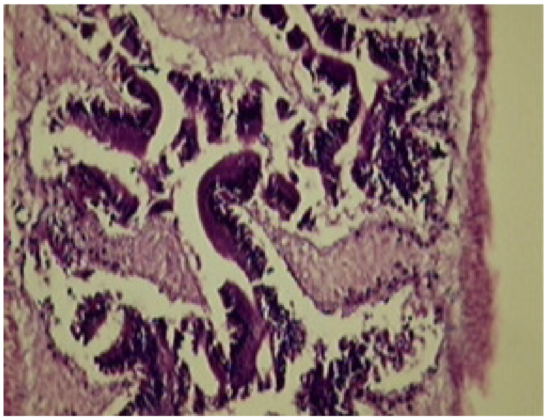


Plate 35: Intestinal tissue of *L. gonius* fingerlings showing marked destruction of sub mucosa and mucosal layer at high temperature 33^{0}C and low acidic pH 5.5 (H&E 160X)

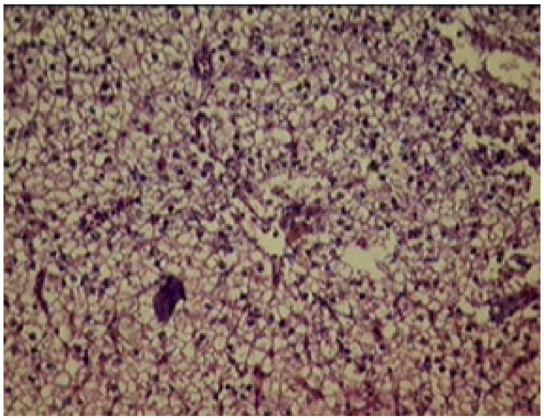


Plate 36: Liver tissue of *L. gonius* fingerlings showing marked necrosis, extensive vacuolation of the hepatocytes and obliteration of the architecture at high temperature 33^{0}C and low acidic pH 5.5 (H&E 160X)

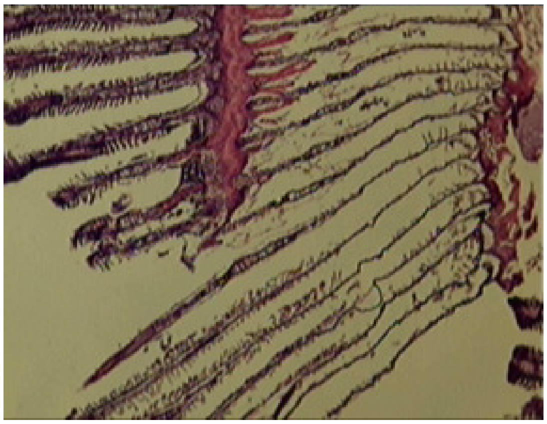


Plate 37: Gill tissue of *L. gonius* fingerlings showed thinning of primary lamellae and marked loss of secondary lamellae at 33^{0}C and high alkaline pH 9.0 (H&E 40X)

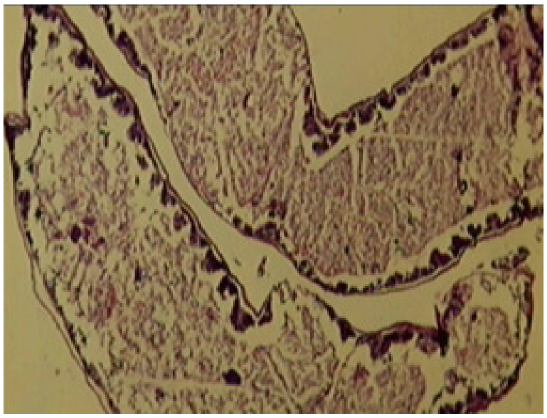


Plate 38: Intestinal tissue of *L. gonius* fingerlings showed severe desquamation of the lamina propria at high temperature 33^{0}C and high alkaline pH 9.0 (H&E 40X)

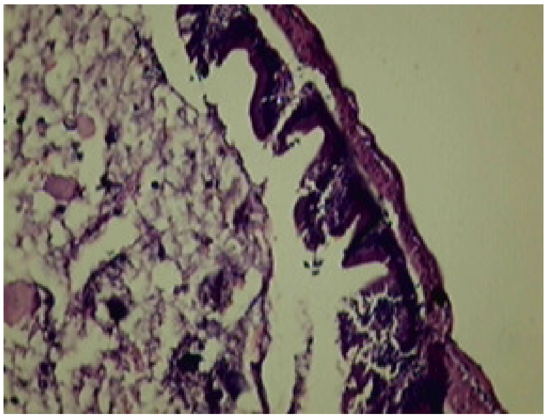


Plate 39: Intestinal tissue of *L. gonius* fingerlings showed desquamation of the intestinal mucosa at high temperature 33^0C and high alkaline pH 9.0 (H&E 160X)

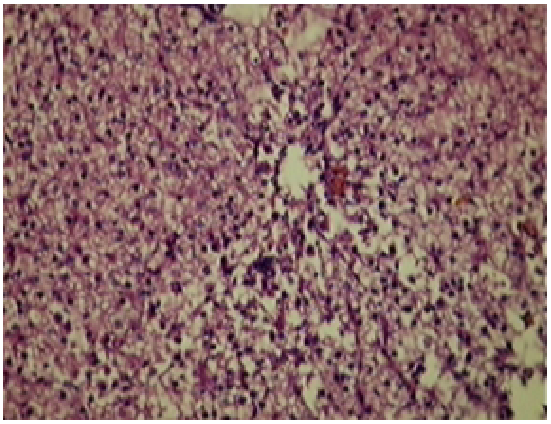


Plate 40: Liver tissue of *L. gonius* fingerlings showed moderately vacuolated haepatocyte at high temperature 33^0C and high alkaline pH 9.0 (H&E 160X)

4.2.17 Scanning Electron Microscopy (SEM) Study

4.2.17.1 Gill tissue

SEM study of gill tissue after 60 days of experimental period, indicate ultrastructural changes in gill tissue of *L. gonius* fingerlings in all the treatments. However, rate of changes varies from treatment to treatment. At T_1 group, SEM photograph (Spot magnification 250X) of gill tissue showed normal structure of primary and secondary gill lamella (Plate 41). At T_2 group, SEM photograph (Spot Magnification 200X) showed the disintegration of lamellar tissues (Plate 42). At T_3 group, SEM photograph (Spot Magnification 200X) showed marked necrotic lamellar tissues with disintegration of the secondary lamellae (Plate 43). At T_4 group, SEM photograph (Spot Magnification 350X) of gill tissue showed loss of secondary lamella alongside the primary damaged cells (Plate 44). At T_5 group, SEM photograph (Spot Magnification 200X) showed the damage primary lamellae and fragmented secondary lamellae (Plate 45). At T_6 group, SEM photograph (Spot Magnification 120X) showed severe loss of secondary lamellae and necrotic primary lamellae are evident as clumped (Plate 46).

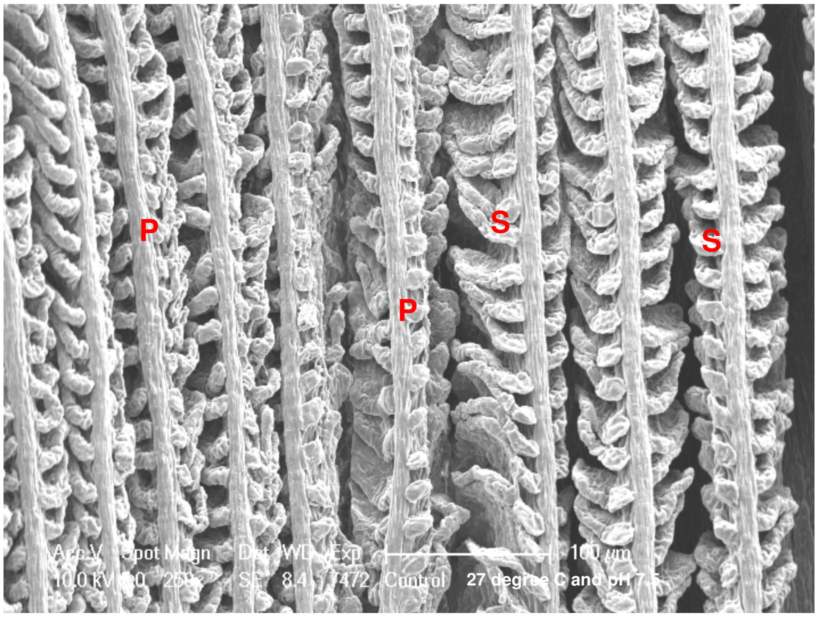


Plate 41: SEM photographs (Spot Magnification 250X) showed the normal gill structure of *L. gonius* fingerlings at ambient temperature 27^{0}C and pH 7.5 (P- Primary lamella and S- Secondary lamella)

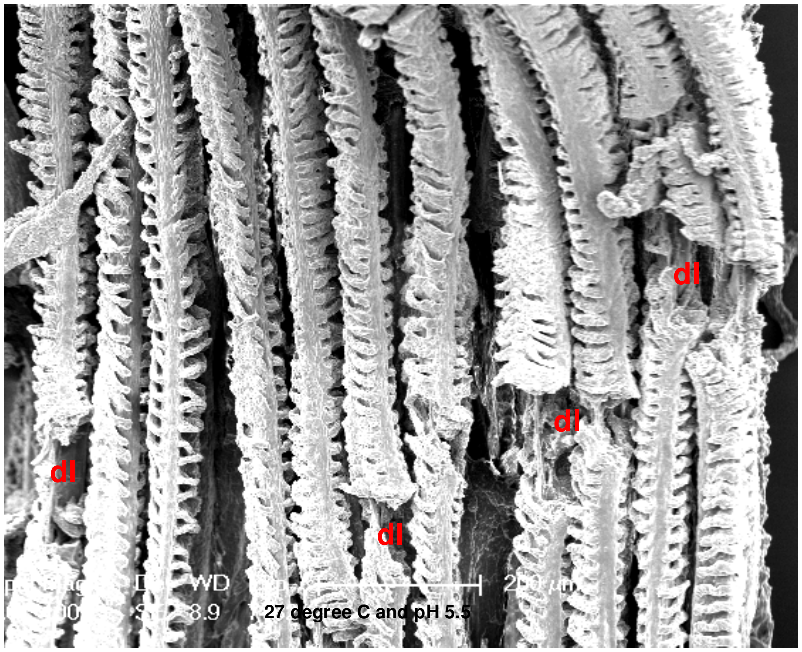


Plate 42: SEM photographs (Spot Magnification 200X) showed the gill structure of *L. gonius* fingerlings at ambient temperature 27^{0}C and low acidic pH 5.5. Disintegration of lamellar tissues (dl) was observed

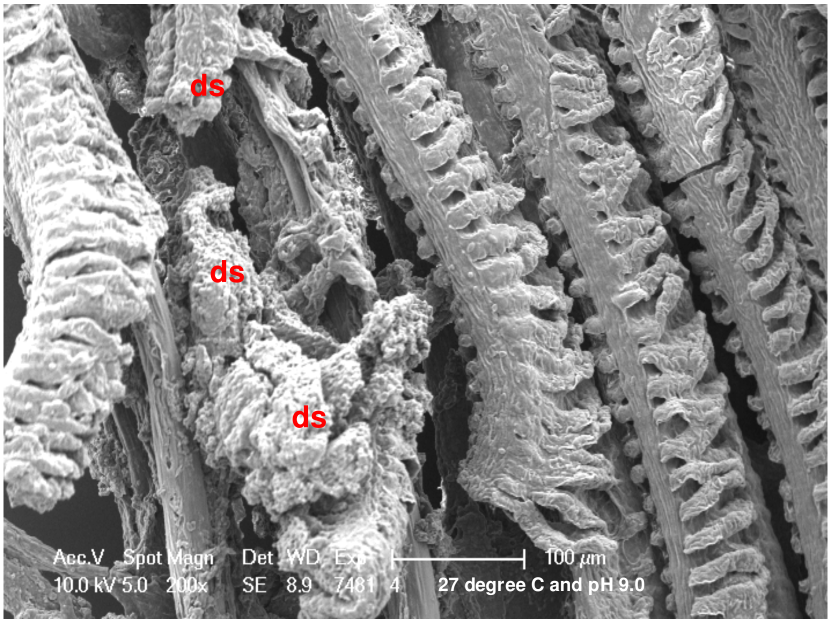


Plate 43: SEM photographs (Spot Magnification 200X) showed the gill structure of *L. gonius* fingerlings at ambient temperature 27^{0}C and high alkaline pH 9.0. Markedly necrotic lamellar tissues with disintegration of secondary lamellae (ds) are evident in the SEM.

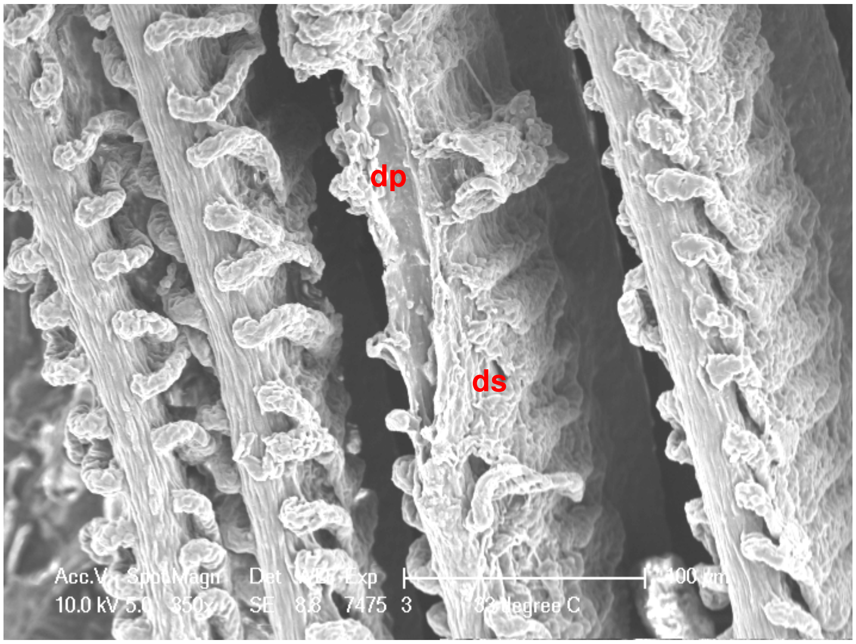


Plate 44: SEM photographs (Spot Magnification 350X) showed the gill structure of *L. gonius* fingerlings at 33^{0}C and ambient pH 7.5. Damaged of the primary lamella (dp). Observe the loss of secondary lamellae alongside the damaged primary cells (ds).

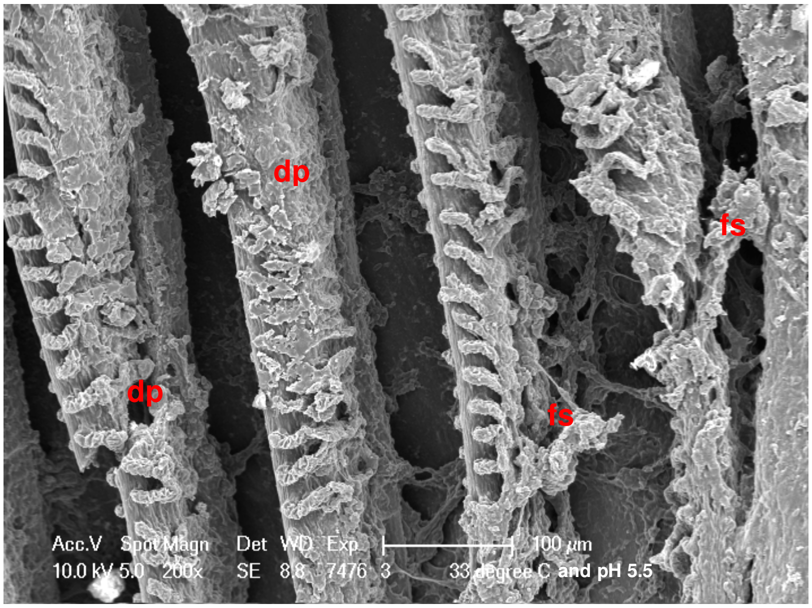


Plate 45: SEM photographs (Spot Magnification 200 X) showed the gill structure of *L. gonius* fingerlings at high temperature 33^{0}C and low acidic pH 5.5. Observe the damaged primary lamellae (dp) and the fragmented secondary lamellae (fs)

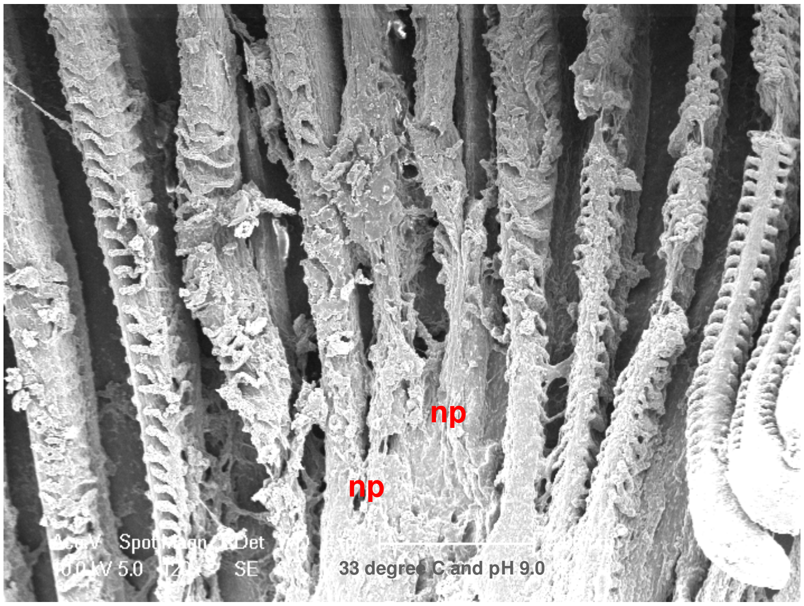


Plate 46: SEM photographs (Spot Magnification 120X) showed the gill structure of *L. gonius* fingerlings at high temperature 33^{0}C and high alkaline pH 9.0. Severe loss of secondary lamellae and necrotic primary lamellae (np) are evident as clumped

4.3 Experiment 3

4.3.1 Physico-chemical Properties of Water

The physico-chemical parameters of water such as temperature (^{0}C), pH, dissolved oxygen (mg/L), free carbon dioxide (mg/L), ammonia (mg/L), Nitrite-N (mg/L) and Nitrate-N (mg/L) were recorded in all the experimental groups are given in Table 34. Water temperature of all the experimental groups was recorded within the range of 27.0 to 27.8^{0}C. There was no variation in pH values and recorded within the range of 7.43 to 7.69. Dissolved oxygen was recorded with in the range of 6.18 to 7.31 mg/L and free carbon dioxide (mg/L) in water was found to be negligible in entire tanks. The total ammonia, nitrite- N and nitrate-N content of all the experimental tubs was recorded before water exchange and found in the range of 0.12 to 0.20, 0.001 to 0.006 and 0.03 to 0.07 mg/L respectively.

Table 34: Water quality parameters in rearing tank during the 60 days of experimental period

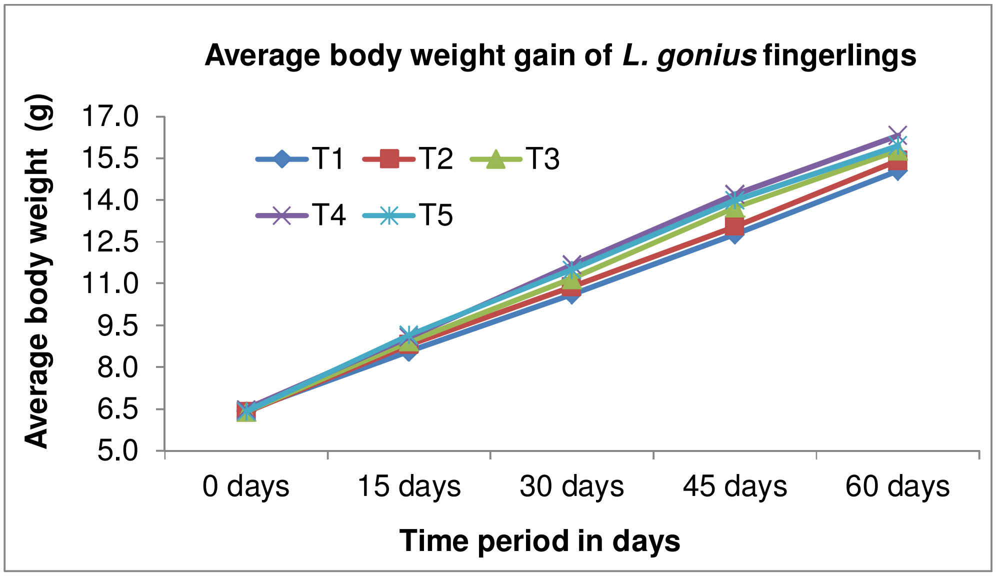
Treatments	^{0}C	pH	DO	Ammonia-N	Nitrite-N	Nitrate-N
T_1	27.0-27.7	7.43-7.62	6.18-7.26	0.12-0.17	0.001-0.004	0.03-0.07
T_2	27.2-27.7	7.29-7.63	6.20 -7.19	0.12-0.19	0.002-0.005	0.04-0.07
T_3	27.1-27.8	7.38-7.69	6.19-7.31	0.15-0.20	0.001-0.004	0.03-0.05
T_4	27.3-27.7	7.52-7.69	6.22-7.26	0.15-0.19	0.002-0.006	0.04-0.06
T_5	27.0-27.6	7.44-7.64	6.28-7.25	0.14-0.18	0.003-0.004	0.03-0.04

Values expressed in range; DO: Dissolved oxygen; Temperature is expressed as ^{0}C; Dissolved oxygen, Ammonia-N, Nitrite-N and Nitrate-N are expressed as mg/L

4.3.2 Growth Parameters

The body weight gain of *L. gonius* fingerlings fed with graded level of L-TRP was recorded at 15 days intervals is shown in Table 35 and Figure 12. The final body weight gain of the fingerlings differed significantly ($p<0.05$) with increase in in L-TRP supplementation. The highest final body weight was recorded in T_4 group and the lowest was in T_1 group. However, there was no significant difference (p>0.05) among T_4 and T_5 group. After 15 days of rearing, body weight gain of the *L. gonius* fingerlings varied significantly among all the treatment groups.

Figure 12: Body weight (g) gain (at 15 days interval) of *L. gonius* fingerlings exposed to dietary L-tryptophan supplementation for a period 60 days



The body weight gain %, SGR, FCR, FER, PER and survival rate of *L. gonius* fingerlings fed with graded level of L-TRP are presented in Table 36. Body weight gain (%), SGR, FER, PER increased significantly ($p<0.05$) with increase in L-TRP supplementation. The highest value was recorded in T_4 group and the lowest was in T_1 group. On the contrary, FCR follows an opposite trend. During the entire experimental period, there was no mortality of fish.

4.3.3 DNA, RNA and RNA/DNA Ratio

DNA, RNA and RNA/DNA ratio in liver and muscle tissue of *L. gonius* fingerlings fed with graded level of L-TRP are presented in Table 37. DNA content in both the tissues did not vary significantly ($p>0.05$) with increase in L-TRP supplementation. RNA and RNA/DNA ratio in both tissue increased significantly ($p<0.05$) with increase in L-TRP supplementation. The lowest value was recorded in T_1. The highest value was recorded in T_4 group, which was not significantly different from T_5 and T_3 group. A linear relationship was observed weight gain % and RNA, RNA/DNA ratio of *L. gonius* fingerlings (Figure 13 & 14).

Table 35: Body weight (g) gain (at 15 days interval) of *L. gonius* fingerlings to dietary L-tryptophan supplementation for a period 60 days

Treatment	Initial	0-15 days	0-30 days	0-45 days	0-60 days
T_1 (0.0% L-TRP)	$6.42^{a}\pm0.07$	$8.56^{a}\pm0.15$	$10.61^{c}\pm0.15$	$12.77^{c}\pm0.24$	$15.05^{c}\pm0.24$
T_2 (0.35% L-TRP)	$6.39^{a}\pm0.04$	$8.81^{ab}\pm0.25$	$10.89^{bc}\pm0.19$	$13.07^{bc}\pm0.39$	$15.42^{bc}\pm0.26$
T_3 (0.70% L-TRP)	$6.40^{a}\pm0.07$	$8.92^{ab}\pm0.17$	$11.18^{abc}\pm0.22$	$13.73^{ab}\pm0.18$	$15.79^{ab}\pm0.18$
T_4 (1.40% L-TRP)	$6.49^{a}\pm0.09$	$9.07^{ab}\pm0.13$	$11.68^{a}\pm0.25$	$14.21^{a}\pm0.30$	$16.33^{a}\pm0.21$
T_5 (2.10% L-TRP)	$6.41^{a}\pm0.09$	$9.16^{ab}\pm0.15$	$11.50^{ab}\pm0.15$	$13.99^{ab}\pm0.25$	$15.96^{ab}\pm0.16$
P-Value	0.405	0.149	0.019	0.021	0.014

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=3 (each replicate is having 12 fish)

Table 36: Growth and survival of *L. gonius* fingerlings to dietary L-tryptophan supplementation for a period 60 days

Treatments	Weight gain %	FCR	FER	PER	SGR	Survival %
T_1 (0.0% L-TRP)	$134.23^{c}\pm1.45$	$1.84^{c}\pm0.03$	$0.54^{b}\pm0.01$	$1.53^{c}\pm0.02$	$1.41^{c}\pm0.01$	100
T_2 (0.35% L-TRP)	$141.29^{b}\pm2.65$	$1.80^{bc}\pm0.01$	$0.56^{ab}\pm0.01$	$1.57^{bc}\pm0.01$	$1.47^{b}\pm0.02$	100
T_3 (0.70% L-TRP)	$146.75^{ab}\pm2.73$	$1.77^{ab}\pm0.02$	$0.57^{a}\pm0.01$	$1.59^{ab}\pm0.02$	$1.51^{ab}\pm0.02$	100
T_4 (1.40% L-TRP)	$151.83^{a}\pm2.43$	$1.74^{a}\pm0.01$	$0.58^{a}\pm0.01$	$1.62^{a}\pm0.01$	$1.54^{a}\pm0.02$	100
T_5 (2.10% L-TRP)	$148.96^{a}\pm1.44$	$1.79^{ab}\pm0.01$	$0.56^{a}\pm0.01$	$1.58^{ab}\pm0.01$	$1.52^{a}\pm0.01$	100
P-Value	0.002	0.019	0.016	0.015	0.001	-

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=3 (each replicate is having 12 fish for growth parameters)

FCR: Feed conversion ratio; FER: Feed efficiency ratio;

PER: Protein efficiency ratio; SGR: Specific growth rate

Figure 13: Relationship between weight gain % and RNA in muscle and liver tissue of *L. gonius* fingerlings to dietary L-tryptophan supplementation for a period of 60 days

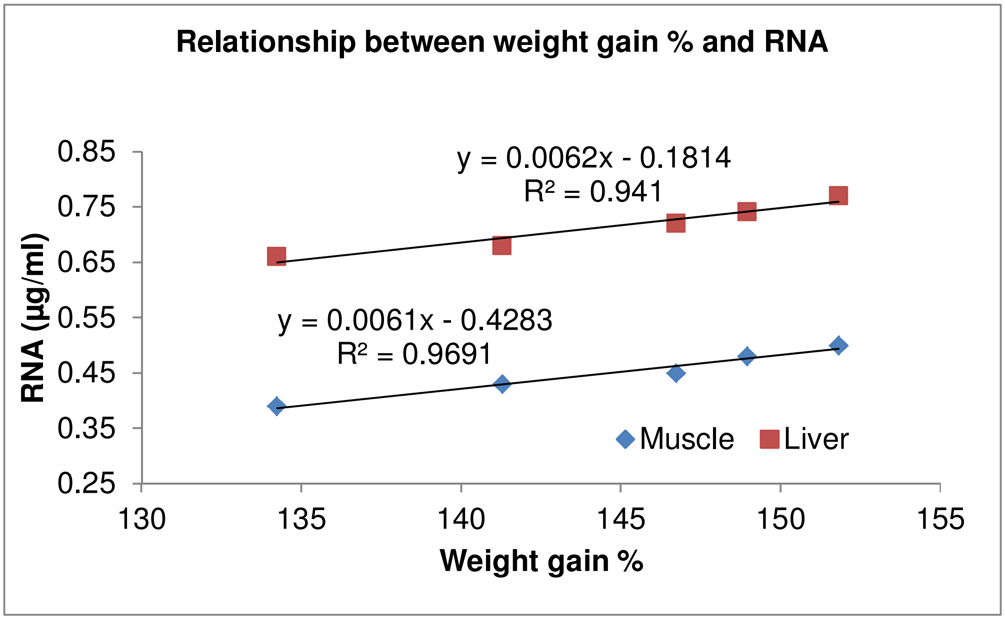


Figure 14: Relationship between weight gain % and RNA/DNA ratio in muscle and liver tissue of *L. gonius* fingerlings to dietary L-tryptophan supplementation for a period of 60 days

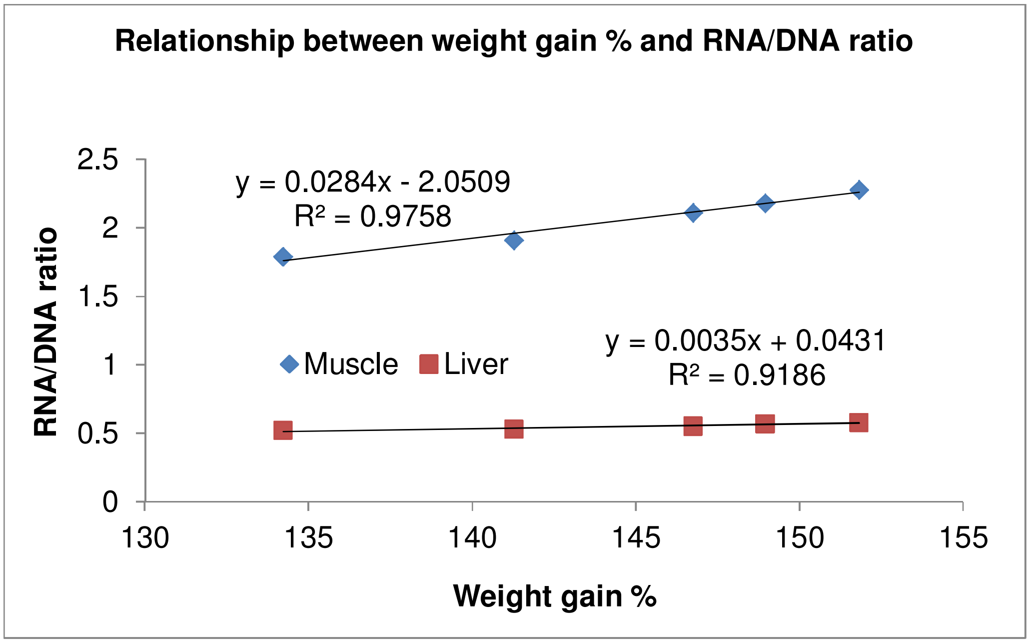


Table 37: DNA, RNA, RNA/DNA ratio and body indices (HSI and GSI) of *L. gonius* fingerlings to dietary L-tryptophan supplementation for a period 60 days

Treatment	DNA and RNA in muscle			DNA and RNA in liver			Body indices	
	µg DNA/ml	µg RNA/ml	RNA/DNA ratio	µg DNA/ml	µg RNA/ml	RNA/DNA ratio	HSI	GSI
T_1 (0.0% L-TRP)	$0.22^{a}\pm0.01$	$0.39^{d}\pm0.02$	$1.79^{c}\pm0.02$	$1.28^{a}\pm0.01$	$0.66^{d}\pm0.01$	$0.52^{d}\pm0.01$	$1.34^{a}\pm0.20$	$2.69^{a}\pm0.36$
T_2 (0.35% L-TRP)	$0.22^{a}\pm0.01$	$0.43^{cd}\pm0.01$	$1.92^{c}\pm0.05$	$1.28^{a}\pm0.01$	$0.68^{cd}\pm0.01$	$0.53^{cd}\pm0.01$	$1.46^{a}\pm0.21$	$2.84^{a}\pm0.22$
T_3 (0.70% L-TRP)	$0.21^{a}\pm0.01$	$0.45^{bc}\pm0.02$	$2.11^{b}\pm0.02$	$1.30^{a}\pm0.01$	$0.72^{bc}\pm0.01$	$0.55^{bc}\pm0.01$	$1.41^{a}\pm0.13$	$2.96^{a}\pm0.41$
T_4 (1.40% L-TRP)	$0.22^{a}\pm0.01$	$0.50^{a}\pm0.01$	$2.28^{a}\pm0.03$	$1.30^{a}\pm0.01$	$0.77^{a}\pm0.02$	$0.58^{a}\pm0.01$	$1.50^{a}\pm0.19$	$3.09^{a}\pm0.28$
T_5 (2.10% L-TRP)	$0.22^{a}\pm0.01$	$0.48^{ab}\pm0.01$	$2.18^{ab}\pm0.06$	$1.29^{a}\pm0.01$	$0.74^{ab}\pm0.01$	$0.57^{ab}\pm0.01$	$1.49^{a}\pm0.30$	$3.07^{a}\pm0.22$
P-Value	0.362	0.002	0.001	0.136	0.002	0.002	0.637	0.411

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6

Table 38: Glycogen, ascorbic acid, AST and ALT in tissues of *L. gonius* fingerlings to dietary L-tryptophan supplementation for a period 60 days

Treatments	Glycogen		Ascorbic acid	GOT/AST		GPT/ALT	
	Muscle	Liver	Muscle	Liver	Muscle	Liver	Muscle
T$_1$ (0.0% L-TRP)	1.32^{a} ±0.10	12.62^{d} ±0.73	131.79^{d} ±3.59	15.39^{d} ±0.73	20.05^{c} ±0.82	5.25^{c} ±0.40	7.26^{d} ±0.37
T$_2$ (0.35% L-TRP)	1.37^{a} ±0.08	12.97^{cd} ±0.95	136.33^{cd} ±4.10	15.29^{cd} ±0.52	19.56^{c} ±1.07	5.07^{bc} ±0.43	6.95^{cd} ±0.39
T$_3$ (0.70% L-TRP)	1.44^{a} ±0.06	13.38^{bc} ±0.20	140.76^{bc}±4.09	12.23^{bc} ±0.99	16.65^{b} ±0.87	3.62^{ab} ±0.58	5.58^{bc} ±0.49
T$_4$ (1.40% L-TRP)	1.52^{a} ±0.05	14.49^{ab} ±0.15	147.26^{ab} ±4.38	10.12^{ab} ±0.14	15.22^{ab} ±0.65	2.80^{a} ±0.54	4.95^{ab} ±0.53
T$_5$ (2.10% L-TRP)	1.55^{a} ±0.09	14.97^{a} ±0.20	151.35^{a} ±4.30	9.18^{a} ±0.77	13.53^{a} ±0.75	2.31^{a} ±0.32	3.83^{a} ±0.57
P-Value	0.255	0.044	0.041	0.001	0.001	0.004	0.002

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6 (n=9 for body indices);

Glycogen: mg glycogen/g tissue; Ascorbic acid: microgram/g wet tissue;

Hepatosomatic Index (HSI) and Gastrosomatic Index (GSI) are expressed as %;

AST activity was expressed as n mol of oxaloacetate released/ min/ mg protein at 37^{o}C;

ALT activity was expressed as n mol of sodium pyruvate released/ min/ mg protein at 37^{o}C

4.3.4 Body Indices

Body indices (HSI and GSI) of *L. gonius* fingerlings fed with graded level of L-TRP are presented in Table 37. HSI and GSI did not show any significance difference. However, higher values of GSI were observed in L-TRP fed groups then the non L-TRP fed counterpart.

4.3.5 Tissue Glycogen

Glycogen content in liver and muscle of *L. gonius* fingerlings fed with graded level of L-TRP is presented in Table 38. Glycogen content in liver tissue increased significantly ($p<0.05$) with increase in L-TRP supplementation. The lowest value was recorded in T_1 group. However, there was no significant difference ($p>0.05$) between T_1 and T_2 group. The highest value was recorded in T_5 group, which did not significantly differ from T_4 group. However, there was no significant ($p>0.05$) difference in glycogen content in muscle tissue.

4.3.6 Ascorbic Acid

Ascorbic acid in muscle of *L. gonius* fingerlings fed with graded level of L-TRP is presented in Table 38. Ascorbic acid in muscle tissue was significantly ($p<0.05$) higher in the L-TRP supplemented groups. The lowest value was recorded in T_1 group. However, there was no significant difference ($p>0.05$) between T_1 and T_2 group. The highest value was recorded in T_5 group, which is not significantly different from T_4 group.

4.3.7 Enzymatic Activity in Tissues

4.3.7.1 Enzymes of protein metabolism (AST and ALT)

AST and ALT activity in liver and muscle of *L. gonius* fingerlings fed with graded level of L-TRP are presented in Table 38. AST and ALT activity in liver and muscle tissue decreased significantly ($p<0.05$) with increase in L-TRP supplementation. The highest value was recorded in T_1 group and the lowest activity was in T_5 group followed by T_4 group. However, there was no significant difference ($p>0.05$) between T_1 and T_2 group.

4.3.7.2 Enzymes of carbohydrate metabolism (LDH and MDH)

LDH and MDH activity in liver and muscle of *L. gonius* fingerlings fed with graded level of L-TRP are presented in Table 39. LDH and MDH activity in liver and muscle tissue decreased significantly ($p<0.05$) with increase in L-TRP supplementation. The highest activity was recorded in T_1 group and the lowest activity was in T_5 group followed by T_4 group. However, there was no significant difference ($p>0.05$) between T_1 and T_2 group.

4.3.7.3 Enzyme of neurotransmission (AchE)

AchE activity in brain of *L. gonius* fingerlings fed with graded level of L-TRP is presented in Table 39. AchE activity in brain increased significantly ($p<0.05$) with increase in L-TRP supplementation. The lowest value was recorded in T_1 group and the highest value was recorded in T_5 group followed by T_4 group. However, there was no significant difference ($p>0.05$) between T_1 and T_2 group.

4.4.7.4 Enzymes of oxidative stress (Catalase, SOD and GST)

Catalase, SOD and GST activity in liver and gill tissue of *L. gonius* fingerlings fed with graded level of L-TRP are presented in Table 40. Catalase activity in liver and gill tissue decreased significantly ($p<0.05$) with increase in L-TRP supplementation. The highest value was recorded in T_1 group and the lowest value was recorded in T_5 group followed by T_4 group. However, there was no significant difference ($p>0.05$) between T_1 and T_2 group.

4.3.7.5 Digestive enzymes (Protease, amylase and lipase)

Protease, amylase and lipase enzyme activity in intestinal tissue of *L. gonius* fingerlings fed with graded level of L-TRP are presented in Table 41. The protease and amylase enzyme activity in intestinal tissue increased significantly ($p<0.05$) with increase in L-TRP supplementation. The lowest value was recorded in T_1 group and the highest value was in T_5 group followed by T_4 group. However, there was no significant difference ($p>0.05$) between T_1 and T_2 group. Lipase activity does not show any significance ($p>0.05$) difference among different treatments.

Table 39: LDH, MDH and AchE enzyme activity in tissues of *L. gonius* fingerlings to dietary L-tryptophan supplementation for a period 60 days

Treatments	LDH		MDH		AchE
	Liver	Muscle	Liver	Muscle	Brain
T_1 (0.0% L-TRP)	4.83^{c} ±0.52	6.29^{c} ±0.52	3.72^{d} ±0.55	4.61^{d} ±0.43	6.25^{b} ±0.10
T_2 (0.35% L-TRP)	4.45^{bc} ±0.40	5.82^{bc} ±0.53	3.43^{cd} ±0.40	4.41^{cd} ±0.37	6.49^{b} ±0.22
T_3 (0.70% L-TRP)	3.33^{ab} ±0.28	4.45^{ab} ±0.65	2.47^{bc} ±0.31	3.35^{bc} ±0.35	7.79^{ab} ±0.46
T_4 (1.40% L-TRP)	2.83^{a} ±0.32	3.41^{a} ±0.41	1.98^{ab} ±0.25	2.46^{a} ±0.30	8.39^{a} ±0.72
T_5 (2.10% L-TRP)	2.59^{a} ±0.18	3.05^{a} ±0.40	1.79^{a} ±0.19	2.32^{a} ±0.26	8.86^{a} ±0.57
P-Value	0.005	0.004	0.012	0.004	0.010

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6;

LDH and MDH specify activity expressed as Unit/ min/ mg protein at 37^0C;

AchE: micromoles acetylcholine hydrolyzed/ mg protein/ min at 37^0C

Table 40: Catalase, SOD and GST enzyme activity in tissues of *L. gonius* fingerlings to dietary L-tryptophan supplementation for a period 60 days

Treatments	Catalase		SOD		GST	
	Liver	**Gill**	**Liver**	**Gill**	**Liver**	**Gill**
T_1 (0.0% L-TRP)	4.56^{d} ±0.36	7.11^{d} ±0.33	11.13^{c} ±0.55	25.28^{c} ±0.67	0.406^{d} ±0.01	0.501^{c}±0.02
T_2 (0.35% L-TRP)	4.22^{cd} ±0.23	6.56^{cd} ±0.35	10.22^{c}±0.49	24.12^{c} ±0.76	0.389^{cd} ±0.01	0.490^{bc} ±0.01
T_3 (0.70% L-TRP)	3.31^{bc} ±0.50	5.25^{bc} ±0.34	8.09^{b} ±0.58	20.77^{b} ±1.19	0.327^{bc} ±0.02	0.407^{ab} ±0.04
T_4 (1.40% L-TRP)	2.45^{ab} ±0.35	4.47^{ab} ±0.56	6.48^{a} ±0.42	18.54^{ab} ±0.76	0.275^{ab} ±0.03	0.362^{a} ±0.03
T_5 (2.10% L-TRP)	2.10^{a} ±0.23	3.85^{a} ±0.48	5.33^{a} ±0.44	16.03^{a} ±0.92	0.239^{a} ±0.03	0.338^{a} ±0.02
P-Value	0.002	0.001	0.001	0.001	0.002	0.006

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6;

Catalase: Nano moles H_2O_2 decomposed/ min/ mg protein at 37^0C;

SOD: micromole/ mg protein/min at 37^0C;

GST: units /mg protein

Table 41: Digestive enzymes activity in tissues of *L. gonius* fingerlings to dietary L-tryptophan supplementation for a period 60 days

Treatments	Protease	Amylase	Lipase
	Intestine	Intestine	Intestine
T_1 (0.0% L-TRP)	18.40^{c} ±0.77	14.19^{c} ±0.22	5.94^{a} ±0.24
T_2 (0.35% L-TRP)	19.89^{c} ±0.92	14.56^{bc} ±0.39	6.25^{a}±0.44
T_3 (0.70% L-TRP)	22.39^{b} ±0.46	15.59^{abc} ±0.97	6.72^{a} ±0.31
T_4 (1.40% L-TRP)	24.27^{ab} ±0.57	16.54^{ab} ±0.74	7.17^{a} ±0.53
T_5 (2.10% L-TRP)	25.27^{a} ±0.38	17.28^{a} ±0.60	7.27^{a} ±0.50
P-Value	0.001	0.031	0.191

Values in the same column with different superscript differ significantly ($p<0.05$);
Data expressed as Mean ± SE, n=6;
Amylase activity as mmole of maltose released/ min/ g protein at 37^{0}C;
Protease activity as mmole of tryosine released/ min/ g protein at 37^{0}C;
Lipase activities expressed as units/ mg protein

4.3.8 Haemato-immunological Parameters (Pre and post challenge)

4.3.8.1 Erythrocyte (RBC) count, Haemoglobin (Hb) and Haematocrit (Hct)

RBC, haemoglobin and haematocrit values of *L. gonius* fingerlings fed with graded level of L-TRP are presented in Table 42. The values increased significantly ($p<0.05$) with increase in L-TRP supplementation. The lowest value was recorded in T_1 group. However, there was no significant difference ($p>0.05$) between T_1 and T_2 group. The highest count was recorded in T_5 group, which is not significantly ($p>0.05$) different from T_3 andT_4 group. RBC count, haemoglobin and haematocrit of different experimental groups challenged with *Aeromonas hydrophilla* are given in Table 42. All the parameters follow the similar trends as of pre-challenge study, though overall lower values were observed in post-challenge.

4.3.8.2 Leucocyte (WBC) count

WBC count of *L. gonius* fingerlings fed with graded level of L-TRP is presented in Table 43. The values increased significantly ($p<0.05$) with increase in L-TRP supplementation. The lowest value was recorded in T_1 group. However, there was no significant difference ($p>0.05$) between T_1 and T_2 group. The highest count was recorded in T_5 group, which is not significantly ($p>0.05$) different from T_3 and T_4 groups. WBC counts of different experimental groups challenged with *A. hydrophilla* are given in Table 43. All the parameters follow the similar trends as of pre-challenge study, though overall higher values were observed in post-challenge.

4.3.8.3 Respiratory burst activity (NBT)

NBT value of *L. gonius* fingerlings fed with graded levels of L-TRP is presented in Table 43. The values increased significantly ($p<0.05$) with increase in L-TRP supplementation. The lowest value was recorded in T_1 group. However, there was no significant difference ($p>0.05$) between T_1 and T_2 group. The highest value was recorded in T_5 group, which is not significantly ($p>0.05$) different from T_4 group. NBT value of different experimental groups challenged with *A. hydrophilla* are given in Table 42 and follows the similar trends as of pre-challenge study, though overall higher values were observed in post-challenge.

4.3.8.4 Blood glucose

Blood glucose levels of *L. gonius* fingerlings fed with graded level of L-TRP is presented in Table 43. Blood glucose level decreased significantly ($p<0.05$) with increase in dietary L-TRP supplementation. The highest value was recorded in T_1 group, which not significantly different from T_2 group. The lowest value was recorded in T_5 followed by T_4 group. Blood glucose of different experimental groups challenged with *A. hydrophilla* are given in Table 42 and follow the similar trends as of pre-challenge study, though overall higher values were observed in post-challenge.

Table 42: Total erythrocyte (RBC), hemoglobin (Hb) and haematocrit (Hct) of *L. gonius* fingerlings to dietary L-tryptophan supplementation for a period 60 days (Pre and post challenge)

Treatments	RBC (10^6cells/ mm^3)		Haemoglobin (g/dL)		Haematocrit (%)	
	Pre	Post	Pre	Post	Pre	Post
T_1 (0.0% L-TRP)	$1.99^{cA} \pm 0.02$	$1.73^{cB} \pm 0.06$	$10.01^{dA} \pm 0.28$	$8.58^{dB} \pm 0.29$	$27.80^{cA} \pm 0.70$	$24.88^{dB} \pm 0.17$
T_2 (0.35% L-TRP)	$2.04^{cA} \pm 0.06$	$1.80^{bcB} \pm 0.05$	$10.32^{cdA} \pm 0.19$	$9.19^{cdB} \pm 0.50$	$29.39^{bcA} \pm 0.89$	$26.93^{cB} \pm 0.44$
T_3 (0.70% L-TRP)	$2.14^{bcA} \pm 0.03$	$1.95^{bA} \pm 0.06$	$10.90^{bcA} \pm 0.31$	$10.12^{bcA} \pm 0.32$	$30.54^{abA} \pm 0.66$	$28.83^{bA} \pm 0.35$
T_4 (1.40% L-TRP)	$2.25^{abA} \pm 0.07$	$2.12^{aA} \pm 0.05$	$11.58^{abA} \pm 0.31$	$10.68^{abA} \pm 0.27$	$31.20^{abA} \pm 0.55$	$29.98^{abA} \pm 0.97$
T_5 (2.10% L-TRP)	$2.31^{aA} \pm 0.05$	$2.19^{aA} \pm 0.04$	$11.94^{aA} \pm 0.08$	$11.03^{aA} \pm 0.49$	$32.59^{aA} \pm 0.90$	$31.45^{aA} \pm 0.13$
P-Value	0.004	0.001	0.003	0.002	0.011	0.001

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6;

Different superscripts (A and B) in the same row (pre and post challenge) signify statistical differences ($p<0.05$) in paired t-test

Table 43: Total leucocytes (WBC), NBT and blood glucose of *L. gonius* fingerlings to dietary L-tryptophan supplementation for a period 60 days (Pre and post challenge)

Treatments	**WBC (10^{3}cells/ mm^3)**		**NBT**		**Blood glucose (mg/100 mL)**	
	Pre	**Post**	**Pre**	**Post**	**Pre**	**Post**
T$_1$ (0.0% L-TRP)	216.13^{cA}±4.72	222.68^{cA}±2.28	0.330^{dA} ±0.01	0.349^{eA} ±0.01	38.23^{cA}±0.19	43.09^{dB}±0.11
T$_2$ (0.35% L-TRP)	222.74^{bcA}±3.85	229.13^{bcA}±4.71	0.341^{dA} ±0.01	0.389^{dA} ±0.01	37.87^{cA}±0.43	41.71^{cB}±0.36
T$_3$ (0.70% L-TRP)	229.05^{abA}±3.23	239.75^{bA}±2.70	0.384^{cA} ±0.01	0.446^{cB} ±0.01	37.01^{bcA}±0.31	37.30^{bA}±0.39
T$_4$ (1.40% L-TRP)	234.98^{aA}±2.64	251.47^{aB}±2.88	0.401^{bA} ±0.01	0.475^{bB} ±0.01	36.39^{abA}±0.63	36.90^{abA}±0.47
T$_5$ (2.10% L-TRP)	237.13^{aA}±2.26	253.02^{aB}±3.87	0.417^{aA} ±0.01	0.495^{aB} ±0.01	35.63^{aA}±0.35	35.99^{aA}±0.19
P-Value	0.008	0.001	0.001	0.001	0.007	0.001

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6;

Different superscripts (A and B) in the same row (pre and post challenge) signify statistical differences ($p<0.05$) in paired t-test

NBT (Respiratory burst activity) expressed in optimal density at 540 nm

Table 44: Total protein, albumin (A) and globulin (G) in serum of *L. gonius* fingerling to dietary L-tryptophan supplementation for a period 60 days (Pre and post challenge)

Treatments	Total protein (g/dL)		Albumin (g/dL)		Globulin (g/dL)	
	Pre	Post	Pre	Post	Pre	Post
T_1 (0.0% L-TRP)	$3.97^{dA}\pm0.03$	$3.40^{cB}\pm0.06$	$0.876^{dA}\pm0.01$	$0.803^{cB}\pm0.01$	$3.10^{cA}\pm0.03$	$2.60^{cB}\pm0.07$
T_2 (0.35% L-TRP)	$4.04^{dA}\pm0.04$	$3.71^{bB}\pm0.09$	$0.890^{cdA}\pm0.01$	$0.837^{bcB}\pm0.01$	$3.17^{cA}\pm0.03$	$2.89^{bB}\pm0.08$
T_3 (0.70% L-TRP)	$4.28^{cA}\pm0.03$	$3.92^{bA}\pm0.12$	$0.917^{bcA}\pm0.01$	$0.877^{abA}\pm0.01$	$3.37^{bA}\pm0.03$	$3.04^{bA}\pm0.11$
T_4 (1.40% L-TRP)	$4.45^{bA}\pm0.05$	$4.26^{aA}\pm0.05$	$0.933^{abA}\pm0.01$	$0.910^{aA}\pm0.01$	$3.52^{aA}\pm0.05$	$3.35^{aA}\pm0.05$
T_5 (2.10% L-TRP)	$4.68^{aA}\pm0.04$	$4.46^{aA}\pm0.05$	$0.955^{aA}\pm0.01$	$0.917^{aA}\pm0.01$	$3.67^{aA}\pm0.06$	$3.54^{aA}\pm0.09$
P-Value	0.001	0.001	0.003	0.002	0.001	0.001

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6;

Different superscripts (A and B) in the same row (pre and post challenge) signify statistical differences ($p<0.05$) in paired t-test

4.3.8.5 Total serum protein, albumin and globulin

Total serum protein, albumin (A) and globulin (G) of *L. gonius* fingerlings fed with graded level of L-TRP are presented in Table 44. Serum protein, albumin and globulin increased significantly ($p<0.05$) with increase in dietary L-TRP supplementation. The lowest value was recorded in T_1 group. However, there was no significant difference ($p>0.05$) between T_1 and T_2 group. The highest value was recorded in T_5 group followed by T_4 group. Total serum protein, albumin and globulin of different experimental groups challenged with *A. hydrophilla* are shown in Table 44. All the parameters followed the similar trends as of pre-challenge study, though overall lower values were observed in post challenge.

Table 45: A/G ratio in serum of *L. gonius* fingerling to dietary L-tryptophan supplementation for a period 60 days (Pre and post challenge)

Treatments	**A/G ratio**	
	Pre	**Post**
T_1 (0.0% L-TRP)	0.287^{cA}±0.01	0.310^{cB} ±0.01
T_2 (0.35% L-TRP)	0.280b^{bcA}±0.01	0.290^{bcB}±0.01
T_3 (0.70% L-TRP)	0.270^{abA}±0.01	0.287^{bA}±0.01
T_4 (1.40% L-TRP)	0.267^{abA} ±0.01	0.273^{abA}±0.01
T_5 (2.10% L-TRP)	0.257^{aA}±0.01	0.261^{aA}±0.01
P-Value	0.006	0.013

Values in the same column with different superscript differ significantly ($p<0.05$);
Data expressed as Mean ± SE, n=6;
Different superscripts (A and B) in the same row (pre and post challenge) signify statistical differences ($p<0.05$) in paired t-test

4.3.8.6 Albumin (A) and globulin (G) ratio

A-G ratio of *L. gonius* fingerlings fed with graded level of L-TRP is presented in Table 45. A-G ratio decreased significantly ($p<0.05$) with increase in dietary L-TRP supplementation. The highest value was recorded in T_1 group. However, there was no significant difference ($p>0.05$) between T_1 and T_2 group.

The lowest value was recorded in T_5 group followed by T_4 group. A-G ratios of different experimental groups challenged with *A. hydrophilla* are shown in Table 45. A-G ratio followed the similar trends as of pre-challenge study, though overall higher A-G ratio was observed in post challenge.

Table 46: Serum lysozyme activity of *L. gonius* fingerling to dietary L-tryptophan supplementation for a period 60 days (Pre and post challenge)

Treatments	Serum lysozyme	
	Pre	Post
T_1 (0.0% L-TRP)	374.59^{dA}±4.66	386.24^{eA}±4.37
T_2 (0.35% L-TRP)	393.25^{dA}±7.51	397.39^{dA}±7.51
T_3 (0.70% L-TRP)	430.87^{cA}±5.76	455.26^{cB}±5.25
T_4 (1.40% L-TRP)	456.73^{bA}±6.48	481.24^{bB}±2.49
T_5 (2.10% L-TRP)	481.55^{aA}±8.42	538.19^{aB}±5.52
P-Value	0.001	0.001

Values in the same column with different superscript differ significantly ($p<0.05$);
Data expressed as Mean ± SE, n=6;
Different superscripts (A and B) in the same row (pre and post challenge) signify statistical differences ($p<0.05$) in paired t-test;
Serum lysozyme activity is expressed as unit/ min/ mg serum protein 37^0C

4.3.8.7 Serum lysozyme activity

Serum lysozyme activity of *L. gonius* fingerlings fed with graded level of L-TRP is presented in Table 46. Lysozyme activities increased significantly ($p<0.05$) with increase in dietary L-TRP supplementation. The lowest value was recorded in T_1 group. However, there was no significant difference ($p>0.05$) between T_1 and T_2 group. The highest value was recorded in T_5 followed by T_4 group. Serum lysozyme activity of different experimental groups challenged with *A. hydrophilla* are shown in Table 46 and follow the similar trends as of pre-challenge study, though overall higher serum lysozyme activity was observed in post challenge.

Table 47: Serum enzyme activity of *L. gonius* fingerling to dietary L-tryptophan supplementation for a period 60 days (Pre and post challenge)

Treatments	sGOT (IU/L)		sGPT (IU/L)		sLDH (IU/L)	
	Pre	Post	Pre	Post	Pre	Post
T_1 (0.0% L-TRP)	25.35^{dA} ±0.48	27.60^{dB} ±0.79	9.51^{dA} ±0.25	12.94^{cB} ±0.48	6.56^{dA} ±0.24	8.29^{dB} ±0.28
T_2 (0.35% L-TRP)	24.43^{dA} ±0.39	26.61^{dB} ±0.28	8.67^{dA} ±0.29	12.04^{cB} ±0.17	5.97^{cdA} ±0.34	7.70^{cdB} ±0.27
T_3 (0.70% L-TRP)	18.84^{cA} ±0.66	20.23^{cB} ±0.56	7.06^{dA} ±0.38	8.58^{bB} ±0.33	4.91^{bcA} ±0.13	6.11^{bcB} ±0.21
T_4 (1.40% L-TRP)	15.41^{bA} ±0.57	16.50^{bA} ±0.29	5.98^{bA} ±0.18	6.52^{aA} ±0.34	4.30^{abA} ±0.31	5.08^{abA} ±0.14
T_5 (2.10% L-TRP)	13.39^{aA} ±0.56	14.61^{aA} ±0.25	5.11^{aA} ±0.22	5.50^{aA} ±0.40	3.57^{aA} ±0.29	4.12^{aA} ±0.60
P-Value	0.001	0.001	0.001	0.001	0.002	0.001

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6;

Different superscripts (A and B) in the same row (pre and post challenge) signify statistical differences ($p<0.05$) in paired t-test

4.3.9 Serum Enzyme Activity (Pre and post challenge)

GOT, GPT and LDH activity in serum of *L. gonius* fingerlings fed with graded level of L-TRP are presented in Table 47. The activities decreased significantly ($p<0.05$) with increase in dietary L-TRP supplementation. The high value was recorded in T_1 group and lowest activity was recorded in T_5 group. Serum enzyme activity of different treatments challenged with *A. hydrophilla* is shown in Table 52. All the parameters follow the similar trends as of pre-challenge study, though overall higher activity of sGOT, sGPT and sLDH were observed in post-challenge.

Table 48: Cortisol hormone levels of *L. gonius* fingerlings to dietary L-tryptophan supplementation for a period 60 days (Pre and Post challenge)

Treatments	Cortisol (ng/mL)	
	Pre	Post
T_1 (0.0% L-TRP)	99.73^{dA} ±3.14	111.51^{dB} ±3.09
T_2 (0.35% L-TRP)	94.22^{dA}±2.70	102.32^{cB} ±4.06
T_3 (0.70% L-TRP)	75.21^{cA} ±1.40	78.84^{bA} ±2.43
T_4 (1.40% L-TRP)	66.85^{bA} ±1.42	71.75^{abA} ±1.93
T_5 (2.10% L-TRP)	58.72^{aA} ±3.37	66.35^{aA} ±2.01
P-Value	0.001	0.001

Values in the same column with different superscript differ significantly ($p<0.05$); Data expressed as Mean ± SE, n=6;

Different superscripts (A and B) in the same row (pre and post challenge) signify statistical differences ($p<0.05$) in paired t-test

4.3.10 Serum Cortisol (Pre and post challenge)

Serum cortisol level of *L. gonius* fingerlings fed with graded level of L-TRP is presented in Table 48. Cortisol decreased significantly ($p<0.05$) with increase in dietary L-TRP supplementation. The highest value was recorded in T_1 group. However, there was no significant difference ($p>0.05$) between T_1 and T_2 group. The lowest value was recorded in T_5 followed by T_4 group. Cortisol follows the similar trends as of pre-challenge study, though overall higher values were observed during post-challenge.

4.3.11 Thyroid Hormones (T3 and T4)

Thyroid hormones (T3 and T4) level of *L. gonius* fingerlings fed with graded level of L-TRP are shown in Table 49. It was found that L-TRP had a significant effect ($p<0.05$) on thyroid hormones. The T3 and T4 hormone levels in serum increased significantly ($p<0.05$) with increase in dietary L-TRP supplementation. The higher level of T3 and T4 hormone was recorded in T_4 group, which is not significantly different from T_3 and T_5 treatment group. The lowest level was recorded in T_1 group followed by T_2 group. A linear relationship was observed between weight gain % and thyroid hormone levels of *L. gonius* fingerlings (Figure 15 & 16).

Figure 15: Relationship between weight gain % and T3 hormone in serum of *L. gonius* fingerlings to dietary L-tryptophan supplementation for a period of 60 days

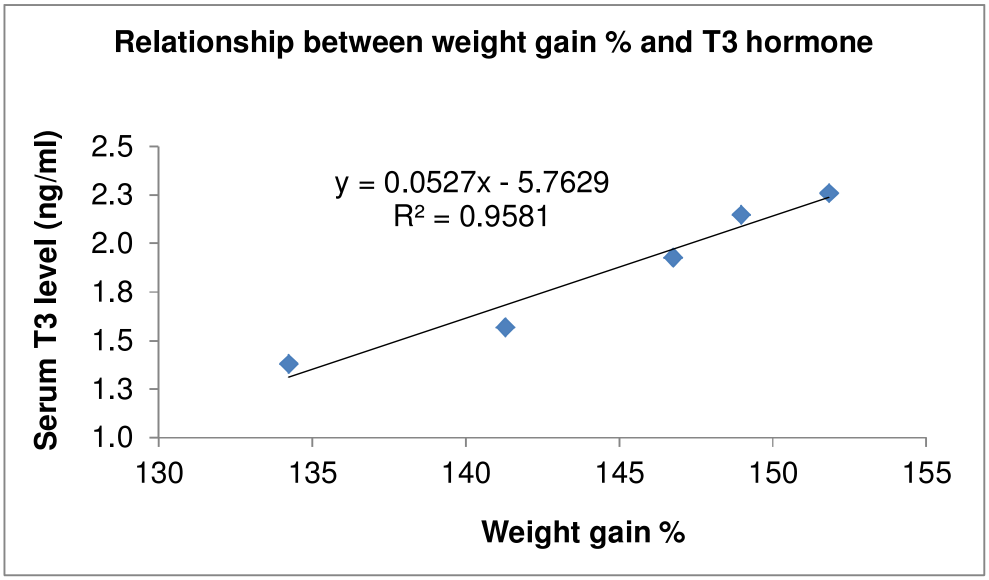
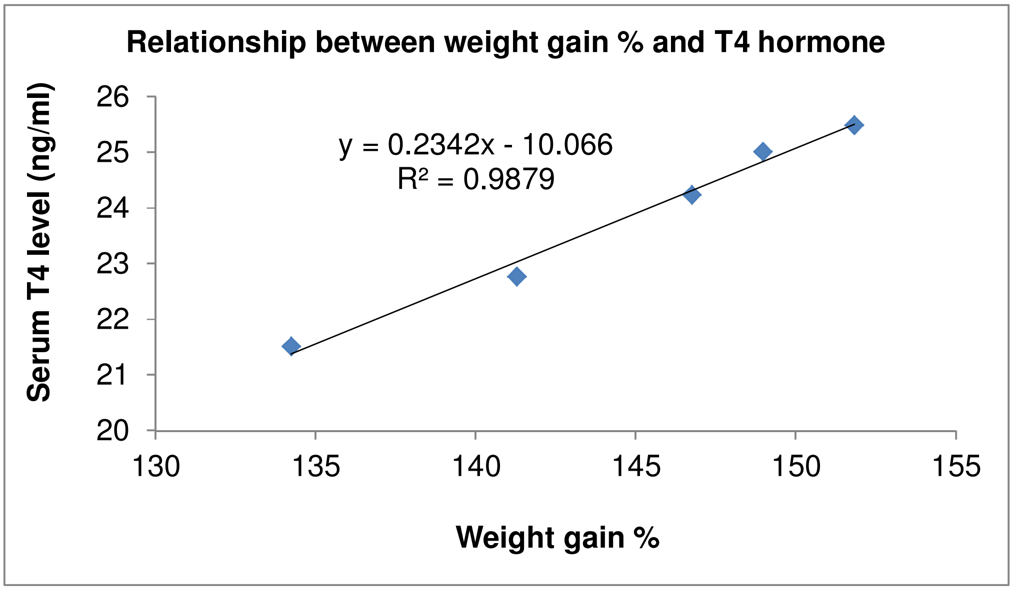


Table 49: Thyroid hormones (T3 and T4) of *L. gonius* fingerlings to dietary L-tryptophan supplementation for a period 60 days

Treatments	Thyroid hormones (ng/mL)	
	T3	T4
T_1 (0.0% L-TRP)	1.38^{c}±0.08	23.29^{c}±0.69
T_2 (0.35% L-TRP)	1.57^{bc}±0.10	23.88^{bc}±0.46
T_3 (0.70% L-TRP)	1.93^{ab}±0.20	25.78^{ab}±0.64
T_4 (1.40% L-TRP)	2.26^{a}±0.18	27.58^{a}±0.63
T_5 (2.10% L-TRP)	2.15^{a}±0.21	26.80^{a}±0.81
P-Value	0.014	0.009

Values in the same column with different superscript differ significantly ($p<0.05$); Data expressed as Mean ± SE, n=6

Figure 16: Relationship between weight gain % and T3 hormone in serum of *L. gonius* fingerlings to dietary L-tryptophan supplementation for a period of 60 days



4.3.12 Survival % in Challenge Study

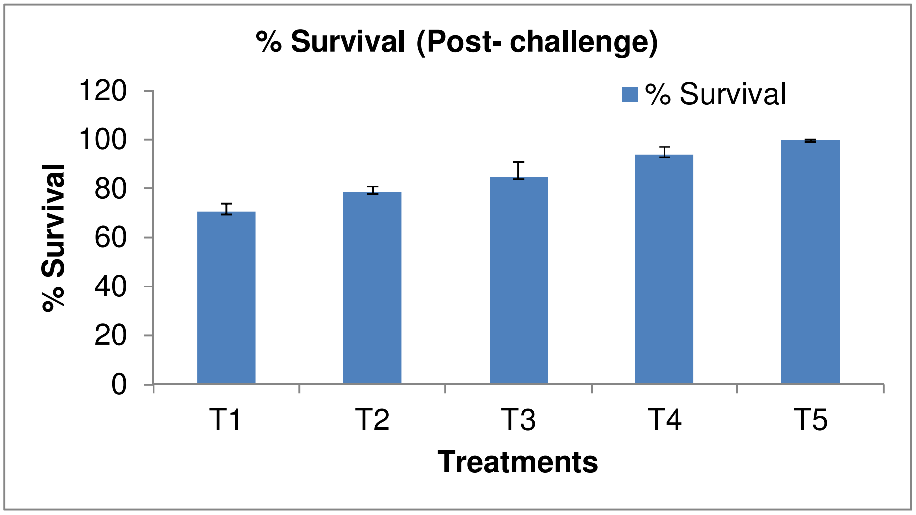
After 60 days of experimental trail all the experimental groups were challenged with *A. hydrophila* and the observation was made for a week. The percentage survival is depicted in Table 50 and Figure 17. The 100% survival was recorded in T_5 followed by T_4 group. The lowest survival was recorded in T_1 group followed T_2 and T_3 groups.

Table 50: Role of dietary L-tryptophan on survival (%) of *L. gonius* fingerlings for a period of 60 days (Post- challenge)

Treatments	Survival %
T_1 (0.0% L-TRP)	70.46 ± 3.47^{d}
T_2 (0.35% L-TRP)	78.94 ± 2.03^{cd}
T_3 (0.70% L-TRP)	84.80 ± 6.05^{bc}
T_4 (1.40% L-TRP)	93.94 ± 3.03^{ab}
T_5 (2.10% L-TRP)	100.0 ± 0.0^{a}
P-value	0.002

Values in the same column with different superscript differ significantly ($p<0.05$); Data expressed as Mean ± SE, n=10

Figure 17: Survival % of different experimental groups fed with dietary L-tryptophan (mean ± SE) after challenge with *Aeromonas hydrophila*



4.4 Experiment 4

4.4.1 Physico-chemical Properties of Water

The physico-chemical parameters of water such as temperature (^{0}C), pH, dissolved oxygen (mg/L), free carbon dioxide (mg/L), ammonia (mg/L), Nitrite-N (mg/L) and Nitrate-N (mg/L) recorded in all the experimental groups are given in Table 51. The ambient water temperature was maintained at 27^{0}C in T_1 experimental group while the temperature in other experimental groups (T_2 to T_9) kept steady at 33^{0}C to ensure chronic thermal stress to the animal during the entire experimental period of 60 days. The ambient pH 7.5 was maintained in T_1 experimental group as control group (without stress). In order to impart chronic pH stress, pH 9.0 was maintained in four experimental groups from T_2 to T_5 and pH 5.5 in four other experimental groups from T_6 to T_9 during the entire experimental period. The dissolved oxygen concentration was recorded with in the range of 5.81 to 7.29 mg/L. Free carbon dioxide (mg/L) in water was found to be negligible in the entire experimental period. The total ammonia, nitrite and nitrate content of experimental tubs was recorded before water exchange and found to be in the range of 0.14 to 0.29, 0.002 to 0.013 and of 0.03 to 0.14 mg/L respectively.

Table 51: Water quality parameters in rearing tank during the 60 days of experimental period

Treatments	DO	Ammonia - N	Nitrite-N	Nitrate-N
T_1	6.21-7.29	0.14-0.19	0.002-0.005	0.03-0.07
T_2	5.90-6.29	0.22-0.28	0.008-0.012	0.10-0.14
T_3	5.89-6.33	0.20-0.27	0.007-0.010	0.11-0.12
T_4	5.87-6.37	0.21- 0.27	0.009-0.012	0.13-0.13
T_5	5.95-6.43	0.23-0.28	0.009-0.012	0.12-0.13
T_6	5.88-6.31	0.24- 0.28	0.009-0.013	0.11-0.14
T_7	5.91-6.32	0.23-0.29	0.007-0.011	0.13-0.13
T_8	5.79-6.37	0.22-0.28	0.009-0.012	0.12-0.14
T_9	5.81-6.40	0.21-0.28	0.008-0.011	0.10-0.14

Values expressed in range; DO; Dissolved oxygen; Hardness, Ammonia-N, Nitrite-N and Nitrate-N are expressed as mg/L

4.4.2 Growth Parameters

The body weight gain of *L. gonius* fingerlings in different experimental groups were recorded at 15 days intervals is shown in Table 52 and Figure 18. Body weight gain decreased upon exposure to multiple stressors, while it increases gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. Lowest values were observed in T_6 and T_2 group, whereas the higher values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups.

The body weight gain %, SGR, FCR, FER, PER and survival rate of *L. gonius* fingerling in different experimental groups are shown in Table 53. Body weight gain %, SGR, FER, PER and survival rate decreased upon exposure to multiple stressors, while it increases gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. Lowest values were observed in T_6 and T_2 group, whereas the higher values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups. On the contrary, FCR values follow an opposite trend.

Figure 18: Role of dietary L-tryptophan on body (g) weight gain (at 15 days interval) of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

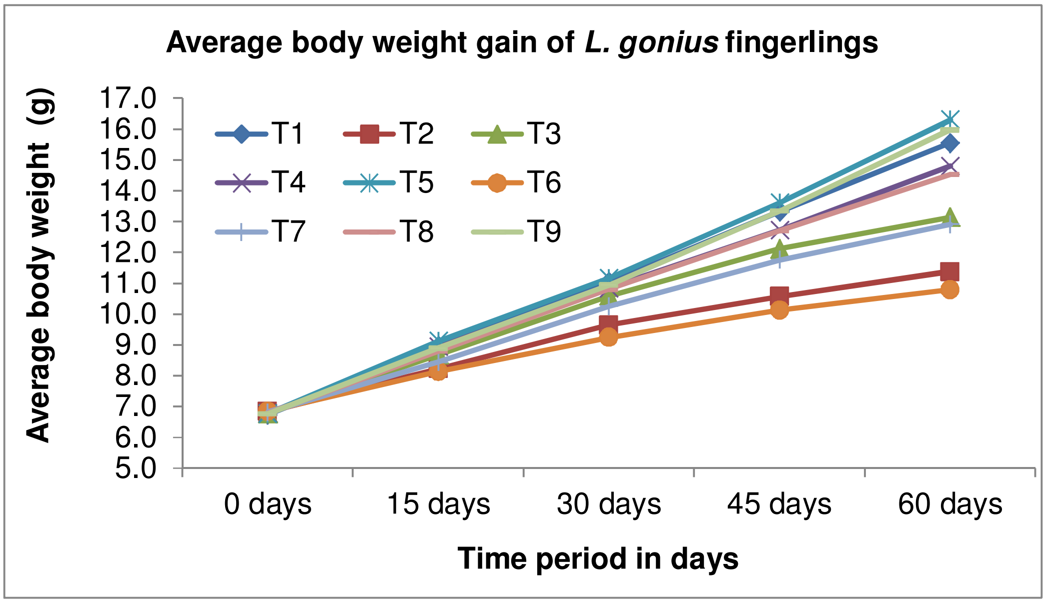


Table 52: Role of dietary L-tryptophan on body weights gain (g) (at 15 days interval) of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

Treatment	Initial	0-15 days	0-30 days	0-45 days	0-60 days
T_1 (No stress)	6.73±0.10	8.97^{c}±0.25	11.04^{cd}±0.21	13.33^{de}±0.39	15.56^{d}±0.25
T_2 (MS_1 x 0.0% L-TRP)	6.84±0.07	8.22^{a}±0.05	9.64^{a}±0.18	10.57^{a}±0.16	11.38^{a}±0.22
T_3 (MS_1 x 0.7% L-TRP)	6.78±0.03	8.67^{bc}±0.06	10.59^{bc}±0.10	12.12^{bc}±0.17^{cd}	13.15^{b}±0.25
T_4 (MS_1 x 1.4% L-TRP)	6.79±0.07	8.95^{c}±0.16	10.85^{cd}±0.11	12.73±0.25	14.81^{c}±0.26
T_5 (MS_1 x 2.1% L-TRP)	6.76±0.04	9.12^{c}±0.13	11.16^{d}±0.20	13.61^{e}±0.25	16.31^{e}±0.26
T_6 (MS_2 x 0.0% L-TRP)	6.85±0.05	8.14^{a}±0.17	9.24^{a}±0.11	10.13^{a}±0.11	10.79^{a}±0.16
T_7 (MS_2 x 0.7% L-TRP)	6.81±0.04	8.45^{ab}±0.08	10.26^{b}±0.11	11.76^{b}±0.17	12.91^{b}±0.18
T_8 (MS_2 x 1.4% L-TRP)	6.77±0.05	8.80^{bc}±0.12	10.81^{cd}±0.08	12.71^{cd}±0.15	14.53^{c}±0.21
T_9 (MS_2 x 2.1% L-TRP)	6.76±0.05	8.90^{c}±0.17	10.94^{cd}±0.23	13.34^{de}±0.31	15.99^{de}±0.23

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=3 (each replicate is having 12 fish)

MS_1: Multiple stressors1 ($33^{0}C$ x pH 9.0); MS_2: Multiple stressors2 ($33^{0}C$ x pH 5.5)

Table 53: Role of dietary L-tryptophan on growth and survival of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

Treatments	Weight gain %	FCR	FER	PER	SGR	% survival
T_1 (No stress)	$131.27^{e}\pm2.33$	$1.88^{ef}\pm0.03$	$0.53^{e}\pm0.01$	$1.50^{e}\pm0.02$	$1.40^{e}\pm0.02$	$100.0^{a}\pm0.00$
T_2 (MS_1 x 0.0% L-TRP)	$66.30^{b}\pm1.50$	$3.25^{b}\pm0.07$	$0.31^{b}\pm0.01$	$0.87^{b}\pm0.02$	$0.85^{b}\pm0.02$	$77.77^{ef}\pm2.77$
T_3 (MS_1 x 0.7% L-TRP)	$99.97^{c}\pm4.25$	$2.50^{c}\pm0.10$	$0.40^{c}\pm0.02$	$1.13^{c}\pm0.05$	$1.10^{c}\pm0.04$	$83.33^{cde}\pm4.81$
T_4 (MS_1 x 1.4% L-TRP)	$117.98^{d}\pm1.87$	$2.04^{de}\pm0.04$	$0.49^{d}\pm0.01$	$1.39^{d}\pm0.03$	$1.30^{d}\pm0.02$	$91.67^{ab}\pm4.81$
T_5 (MS_1 x 2.1% L-TRP)	$141.11^{f}\pm2.92$	$1.77^{f}\pm0.03$	$0.57^{f}\pm0.01$	$1.60^{f}\pm0.03$	$1.47^{e}\pm0.02$	$97.22^{ab}\pm2.77$
T_6 (MS_2 x 0.0% L-TRP)	$57.43^{a}\pm1.29$	$3.65^{a}\pm0.10$	$0.27^{a}\pm0.01$	$0.77^{a}\pm0.02$	$0.75^{a}\pm0.01$	$72.22^{f}\ \pm2.77$
T_7 (MS_2 x 0.7% L-TRP)	$89.64^{c}\pm3.30$	$2.55^{c}\pm0.08$	$0.40^{c}\pm0.02$	$1.11^{c}\pm0.04$	$1.07^{c}\pm0.05$	$80.55^{ef}\pm2.77$
T_8 (MS_2 x 1.4% L-TRP)	$114.57^{d}\pm2.40$	$2.09^{d}\pm0.05$	$0.48^{d}\pm0.01$	$1.35^{d}\pm0.03$	$1.27^{d}\pm0.02$	$88.89^{cd}\pm2.78b$
T_9 (MS_2 x 2.1% L-TRP)	$136.42^{ef}\pm4.50$	$1.81^{f}\pm0.04$	$0.55^{ef}\pm0.02$	$1.56^{ef}\pm0.04$	$1.43^{e}\pm0.03$	$94.45^{ab}\ 2.77$

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=3 (each replicate is having 12 fish for growth parameters);

FCR: Feed conversion ratio; FER: Feed efficiency ratio;

PER: Protein efficiency ratio; SGR: Specific growth rate

MS_1: Multiple stressors1 (33^0C x pH 9.0); MS_2: Multiple stressors2 (33^0C x pH 5.5)

4.4.3 DNA, RNA and RNA/DNA Ratio

DNA, RNA and RNA/DNA ratio in liver and muscle tissue of of *L. gonius* fingerlings in different experimental groups are shown in Table 54. DNA content in both the tissue does not vary significantly ($p>0.05$) upon exposure to multiple stressors and dietary L-tryptophan supplementation. RNA and RNA/DNA ratio in both the tissues decreases upon exposure to multiple stressors, while it increases gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. Lowest values of RNA and RNA/DNA ratio were observed in T_6 and T_2 group, whereas the higher values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups. A linear relationship was observed between weight gain % and RNA, RNA/DNA ratio of *L. gonius* fingerlings (Figure 19 & 20).

Figure 19: Role of L-tryptophan on relationship between weight gain % and RNA in muscle and liver tissue of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

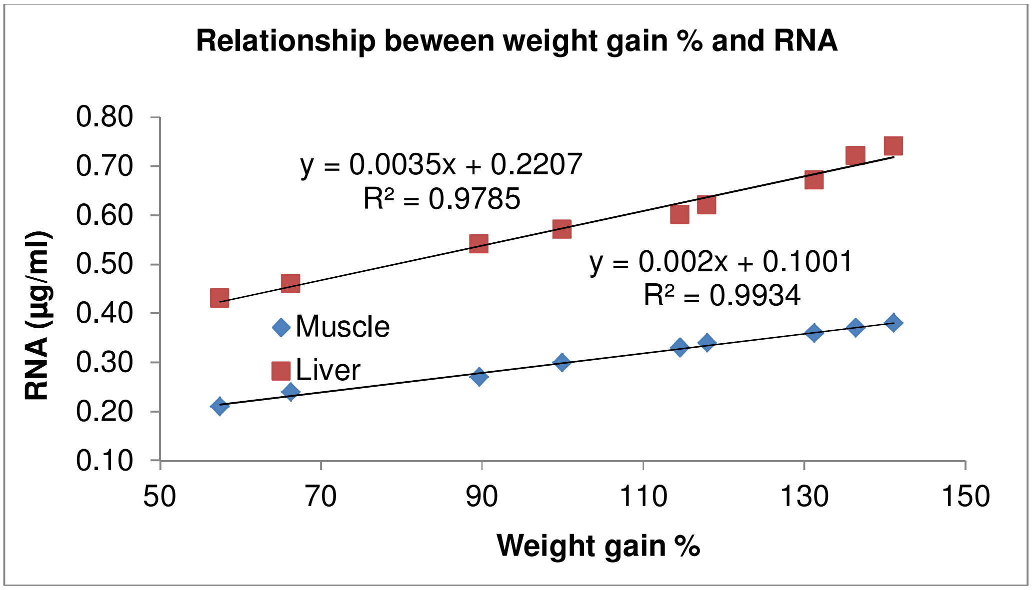
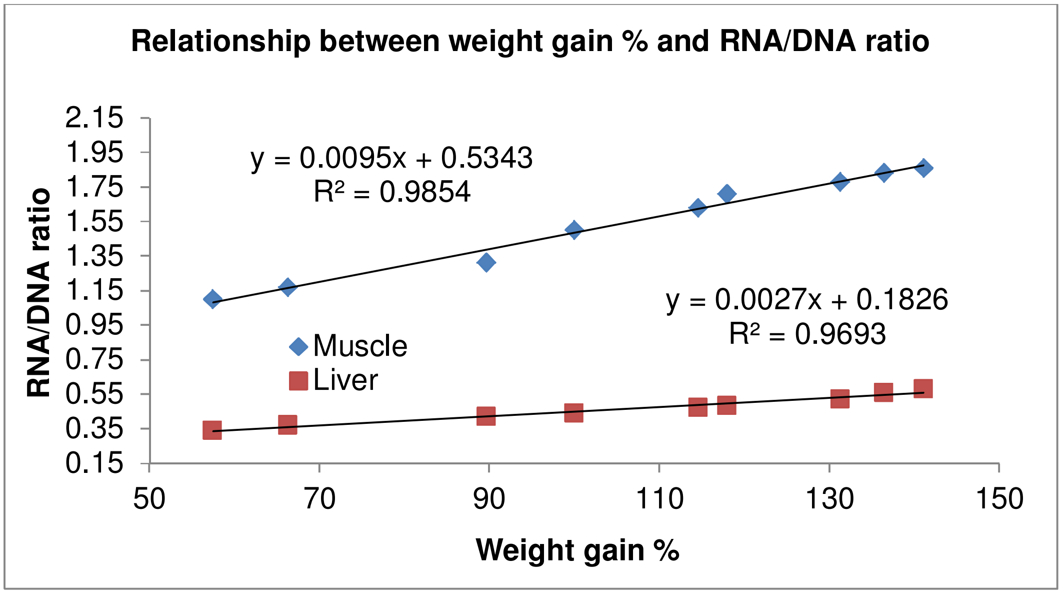


Figure 20: Role of L-tryptophan on relationship between weight gain % and RNA/DNA ratio in muscle and liver tissue of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days



4.4.4 Tissue Glycogen

Glycogen content in liver and muscle tissue of *L. gonius* fingerling in different experimental groups is shown in Table 55. Glycogen content in liver and muscle tissues decreases upon exposure to multiple stressors, while it increases gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. Lowest values were observed in T_6 and T_2 group, whereas the higher values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups.

4.4.5 Ascorbic Acid

Ascorbic acid in muscle tissue of *L. gonius* fingerling in different experimental groups is shown in Table 55. Ascorbic acid in muscle decreases upon exposure to multiple stressors, while it increases gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. Lowest values were observed in T_6 and T_2 group, whereas the higher values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups.

Table 54: Role of dietary L-tryptophan on DNA, RNA and RNA/DNA ratio of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

Treatment	Liver			Muscle		
	µg DNA/ ml	µg RNA/ ml	RNA/ DNA ratio	µg DNA/ ml	µg RNA/ ml	RNA/ DNA ratio
T_1 (No stress)	$0.20^{a}\pm0.01$	$0.36^{ef}\pm0.01$	$1.78^{ef}\pm0.03$	$1.29^{a}\pm0.01$	$0.67^{e}\pm0.01$	$0.52^{d}\pm0.01$
T_2 (MS_1 x 0.0% L-TRP)	$0.20^{a}\pm0.01$	$0.24^{a}\pm0.01$	$1.17^{a}\pm0.01$	$1.27^{a}\pm0.01$	$0.46^{b}\pm0.01$	$0.37^{a}\pm0.01$
T_3 (MS_1 x 0.7% L-TRP)	$0.20^{a}\pm0.01$	$0.30^{c}\pm0.01$	$1.50^{b}\pm0.05$	$1.29^{a}\pm0.01$	$0.57^{c}\pm0.01$	$0.44^{b}\pm0.01$
T_4 (MS_1 x 1.4% L-TRP)	$0.20^{a}\pm0.01$	$0.34^{de}\pm0.01$	$1.71^{de}\pm0.02$	$1.29^{a}\pm0.02$	$0.62^{d}\pm0.01$	$0.48^{c}\pm0.01$
T_5 (MS_1 x 2.1% L-TRP)	$0.20^{a}\pm0.01$	$0.38^{f}\pm0.01$	$1.86^{f}\pm0.03$	$1.26^{a}\pm0.01$	$0.74^{f}\pm0.01$	$0.58^{e}\pm0.01$
T_6 (MS_2 x 0.0% L-TRP)	$0.20^{a}\pm0.01$	$0.21^{a}\pm0.01$	$1.10^{a}\pm0.03$	$1.28^{a}\pm0.01$	$0.43^{a}\pm0.01$	$0.34^{a}\pm0.01$
T_7 (MS_2 x 0.7% L-TRP)	$0.21^{a}\pm0.01$	$0.27^{b}\pm0.01$	$1.31^{c}\pm0.04$	$1.28^{a}\pm0.01$	$0.54^{c}\pm0.01$	$0.42^{b}\pm0.01$
T_8 (MS_2 x 1.4% L-TRP)	$0.21^{a}\pm0.01$	$0.33^{d}\pm0.01$	$1.63^{d}\pm0.04$	$1.28^{a}\pm0.01$	$0.60^{d}\pm0.01$	$0.47^{c}\pm0.02$
T_9 (MS_2 x 2.1% L-TRP)	$0.20^{a}\pm0.01$	$0.37^{ef}\pm0.01$	$1.83^{f}\pm0.03$	$1.28^{a}\pm0.01$	$0.72^{f}\pm0.01$	$0.56^{e}\pm0.01$

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6;

MS_1: Multiple stressors1 (33^{0}C x pH 9.0); MS_2: Multiple stressors2 (33^{0}C x pH 5.5)

Table 55: Role of dietary L-tryptophan on glycogen and ascorbic acid content in tissues of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

Treatments	Glycogen		Ascorbic acid
	Muscle	Liver	Muscle
T_1 (No stress)	1.33^{de} ±0.12	12.84^{efg} ±0.57	131.90^{g}±2.27
T_2 (MS_1 x 0.0% L-TRP)	0.64^{ab} ±0.04	5.77^{b} ±0.39	63.90^{b}±2.87
T_3 (MS_1 x 0.7% L-TRP)	1.01^{cd} ±0.08	9.62^{cd} ±0.59	93.83^{d}±1.15
T_4 (MS_1 x 1.4% L-TRP)	1.24^{de} ±0.05	11.81^{ef} ±0.88	125.01^{f}±2.44
T_5 (MS_1 x 2.1% L-TRP)	1.45^{e} ±0.12	13.86^{g} ±0.61	138.77^{g}±2.98
T_6 (MS_2 x 0.0% L-TRP)	0.53^{a} ±0.03	4.12^{a} ±0.50	53.73^{a}±1.52
T_7 (MS_2 x 0.7% L-TRP)	0.84^{b} ±0.02	8.01^{c} ±0.28	80.71^{c}±2.01
T_8 (MS_2 x 1.4% L-TRP)	1.20^{de} ±0.04	11.19^{de} ±0.57	117.94^{e} ±1.93
T_9 (MS_2 x 2.1% L-TRP)	1.38^{de} ±0.05	13.50^{fg} ±0.33	134.96^{g}±2.70

Values in the same column with different superscript differ significantly ($p<0.05$); Data expressed as Mean ± SE, n=6;

Glycogen: mg glycogen/g tissue; Ascorbic acid: microgram/g wet tissue;

MS_1: Multiple stressors1 (33^0C x pH 9.0); MS_2: Multiple stressors2 (33^0C x pH 5.5)

4.4.6 Enzymatic Activity in Tissues

4.4.6.1 Enzymes of protein metabolism (AST and ALT)

AST and ALT activity in liver and muscle tissue of *L. gonius* fingerlings in different experimental groups are shown in Table 56. AST and ALT activity increases upon exposure to multiple stressors in both the tissue, while it decreases gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. Highest values of AST and ALT were observed in T_6 and T_2 group, whereas the lower values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups.

4.4.6.2 Enzymes of Carbohydrate Metabolism (LDH and MDH)

LDH and MDH activity in liver and muscle tissue of *L. gonius* fingerlings in different experimental groups are shown in Table 57. LDH and MDH activity increases upon exposure to multiple stressors in both the tissue, while it decreases gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. Highest values of LDH and MDH were observed in T_6 and T_2 group, whereas the lower values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups.

4.4.6.3 Enzyme of neurotransmission (AchE)

AchE activity in brain tissue of *L. gonius* fingerlings in different experimental groups is shown in Table 57. AchE activity decreases upon exposure to multiple stressors in brain, while it increases gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. Lowest values were observed in T_6 and T_2 group, whereas the higher values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups.

4.4.6.4 Enzymes of oxidative stress (Catalase, SOD and GST)

Catalase, SOD and GST enzyme activity in liver and gill of *L. gonius* fingerlings in different experimental groups is shown in Table 58. The enzyme activities increase upon exposure to multiple stressors in both the tissues, while it decrease gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. Highest values were observed in T_6 and T_2 group, whereas the lower values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups.

4.4.6.5 Enzyme of gluconeogenic pathways (FDPase)

FDPase activity in liver and gill of *L. gonius* fingerlings in different experimental groups is shown in Table 59. FDPase activity increases upon exposure to multiple stressors in both the tissues, while it decreases gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. Highest values were observed in T_6 and T_2 group, whereas the lower values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups.

Table 56: Role of dietary L-tryptophan on AST and ALT in tissues of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

Treatments	GOT/AST		GPT/ALT	
	Liver	Muscle	Liver	Muscle
T$_1$ (No stress)	15.39^{fg} ±0.76	19.96^{fg} ±0.76	4.91^{fg} ±0.29	7.06^{f} ±0.31
T$_2$ (MS$_1$ x 0.0% L-TRP)	46.16^{b} ±1.04	50.82^{b} ±1.41	16.03b ±1.08	27.55^{b}±1.11
T$_3$ (MS$_1$ x 0.7% L-TRP)	27.74^{d} ±0.52	31.97^{d} ±0.75	9.65^{cd} ±0.76	14.85^{d}±0.97
T$_4$ (MS$_1$ x 1.4% L-TRP)	17.95^{ef} ±1.07	22.87^{ef} ±0.86	6.76^{ef} ±0.46	9.39^{ef}±0.71
T$_5$ (MS$_1$ x 2.1% L-TRP)	13.97^{g} ±0.81	18.80^{g} ±0.75	3.93^{g} ±0.61	6.76f±0.59
T$_6$ (MS$_2$ x 0.0% L-TRP)	57.17^{a} ±1.56	61.57^{a} ±1.38	20.29^{a} ±0.91	31.96^{a}±0.74
T$_7$ (MS$_2$ x 0.7% L-TRP)	32.18^{c} ±0.83	37.48^{c} ±1.33	12.58^{c} ±0.50	17.42^{c}±0.77
T$_8$ (MS$_2$ x 1.4% L-TRP)	20.68e ±0.61	25.69^{e} ±0.70	8.05^{de} ±0.53	11.56^{e}±0.97
T$_9$ (MS$_2$ x 2.1% L-TRP)	14.81^{g} ±0.75	19.57^{g} ±0.90	4.24^{g} ±0.50	7.40^{f} ±0.96

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6;

AST activity was expressed as n mol of oxaloacetate released/ min/ mg protein at 37^{o}C;

ALT activity was expressed as n mol of sodium pyruvate released/ min/ mg protein at 37^{o}C;

MS$_1$: Multiple stressors1 (33^{0}C x pH 9.0); MS$_2$: Multiple stressors2 (33^{0}C x pH 5.5)

Table 57: Role of dietary L-tryptophan on LDH, MDH and AchE enzyme activity in tissues of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

Treatments	LDH		MDH		AchE
	Liver	Muscle	Liver	Muscle	Brain
T_1 (No stress)	4.10^{fg} ±0.16	5.53^{fg} ±0.19	2.33^{f} ±0.12	3.98f ±0.11	6.11^{ef} ±0.12
T_2 (MS_1 x 0.0% L-TRP)	15.59^{b} ±0.87	16.69^{b} ±0.66	12.55^{b} ±0.72	14.11^{b} ±0.64	3.74^{ab} ±0.25
T_3 (MS_1 x 0.7% L-TRP)	9.17^{d} ±0.67	10.53^{d} ±0.81	7.94^{de} ±0.61	9.78^{c} ±0.76	4.99^{cd} ±0.18
T_4 (MS_1 x 1.4% L-TRP)	5.95^{ef} ±0.48	7.32^{ef} ±0.73	4.41^{ef} ±0.44	6.14^{de} ±0.48	5.7^{de} ±0.36
T_5 (MS_1 x 2.1% L-TRP)	3.96^{g} ±0.67	4.55^{g} ±0.82	2.79^{f} ±0.84	4.11^{f} ±0.83	6.65^{f} ±0.36
T_6 (MS_2 x 0.0% L-TRP)	17.78^{a} ±0.51	19.90^{a} ±0.54	14.84^{a} ±0.79	17.53^{a} ±0.60	3.16^{a} ±0.18
T_7 (MS_2 x 0.7% L-TRP)	12.57^{c} ±0.72	13.88^{c} ±0.60	10.13^{c} ±0.90	12.20^{b} ±0.51	4.48^{bc} ±0.38
T_8 (MS_2 x 1.4% L-TRP)	6.98^{e} ±0.51	7.96^{e} ±0.38	6.09^{ef} ±0.48	8.04^{cd} ±0.73	5.53^{de} ±0.35
T_9 (MS_2 x 2.1% L-TRP)	4.26^{fg} ±0.72	5.37^{fg} ±0.71	3.64^{f} ±0.72	5.09^{ef} ±0.79	6.31^{ef} ±0.32

Values in the same column with different superscript differ significantly ($p<0.05$); Data expressed as Mean ± SE, n=6;

LDH and MDH activity expressed as Unit/min/mg protein at 37^{0}C;

AchE: micromoles acetylcholine hydrolyzed / mg protein / min at 37^{0}C;

MS_1: Multiple stressors1 (33^{0}C x pH 9.0); MS_2: Multiple stressors2 (33^{0}C x pH 5.5)

4.4.6.6 Enzyme of pentose phosphate pathway (G6PDH)

G6PDH activity in liver and muscle of *L. gonius* fingerlings in different experimental groups is shown in Table 59. G6PDH activity decreases upon exposure to multiple stressors in both the tissue, while it increases gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. Lowest values were observed in T_6 and T_2 group, whereas the higher values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups.

4.4.6.7 Enzyme of energy metabolism (ATPase)

ATPase activity in liver and gill of *L. gonius* fingerlings in different experimental groups is shown in Table 59. ATPase activity decreases upon exposure to multiple stressors in both the tissue, while it increases gradually ($p<0.05$) with increases in dietary L-tryptophan supplementation. Lowest values were observed in T_6 and T_2 group, whereas the higher values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups.

4.4.6.8 Enzyme of phospho monoesterase (ALP)

ALP activity in liver and muscle tissues of *L. gonius* fingerlings in different experimental groups is shown in Table 60. ALP activity increases upon exposure to multiple stressors in tissues, while it decreases gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. Highest values were observed in T_6 and T_2 group, whereas the lower values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups.

4.4.6.9 Digestive enzyme (Protease)

Protease activity in intestinal tissue of *L. gonius* fingerlings in different experimental groups is shown in Table 60. The enzyme activity decreases upon exposure to multiple stressors in intestine, while it increases gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. Lowest values were observed in T_6 and T_2 group, whereas the higher values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups.

Table 58: Role of dietary L-tryptophan on catalase, SOD and GST enzyme activity in tissues of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

Treatments	Catalase		SOD		GST	
	Liver	Gill	Liver	Gill	Liver	Gill
T$_1$ (No stress)	3.59^{fg} ±0.21	6.85^{fg} ±0.23	11.04^{f} ±0.55	25.22^{fg} ±0.67	0.386^{g} ±0.01	0.492^{fg} ±0.01
T$_2$ (MS$_1$ x 0.0% L-TRP)	11.68^{b} ±0.92	18.99^{b} ±0.54	28.13^{b} ±1.05	45.37^{b} ±0.76	0.837^{b} ±0.02	1.048^{b} ±0.02
T$_3$ (MS$_1$ x 0.7% L-TRP)	6.51^{d} ±0.21	10.74^{d} ±0.73	17.90^{d} ±0.72	33.13^{d} ±0.87	0.593^{d} ±0.01	0.717^{d} ±0.01
T$_4$ (MS$_1$ x 1.4% L-TRP)	4.45^{ef} ±0.44	7.51^{ef} ±0.75	12.66^{ef} ±0.44	27.27^{ef} ±0.43	0.402^{f} ±0.02	0.512^{f} ±0.01
T$_5$ (MS$_1$ x 2.1% L-TRP)	2.95^{g} ±0.21	5.36^{g} ±0.78	6.88^{g} ±0.69	20.68^{h} ±0.87	0.370^{gh} ±0.01	0.465^{h} ±0.02
T$_6$ (MS$_2$ x 0.0% L-TRP)	14.64^{a} ±0.46	21.45^{a} ±0.63	32.11^{a} ±0.73	54.26^{a} ±2.24	0.897^{a} ±0.02	1.106^{a} ±0.02
T$_7$ (MS$_2$ x 0.7% L-TRP)	7.98^{c} ±0.22	12.95^{c} ±0.51	22.40^{c} ±0.72	37.66^{c} ±0.59	0.629^{c} ±0.01	0.755^{c} ±0.01
T$_8$ (MS$_2$ x 1.4% L-TRP)	4.98^{e} ±0.44	8.77^{e} ±0.50	14.57^{e} ±0.50	29.18^{e} ±0.95	0.416^{e} ±0.01	0.551^{e} ±0.01
T$_9$ (MS$_2$ x 2.1% L-TRP)	3.47^{fg} ±0.23	6.45^{fg} ±0.47	7.71^{g} ±0.71	22.31^{gh} ±0.55	0.376^{gh} ±0.02	0.482^{gh} ±0.02

Values in the same column with different superscript differ significantly ($p<0.05$); Data expressed as Mean ± SE, n=6;

Catalase: Nano moles H_2O_2 decomposed / min / mg protein at 37^{0}C;

SOD: micromole /mg protein/min at 37^{0}C); GST: units /mg protein;

MS$_1$: Multiple stressors1 (33^{0}C x pH 9.0); MS$_2$: Multiple stressors2 (33^{0}C x pH 5.5)

Table 59: Role of dietary L-tryptophan on FDPase, G6PDH and ATPase enzyme activity in tissues of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

Treatments	FDPase		G6PDH		ATPase	
	Liver	Gill	Liver	Muscle	Liver	Gill
T_1 (No stress)	9.26^{f}±0.52	29.11^{efg}±0.97	11.54^{e}±0.35	6.76^{cd}±0.54	2.94^{ef}±0.10	5.36^{e}±0.29
T_2 (MS_1 x 0.0% L-TRP)	41.93^{b}±3.58	91.29^{b}±3.05	6.71^{a}±0.40	3.23^{a}±0.42	1.40^{ab}±0.05	3.31^{ab}±0.13
T_3 (MS_1 x 0.7% L-TRP)	22.77^{d}±1.05	50.19^{d}±0.94	8.46^{b}±0.63	4.49ab±0.41	1.91^{b}±0.13	3.95^{bc}±0.32
T_4 (MS_1 x 1.4% L-TRP)	11.17^{ef}±0.84	32.49^{ef}±1.82	10.90^{cde}±0.69	6.37^{cd}±0.46	2.45^{cde}±0.24	4.71^{cde}±0.32
T_5 (MS_1 x 2.1% L-TRP)	8.31^{f}±0.65	24.83^{g}±1.78	12.26^{e}±0.61	7.60^{d}±0.33	3.03^{f}±0.17	5.46^{e}±0.27
T_6 (MS_2 x 0.0% L-TRP)	49.19^{a}±1.51	99.89^{a}±3.66	6.26^{a}±0.47	3.13^{a}±0.35	1.26^{a}±0.07	2.83^{a}±0.22
T_7 (MS_2 x 0.7% L-TRP)	31.40^{c}±2.58	60.92^{c}±3.63	7.43^{ab}±0.75	4.15^{a}±0.38	1.84^{b}±0.21	3.68^{ab}±0.26
T_8 (MS_2 x 1.4% L-TRP)	15.14^{e}±1.26	34.18^{e}±1.92	9.60^{cd}±0.63	5.48^{bc}±0.40	2.37^{cd}±0.20	4.19^{bcd}±0.39
T_9 (MS_2 x 2.1% L-TRP)	9.21^{f}±0.44	25.91^{fg}±1.49	11.69^{e}±0.29	6.81^{cd}±0.48	2.73^{def}±0.21	4.95^{de}±0.33

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6;

FDPase: microgram phosphorous released / mg protein / minute at 37^{0}C;

G6PDH: Δ 0.01 optical density / mg protein / minute at 37^{0}C;

ATPase: microgram phosphorous released / mg protein / minute at 37^{0}C;

MS_1: Multiple stressors1 (33^{0}C x pH 9.0); MS_2: Multiple stressors2 (33^{0}C x pH 5.5)

Table 60: Role of dietary L-tryptophan on ALP and protease enzyme activity in tissues of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

Treatments	ALP		Protease
	Muscle	Liver	Intestine
T_1 (No stress)	12.06^{f} ±0.85	29.68^{fg} ±1.89	18.17^{ef}±0.69
T_2 (MS_1 x 0.0% L-TRP)	52.70^{b} ±2.96	102.64^{b} ±2.73	13.34^{ab}±0.36
T_3 (MS_1 x 0.7% L-TRP)	29.01^{d} ±0.99	63.87^{d} ±3.30	15.71^{cd}±0.74
T_4 (MS_1 x 1.4% L-TRP)	17.03^{e} ±0.93	36.27^{ef} ±1.51	17.11^{de}±0.38
T_5 (MS_1 x 2.1% L-TRP)	8.98^{f} ±0.64	22.01^{g} ±2.00	19.45^{f}±0.40
T_6 (MS_2 x 0.0% L-TRP)	60.54^{a} ±2.93	112.01^{a} ±3.36	12.47^{a}±0.29
T_7 (MS_2 x 0.7% L-TRP)	35.38^{c} ±1.16	72.55^{c} ±3.80	14.40^{bc}±0.61
T_8 (MS_2 x 1.4% L-TRP)	21.56^{e} ±1.02	42.48^{e} ±3.38	16.37^{d}±0.41
T_9 (MS_2 x 2.1% L-TRP)	10.56^{f} ±0.41	26.21^{g} ±1.20	18.86^{f}±0.49

Values in the same column with different superscript differ significantly ($p<0.05$); Data expressed as Mean ± SE, n=6;

ALP: nanomoles P-nitrophenol released/ mg protein/ minute at 37^{0}C;

Protease activity as mmole of tryosine released/ min/ g protein at 37^{0}C;

MS_1: Multiple stressors1 (33^{0}C x pH 9.0); MS_2: Multiple stressors2 (33^{0}C x pH 5.5)

4.4.7 Haemato-immunological Parameters (Pre and post challenge)

4.4.7.1 Erythrocyte (RBC) count, haemoglobin (Hb) and haematocirt (Hct)

Erythrocyte (RBC) count, haemoglobin (Hb) and haematocirt (Hct) count of *L. gonius* fingerlings in different experimental groups are shown in Table 61. The values decreased significantly upon exposure to multiple stressors, while it increases gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. Lowest values were observed in T_6 and T_2 group, whereas the higher values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups. RBC count, haemoglobin and haematocrit of different experimental groups challenged with *Aeromonas hydrophila* are given in Table 61. All the parameters followed the similar trends as of pre-challenge study, though overall lower values were observed in post-challenge.

4.4.7.2 Leucocyte (WBC) count

Leucocyte (WBC) count of *L. gonius* fingerlings in different experimental groups is shown in Table 62. The values decreased significantly upon exposure to multiple stressors, while it increased gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. Lowest values were observed in T_6 and T_2 group, whereas the higher values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups. Total leucocyte (WBC) count of different experimental groups challenged with *Aeromonas hydrophila* are given in Table 62 and followed the similar trends as of pre-challenge study, though overall higher values were observed in post-challenge.

4.4.7.3 Respiratory burst activity (NBT)

NBT value of *L. gonius* fingerling in different experimental groups is shown in Table 62. The values decreased significantly upon exposure to multiple stressors, while it increases gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. Lowest values were observed in T_6 and T_2 group, whereas the higher values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups. NBT activity of different experimental groups challenged with *A. hydrophila* are given in Table 62 and follows the similar trends as of pre-challenge study, though values higher values were observed in post-challenge.

4.4.7.4 Blood glucose

Blood glucose level of *L. gonius* fingerlings in different experimental groups is shown in Table 62. Blood glucose levels increased significantly upon exposure to multiple stressors, while it decreased gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. Highest values were observed in T_6 and T_2 group, whereas the lower values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups. Blood glucose of different experimental groups challenged with *A. hydrophila* are given in Table 62 and follow the similar trends as of pre-challenge study, though overall higher values were observed in post-challenge.

4.4.7.5 Total serum protein, albumin (A), globulin (G) and A-G ratio

Total serum protein, albumin, globulin and A-G ratio of *L. gonius* fingerlings in different experimental groups are shown in Table 63 and 64. Total serum protein, albumin and globulin decreased significantly upon exposure to multiple stressors, while it increased gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. Lowest values were observed in T_6 and T_2 group, whereas the higher values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups. On the contrary, A-G ratio follows an opposite trend. Serum albumin, globulin, total serum protein and A-G ratio and lysozyme activity of different experimental groups challenged with *A. hydrophila* are shown in Table 63 and 64. All the parameters follow the similar trends as of pre-challenge study, though overall higher A-G ratio and lower serum albumin, globulin and total serum protein was observed in post challenge.

4.4.7.6 Serum lysozyme activity

Serum lysozyme activity of *L. gonius* fingerlings in different experimental groups is shown in Table 64. The activity decreased significantly upon exposure to multiple stressors, while it increased gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. Lowest values were observed in T_6 and T_2 group, whereas the higher values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups. Serum lysozyme activity of different experimental groups challenged with *A. hydrophila* are shown in Table 64 and follow the similar trends as of pre-challenge study, though overall higher lysozyme activity was observed in post challenge.

4.4.8 Serum Enzyme Activity

GOT, GPT, ALP, ACP and LDH enzyme activity in serum of *L. gonius* fingerling in different experimental groups are shown in Table 65. Serum GOT, GPT, ALP, ACP and LDH increased significantly upon exposure to multiple stressors, while it decreased gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. Highest values were observed in T_6 and T_2 group, whereas the lower values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups.

Table 61: Role of dietary L-tryptophan on RBC, hemoglobin (Hb) and haematocrit of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days (Pre and post challenge)

Treatments	RBC (10^6cells/ mm^3)		Hb (g/dL)		Hct (%)	
	Pre	Post	Pre	Post	Pre	Post
T_1 (No stress)	2.06^{deA}±0.02	1.81^{dB}±0.04	9.84^{deA}±02.3	8.20^{cB}±0.09	29.07^{deA}±0.51	24.23^{cB}±0.53
T_2 (MS_1 x 0.0% L-TRP)	1.55^{aA}±0.01	1.37^{aB}±0.03	5.99^{aA}±0.12	5.71^{aB}±0.11	14.26^{aA}±0.25	12.53^{aB}±0.18
T_3 (MS_1 x 0.7% L-TRP)	1.79^{bA}±0.04	1.69^{bA}±0.03	8.03^{bA}±0.23	7.12^{bA}±0.14	20.58^{bA}±0.47	18.79^{bA}±0.36
T_4 (MS_1 x 1.4% L-TRP)	1.99^{cdA}±0.03	1.94^{eA}±0.01	9.34^{cdA}±0.23	8.61^{cdA}±0.26	27.71^{cdA}±0.66	25.88^{dA}±0.71
T_5 (MS_1 x 2.1% L-TRP)	2.11^{eA}±0.02	2.07^{fA}±0.03	10.45^{eA}±0.22	9.63^{eA}±0.05	30.54^{fA}±0.43	28.16^{eA}±0.41
T_6 (MS_2 x 0.0% L-TRP)	1.47^{aA}±0.02	1.27^{aB}±0.03	5.77^{aA}±0.29	5.19^{aB}±0.22	13.14^{aA}±0.24	11.27^{aB}±0.36
T_7 (MS_2 x 0.7% L-TRP)	1.78^{bA}±0.03	1.72^{bA}±0.04	7.68^{bA}±0.28	6.94^{bA}±0.07	19.67^{bA}±0.48	18.18^{bA}±0.14
T_8 (MS_2 x 1.4% L-TRP)	1.93^{cA}±0.05	1.91d^{eA}±0.03	8.77^{cA}±0.20	8.34^{cA}±0.27	26.43^{cA}±0.45	24.91^{cdA}±0.86
T_9 (MS_2 x 2.1% L-TRP)	2.08^{eA}±0.03	2.04^{fA}±0.03	9.88^{deA}±0.30	9.12^{deA}±0.06	29.68^{efA}±0.52	27.79^{eA}±0.45

Values in the same column with different superscript differ significantly ($P<0.05$); Data expressed as Mean ± SE, n=6;

Different superscripts (A and B) in the same row (pre and post challenge) signify statistical differences ($p<0.05$) in paired t-test;

MS_1: Multiple stressors1 (33^0C x pH 9.0); MS_2: Multiple stressors2 (33^0C x pH 5.5)

Table 62: Role of dietary L-tryptophan on WBC, NBT and blood glucose of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days (Pre and Post challenge)

Treatments	WBC (10^3cells/ mm^3)		NBT		Blood glucose (mg/100 mL)	
	Pre	Post	Pre	Post	Pre	Post
T$_1$ (No stress)	$215.77^{deA}\pm2.67$	$218.86^{cA}\pm1.41$	$0.329^{fA}\pm0.01$	$0.347^{cA}\pm0.01$	$38.83^{fA}\pm0.32$	$43.15^{dB}\pm0.39$
T$_2$ (MS$_1$ x 0.0% L-TRP)	$156.83^{aA}\pm1.54$	$158.09^{aA}\pm1.53$	$0.182^{bA}\pm0.01$	$0.196^{aA}\pm0.01$	$64.70^{bA}\pm0.36$	$67.53^{bB}\pm0.63$
T$_3$ (MS$_1$ x 0.7% L-TRP)	$188.34^{bA}\pm2.79$	$198.49^{bA}\pm4.59$	$0.254^{dA}\pm0.01$	$0.307^{bB}\pm0.01$	$51.99^{dA}\pm0.13$	$53.56^{cA}\pm0.75$
T$_4$ (MS$_1$ x 1.4% L-TRP)	$208.32cd^{A}\pm3.44$	$225.50^{cdB}\pm2.42$	$0.319^{eA}\pm0.01$	$0.375^{dB}\pm0.01$	$50.83^{eA}\pm0.53$	$41.95^{deA}\pm0.86$
T$_5$ (MS$_1$ x 2.1% L-TRP)	$224.61^{eA}\pm3.58$	$238.22^{eB}\pm4.26$	$0.342^{gA}\pm0.01$	$0.406^{eB}\pm0.01$	$37.58^{fA}\pm0.35$	$38.95^{fA}\pm1.29$
T$_6$ (MS$_2$ x 0.0% L-TRP)	$150.84^{aA}\pm2.77$	$153.15^{aA}\pm3.67$	$0.168^{aA}\pm0.01$	$0.182^{aA}\pm0.01$	$66.87^{aA}\pm0.41$	$70.42^{aB}\pm0.69$
T$_7$ (MS$_2$ x 0.7% L-TRP)	$179.69^{bA}\pm5.12$	$191.97^{bA}\pm2.18$	$0.236^{cA}\pm0.01$	$0.297^{bB}\pm0.01$	$53.52^{cA}\pm0.42$	$55.39^{cA}\pm0.74$
T$_8$ (MS$_2$ x 1.4% L-TRP)	$204.44^{cA}\pm3.34$	$220.87^{cB}\pm6.08$	$0.311^{eA}\pm0.01$	$0.365^{dB}\pm0.01$	$41.82^{eA}\pm0.85$	$42.99^{dA}\pm0.58$
T$_9$ (MS$_2$ x 2.1% L-TRP)	$218.10^{deA}\pm2.28$	$235.99^{deB}\pm3.15$	$0.337^{fgA}\pm0.01$	$0.397^{eB}\pm0.01$	$38.03^{fA}\pm0.52$	$39.72^{efA}\pm0.43$

Values in the same column with different superscript differ significantly ($p<0.05$); Data expressed as Mean ± SE, n=6;

Different superscripts (A and B) in the same row (pre and post challenge) signify statistical differences ($p<0.05$) in paired t-test;

NBT (Respiratory burst activity) expressed in optimal density at 540 nm;

MS$_1$: Multiple stressors1 (33^0C x pH 9.0); MS$_2$: Multiple stressors2 (33^0C x pH 5.5)

Table 63: Role of dietary L-tryptophan on total serum protein, albumin (A) and globulin (G) of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days (Pre and post challenge)

Treatments	Total protein (g/dL)		Albumin (g/dL)		Globulin (g/dL)	
	Pre	Post	Pre	Post	Pre	Post
T_1 (No stress)	$3.94^{fA}\pm0.06$	$2.86^{dB}\pm0.06$	$0.850^{fA}\pm0.01$	$0.713^{dB}\pm0.01$	$3.09^{eA}\pm0.05$	$2.15^{cB}\pm0.08$
T_2 (MS_1 x 0.0% L-TRP)	$2.09^{bA}\pm0.03$	$1.68^{bB}\pm0.02$	$0.613^{bA}\pm0.01$	$0.507^{bB}\pm0.01$	$1.48^{bA}\pm0.03$	$1.17^{aB}\pm0.03$
T_3 (MS_1 x 0.7% L-TRP)	$2.75^{dA}\pm0.04$	$2.39^{cA}\pm0.09$	$0.690^{dA}\pm0.01$	$0.610^{cA}\pm0.01$	$2.06^{cA}\pm0.05$	$1.78^{bA}\pm0.01$
T_4 (MS_1 x 1.4% L-TRP)	$3.61^{eA}\pm0.07$	$3.33^{eA}\pm0.08$	$0.793^{eA}\pm0.01$	$0.776^{eA}\pm0.01$	$2.81^{dA}\pm0.06$	$2.55^{dA}\pm0.06$
T_5 (MS_1 x 2.1% L-TRP)	$4.16^{gA}\pm0.04$	$3.80^{fA}\pm0.11$	$0.863^{fA}\pm0.01$	$0.840^{fA}\pm0.02$	$3.29^{fA}\pm0.03$	$2.96^{eA}\pm0.09$
T_6 (MS_2 x 0.0% L-TRP)	$1.84^{aA}\pm0.03$	$1.44^{aB}\pm0.02$	$0.560^{aA}\pm0.01$	$0.420^{aB}\pm0.01$	$1.28^{aA}\pm0.03$	$1.02^{aB}\pm0.04$
T_7 (MS_2 x 0.7% L-TRP)	$2.61^{cA}\pm0.04$	$2.37^{cA}\pm0.07$	$0.660^{cA}\pm0.01$	$0.617^{cA}\pm0.01$	$1.95^{cA}\pm0.05$	$1.75^{bA}\pm0.08$
T_8 (MS_2 x 1.4% L-TRP)	$3.52^{eA}\pm0.07$	$3.20^{eA}\pm0.08$	$0.773^{eA}\pm0.01$	$0.757^{eA}\pm0.02$	$2.74^{dA}\pm0.07$	$2.44^{dA}\pm0.08$
T_9 (MS_2 x 2.1% L-TRP)	$4.03^{fgA}\pm0.04$	$3.71^{fA}\pm0.11$	$0.853^{fA}\pm0.01$	$0.827^{fA}\pm0.01$	$3.17^{efA}\pm0.04$	$2.88^{eA}\pm0.11$

Values in the same column with different superscript differ significantly ($p<0.05$); Data expressed as Mean ± SE, n=6;
Different superscripts (A and B) in the same row (pre and post challenge) signify statistical differences ($p<0.05$) in paired t-test;
MS_1: Multiple stressors1 (33^0C x pH 9.0); MS_2: Multiple stressors2 (33^0C x pH 5.5)

Table 64: Role of dietary L-tryptophan on A/G ratio and lysozyme activity of *L. gonius* fingerling exposed to multiple stressors for a period of 60 days (Pre and post challenge)

Treatments	A/G ratio		Serum lysozyme activity	
	Pre	**Post**	**Pre**	**Post**
T_1 (No stress)	0.273^{cA}±0.01	0.333^{bcdB}±0.02	340.89^{eA}±4.16	366.58^{eA}±6.11
T_2 (MS_1 x 0.0% L-TRP)	0.470^{aA}±0.01	0.430^{aB}±0.02	195.18^{bA}±3.95	209.25^{bA}±1.66
T_3 (MS_1 x 0.7% L-TRP)	0.333^{bA}±0.01	0.347^{bcA}±0.01	260.26^{cA}±4.56	293.19^{dB}±3.25
T_4 (MS_1 x 1.4% L-TRP)	0.280^{cA}±0.01	0.307^{cdA}±0.01	320.26^{dA}±2.97	389.68^{fgB}±3.77
T_5 (MS_1 x 2.1% L-TRP)	0.263^{cA}±0.01	0.283^{dA}±0.03	353.39^{fA}±4.68	403.36^{hB}±3.07
T_6 (MS_2 x 0.0% L-TRP)	0.470^{aA}±0.01	0.413^{aB}±0.03	176.42^{aA}±2.32	186.44^{aA}±1.22
T_7 (MS_2 x 0.7% L-TRP)	0.340^{bA}±0.02	0.353^{bA}±0.02	254.13^{cA}±2.60	279.09^{cB}±1.97
T_8 (MS_2 x 1.4% L-TRP)	0.280^{cA}±0.01	0.310^{bcdA}±0.01	310.13^{dA}±2.60	381.45^{fB}±2.19
T_9 (MS_2 x 2.1% L-TRP)	0.266^{cA}±0.01	0.290^{dA}±0.01	346.31^{efA}±3.76	397.25^{ghB}±2.30

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6;

Serum lysozyme expressed as unit/ min/ mg/ serum protein;

MS_1: Multiple stressors1 (33^0C x pH 9.0); MS_2: Multiple stressors2 (33^0C x pH 5.5)

Table 65: Role of dietary L-tryptophan on serum enzyme activity of *L. gonius* fingerling exposed to multiple stressors for a period of 60 days

Treatments	sGOT (IU/L)	sGPT (IU/L)	sALP (IU/L)	sACP (IU/L)	sLDH (IU/L)
T_1 (No stress)	$25.72^{e}\pm0.91$	$9.17^{fg}\pm0.34$	$94.88^{fg}\pm1.63$	$20.18^{f}\pm0.58$	$6.91^{fg}\pm0.33$
T_2 (MS_1 x 0.0% L-TRP)	$53.69^{b}\pm1.39$	$29.28^{b}\pm0.84$	$167.02^{b}\pm2.27$	$39.37^{b}\pm1.22$	$21.11^{b}\pm0.64$
T_3 (MS_1 x 0.7% L-TRP)	$43.06^{c}\pm1.60$	$19.56^{d}\pm0.57$	$130.79^{d}\pm1.76$	$30.56^{d}\pm1.81$	$12.71^{d}\pm0.39$
T_4 (MS_1 x 1.4% L-TRP)	$29.05d^{e}\pm0.70$	$10.96^{ef}\pm0.99$	$99.60^{ef}\pm2.01$	$21.95^{e}\pm0.89$	$8.38^{ef}\pm0.57$
T_5 (MS_1 x 2.1% L-TRP)	$24.98^{e}\pm0.66$	$8.58^{g}\pm0.69$	$89.64^{g}\pm2.85$	$18.62^{f}\pm1.16$	$6.53^{g}\pm0.43$
T_6 (MS_2 x 0.0% L-TRP)	$65.70^{a}\pm1.71$	$33.11^{a}\pm0.64$	$177.61^{a}\pm2.48$	$43.92^{a}\pm0.64$	$24.40^{a}\pm0.49$
T_7 (MS_2 x 0.7% L-TRP)	$46.82^{c}\pm1.53$	$21.98^{c}\pm0.66$	$138.80^{c}\pm1.82$	$35.02^{c}\pm1.25$	$15.57^{c}\pm0.78$
T_8 (MS_2 x 1.4% L-TRP)	$31.63^{d}\pm1.95$	$12.74^{e}\pm0.45$	$104.98^{e}\pm1.60$	$23.80^{e}\pm0.71$	$9.39^{e}\pm0.32$
T_9 (MS_2 x 2.1% L-TRP)	$25.38^{e}\pm0.58$	$8.84^{fg}\pm0.86$	$93.01^{g}\pm1.74$	$19.98^{f}\pm1.22$	$6.86^{fg}\pm0.68$

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6;

MS_1: Multiple stressors1 (33^0C x pH 9.0); MS_2: Multiple stressors2 (33^0C x pH 5.5)

4.4.9 Serum Cortisol

The serum cortisol level of *L. gonius* fingerlings in different experimental groups is shown in Table 66. Cortisol level increased significantly upon exposure to multiple stressors, while it decreased gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. Highest values were observed in T_6 and T_2 group, whereas the lower values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups. Cortisol level of different experimental groups challenged with *A. hydrophila* is shown in Table 66 and follows the similar trends as of pre-challenge study, though overall higher value was observed in post- challenge.

Table 66: Role of dietary L-tryptophan on cortisol of *L. gonius* fingerling exposed to multiple stressors for a period of 60 days (Pre and post challenge)

Treatments	Cortisol (ng/mL)	
	Pre	Post
T_1 (No stress)	100.56^{efA}±3.38	117.81^{efB}±2.58
T_2 (MS_1 x 0.0% L-TRP)	187.68^{bA}±4.59	210.41^{bB}±4.96
T_3 (MS_1 x 0.7% L-TRP)	152.26^{cA}±4.33	159.67^{dB}±5.36
T_4 (MS_1 x 1.4% L-TRP)	109.50^{deA}±3.44	111.01^{fA}±3.94
T_5 (MS_1 x 2.1% L-TRP)	87.35^{gA}±2.88	89.82^{gA}±1.91
T_6 (MS_2 x 0.0% L-TRP)	206.67^{aA}±5.28	224.93^{aB}±6.10
T_7 (MS_2 x 0.7% L-TRP)	163.43^{cA}±3.31	179.08^{cB}±3.27
T_8 (MS_2 x 1.4% L-TRP)	119.31^{dA}±5.69	127.28^{eA}±2.42
T_9 (MS_2 x 2.1% L-TRP)	94.09^{fgA}±2.87	97.51^{gA}±4.09

Values in the same column with different superscript differ significantly ($p<0.05$); Data expressed as Mean ± SE, n=6; Different superscripts (A and B) in the same row (pre and post challenge) signify statistical differences ($p<0.05$) in paired t-test;
MS_1: Multiple stressors1 (33^0C x pH 9.0); MS_2: Multiple stressors2 (33^0C x pH 5.5)

4.4.10 Hsp-70 Expression

Hsp-70 level of *L. gonius* fingerling in different experimental groups is shown in Table 67. Hsp-70 levels increased significantly upon exposure to multiple stressors, while it decreased gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. The high level of Hsp-70 was recorded in T_6 and T_2 treatments whereas the lowest was in T_5 and T_9 treatment groups. Serum cortisol does not show significant difference ($p>0.05$) among T_1, T_5, and T_9 treatments.

Table 67: Role of dietary L-tryptophan on expression of Hsp-70 of *L. gonius* fingerling exposed to multiple stressors for a period of 60 days

Treatments	Hsp-70 expression (ng/mL)		
	Gill	Liver	Serum
T_1 (No stress)	$3.01^{d}\pm0.07$	$2.50^{d}\pm0.10$	$5.81^{fg}\pm0.26$
T_2 (MS_1 x 0.0% L-TRP)	$8.71^{a}\pm0.46$	$6.42^{a}\pm0.54$	$22.89^{b}\pm0.69$
T_3 (MS_1 x 0.7% L-TRP)	$5.30^{b}\pm0.48$	$4.18^{c}\pm0.43$	$14.28^{d}\pm0.33$
T_4 (MS_1 x 1.4% L-TRP)	$3.75^{c}\pm0.37$	$2.90^{d}\pm0.21$	$7.37^{ef}\pm0.50$
T_5 (MS_1 x 2.1% L-TRP)	$2.88^{d}\pm0.29$	$2.50^{d}\pm0.19$	$5.27^{g}\pm0.26$
T_6 (MS_2 x 0.0% L-TRP)	$9.65^{a}\pm0.53$	$6.95^{a}\pm0.51$	$26.13^{a}\pm0.91$
T_7 (MS_2 x 0.7% L-TRP)	$6.45^{b}\pm0.54$	$5.23^{b}\pm0.23$	$17.57^{c}\pm0.73$
T_8 (MS_2 x 1.4% L-TRP)	$4.15^{cd}\pm0.47$	$3.11^{d}\pm0.37$	$8.06^{e}\pm0.30$
T_9 (MS_2 x 2.1% L-TRP)	$2.85^{d}\pm0.14$	$2.47^{d}\pm0.15$	$5.73^{fg}\pm0.32$

Values in the same column with different superscript differ significantly ($p<0.05$); Data expressed as Mean ± SE, n=6;
MS_1: Multiple stressors1 (33^{0}C x pH 9.0); MS_2: Multiple stressors2 (33^{0}C x pH 5.5)

4.4.11 Thyroid Hormones (T3 and T4)

Thyroid hormones (T3 and T4) levels of *L. gonius* fingerlings in different experimental groups are shown in Table 68. The serum T3 and T4 levels decreased significantly upon exposure to multiple stressors, while it increased gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. It was found that the T3 and T4 hormone levels varied significantly ($p<0.05$) among different experimental groups. Lowest values of T3 and T4 hormone were observed in T_6 and T_2 group, whereas the higher values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups. A linear relationship was observed between weight gain % and thyroid hormone levels of *L. gonius* fingerlings (Figure 21 & 22).

Figure 21: Role of L-tryptophan on relationship between weight gain % and T3 hormone in serum of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

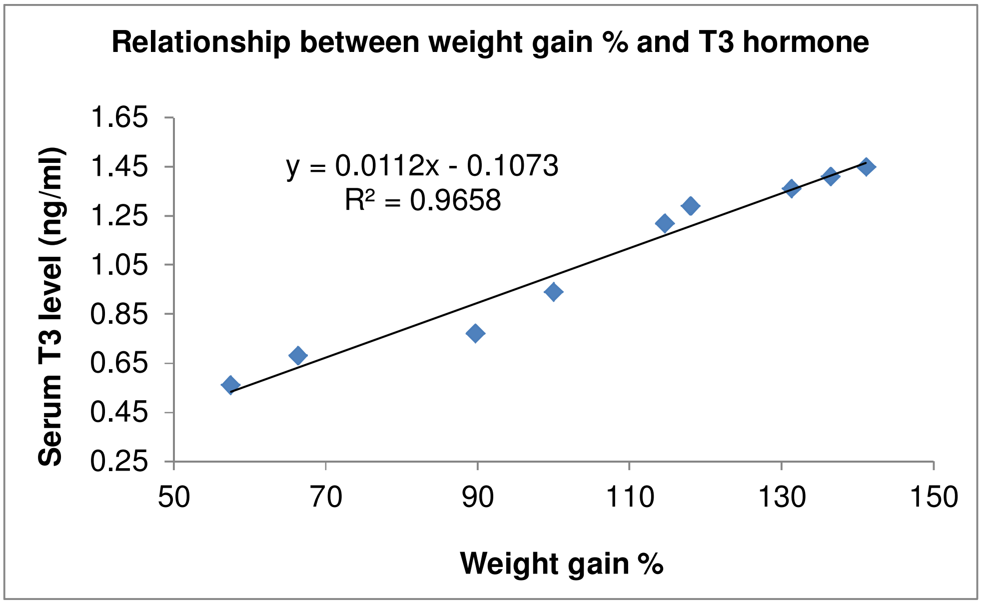


Figure 22: Role of L-tryptophan on relationship between weight gain % and T4 hormone in serum of *L. gonius* fingerlings exposed to multiple stressors a period of 60 days

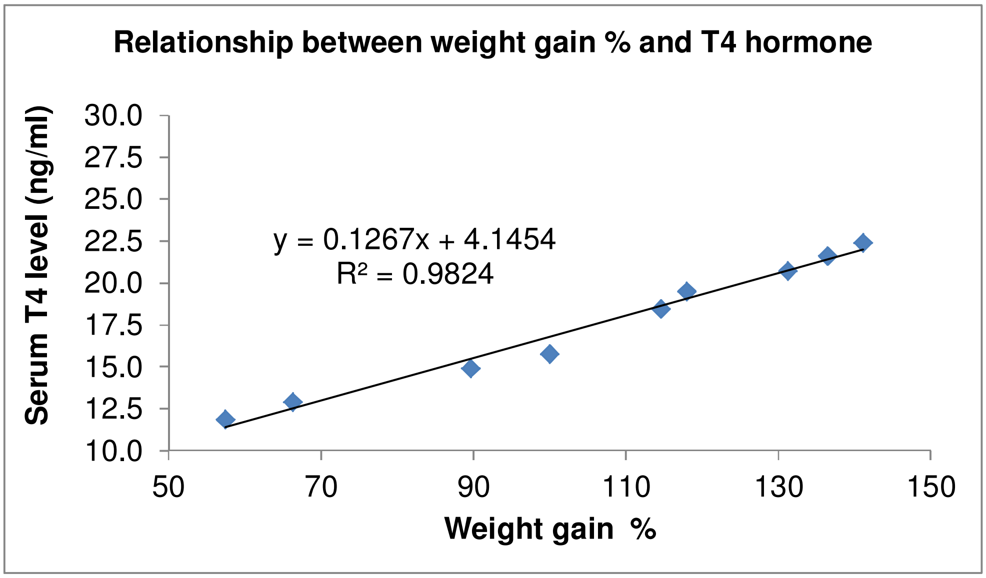


Table 68: Role of dietary L-tryptophan on Thyroid hormones (T3 and T4), electrolytes and Osmolality of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days

Treatments	Thyroid hormones (ng/mL)		Electrolytes (mEq/L)			Osmolality
	T3	T4	Na^+	K^+	Cl^-	(Osmol/L)
T_1 (No stress)	$1.36^{de}\pm0.04$	$21.42^{de}\pm0.34$	$146.33^{f}\pm2.19$	$3.33^{d}\pm0.43$	$134.33^{e}\pm0.88$	$0.294^{e}\pm0.016$
T_2 (MS_1 x 0.0% L-TRP)	$0.68^{ab}\pm0.03$	$12.92^{a}\pm0.50$	$128.00^{ab}\pm4.58$	$5.20^{a}\pm0.26$	$119.33^{ab}\pm2.03$	$0.243^{ab}\pm0.012$
T_3 (MS_1 x 0.7% L-TRP)	$0.94^{c}\pm0.13$	$15.79^{b}\pm0.70$	$135.00^{bcd}\pm2.64$	$4.53^{abc}\pm0.35$	$124.00^{bc}\pm1.53$	$0.263^{abcd}\pm0.004$
T_4 (MS_1 x 1.4% L-TRP)	$1.29^{de}\pm0.05$	$19.50^{cd}\pm0.85$	$138.67^{cdef}\pm1.76$	$4.03^{bcd}\pm0.18$	$128.67^{cd}\pm2.03$	$0.272^{bcde}\pm0.007$
T_5 (MS_1 x 2.1% L-TRP)	$1.45^{e}\pm0.06$	$22.40^{e}\pm0.33$	$144.33^{ef}\pm1.45$	$3.63^{cd}\pm0.29$	$132.00^{de}\pm1.55$	$0.287^{de}\pm0.005$
T_6 (MS_2 x 0.0% L-TRP)	$0.56^{a}\pm0.04$	$11.88^{a}\pm0.64$	$124.67^{a}\pm2.03$	$5.37^{a}\pm0.30$	$117.67^{a}\pm2.91$	$0.238^{a}\pm0.007$
T_7 (MS_2 x 0.7% L-TRP)	$0.77^{b}\pm0.08$	$14.94^{b}\pm0.58$	$133.67^{bc}\pm2.91$	$4.77^{ab}\pm0.35$	$124.67^{bc}\pm2.91$	$0.254^{abc}\pm0.010$
T_8 (MS_2 x 1.4% L-TRP)	$1.22^{d}\pm0.05$	$18.49^{c}\pm0.70$	$136.67^{cde}\pm2.71$	$4.10^{bcd}\pm0.46$	$126.67^{cd}\pm1.45$	$0.270^{bcde}\pm0.004$
T_9 (MS_2 x 2.1% L-TRP)	$1.41^{de}\pm0.06$	$21.63^{e}\pm0.73$	$142.67^{def}\pm2.33$	$3.70^{bcd}\pm0.29$	$131.33^{de}\pm1.20$	$0.281^{cde}\pm0.009$

Values in the same column with different superscript differ significantly ($p<0.05$);

Data expressed as Mean ± SE, n=6;

MS_1: Multiple stressors1 (33^0C x pH 9.0); MS_2: Multiple stressors2 (33^0C x pH 5.5)

4.4.12 Serum Electrolytes (Na^+, K^+ and Cl^-)

Serum electrolytes (Na^+, K^+ and Cl^-) level of *L. gonius* fingerlings in different experimental groups are presented in Table 68. Na^+ and Cl^- levels in serum decreased significantly ($p<0.05$) significantly upon exposure to multiple stressors ($p<0.05$), while it prevented the loss of ions with increase in dietary L-tryptophan supplementation. Lowest values of Na^+ and Cl^- were observed in T_6 and T_2 group, whereas the higher values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups. On the contrary, K^+ levels in serum increased significantly ($p<0.05$) significantly upon exposure to multiple stressors ($p<0.05$), while it prevent the increase with increase in dietary L-tryptophan supplementation. Highest values of K^+ was observed in T_6 and T_2 group, whereas the lower values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups.

4.4.13 Serum Osmolality

Serum osmolality level of *L. gonius* fingerlings in different experimental groups is presented in Table 68.The serum osmolality decreased significantly ($p<0.05$) upon exposure to multiple stressors, while it prevented the loss with increase in dietary L-tryptophan supplementation. Lowest values of osmolality was observed in T_6 and T_2 group, whereas the higher values were observed in T_5, T_9, T_1, T_4 and T_8 treatment groups.

4.4.14 Survival % in Challenge Study

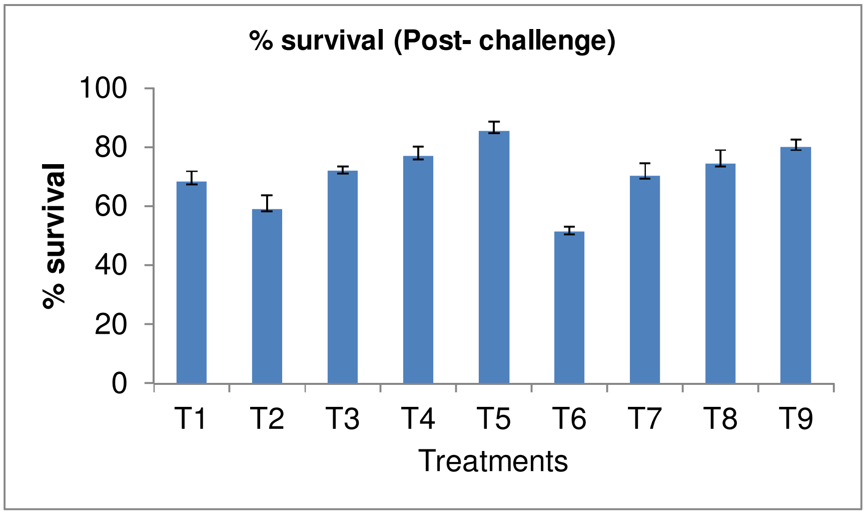
After 60 days of experimental trial, all the experimental groups were challenged with *Aeromonas hydrophila* and the observation was made for a week. The percentage survival of *L. gonius* fingerlings in different experimental groups is presented in Table 69 and Figure 23. The percentage survival decreased significantly ($p<0.05$) upon exposure to multiple stressors, while it increased gradually ($p<0.05$) with increase in dietary L-tryptophan supplementation. Lowest survival % was recorded in T_6 and T_2 treatments whereas the highest was in T_5 and T_9 groups followed by T_4 and T_8 group.

Table 69: Role of dietary L-tryptophan on % survival of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days (Post challenge)

Treatments	% survival
T_1 (No stress)	$68.44^{cd} \pm 3.40$
T_2 (MS_1 x 0.0% L-TRP)	$59.09^{de} \pm 4.55$
T_3 (MS_1 x 0.7% L-TRP)	$72.58^{bc} \pm 1.45$
T_4 (MS_1 x 1.4% L-TRP)	$77.02^{abc} \pm 3.22$
T_5 (MS_1 x 2.1% L-TRP)	$85.60^{a} \pm 3.06$
T_6 (MS_2 x 0.0% L-TRP)	$51.52^{e} \pm 1.51$
T_7 (MS_2 x 0.7% L-TRP)	$70.47^{bc} \pm 4.09$
T_8 (MS_2 x 1.4% L-TRP)	$74.50^{bc} \pm 4.38$
T_9 (MS_2 x 2.1% L-TRP)	$80.05^{ab} \pm 2.56$

Values in the same column with different superscript differ significantly ($p<0.05$); Data expressed as Mean ± SE, n=10; MS_1: Multiple stressors1 (33^0C x pH 9.0); MS_2: Multiple stressors2 (33^0C x pH 5.5)

Figure 23: Role of L-tryptophan on % survival of *L. gonius* fingerlings exposed to multiple stressors for a period of 60 days (Post challenge)



Discussion

5. DISCUSSION

5.1 Experiment 1

Temperature is a major factor, which directly influences metabolism affecting all physiological processes in ectotherms such as food intake, metabolism and nutritional efficiency. Therefore, knowledge of suitable temperatures at which fish have a faster growth rate is very important for effective management of aquaculture systems. Fish physiology is inexplicably linked to temperature and fish have evolved so as to cope with specific hydrologic regimes and habitat niches. Therefore, their physiology and life histories will be affected by alterations induced by climate change. As it strengthens over time, global climate change will become a more powerful stressor for fish living in natural or artificial systems. Therefore, increasing global temperatures can affect individual fish by altering physiological functions such as thermal tolerance, growth, metabolism, food consumption, reproductive success and the ability to maintain internal homeostasis in the face of a variable external environment. In this backdrop, the present experiment was designed to study the growth and physio-biochemical responses of *Labeo gonius* fingerlings exposed to four different temperatures.

5.1.1 Physico-Chemical Properties of Water

In our experiment, we maintained a low stocking density and continuous aeration in order to avoid any confinement stress and ammonia accumulation in rearing tanks. Along with these, regular exchange of water in the rearing tank helps us to maintain the physico-chemical parameters of water such as pH, dissolved oxygen, free carbon dioxide, ammonia, nitrite-N and nitrate-N within the optimum range of requirements for the fish as suggested by many authors.

Temperature plays an important role in regulating the metabolism of animals; therefore an optimum range of temperature is required for optimum metabolic activity, which in turn gives maximum yield. As our experiment is temperature dependent, so we maintained desired water temperatures (27, 30, 33 and 36^0C) required for our experiment. Water pH is considered as one of the important parameters for successful aquaculture production. The optimum pH range for culture of carps is 7.5-8.5 (Banerjea, 1967), and the majority of the Indian carp culture ponds have a pH in this range. In the present study also, water pH was within the range of 6.87-7.61 in all the experimental groups, which is quite favorable for fish culture. The dissolved oxygen level in water varies with a large number of factors such as water temperature, metabolic rate, biomass density, etc. The dissolved oxygen in different experimental tubs was recorded to be within the range of 5.89-7.29 mg/L, which is within the optimum range of 6-7 mg/L for cyprinids suggested by Huet (1975). From the above result, it is assumed that dissolved oxygen content was optimum throughout the experimental period, which might be due to efficient aeration. Toxic concentrations of ammonia for short-term exposure are between 0.6 and 2 mg/L (EIFAC, 1973). The suggested value of ammonia in water ranges from 0 to 0.1 mg/L (Jhingran, 1991), which is in agreement with the recorded range of 0.15-0.27 mg/L in the present study. Nitrite-N concentration was recorded in the range of 0.001-0.009 mg/L, which is well within the permissible range for pond aquaculture (Boyd and Trucker, 1998). Nitrate-N level in a productive pond can be within 0.1-5.0 mg/L (Boyd and Trucker, 1998). In the present study, nitrate-N (0.03 to 0.14 mg/L) was below the toxic level. Nitrite-N and nitrate-N levels were within the permissible limits for warm water fish (Boyd, 1982).

5.1.2 Growth and Survival

In the present study, weight gain %, SGR, FER, PER and survival % decreased, whereas FCR values increased significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. The decrease in growth rate upon exposure to higher temperature can be attributed to re-allocation of energy towards maintenance and repair under stress (Kumaraguru and Beamish, 1986). Das *et al.* (2005) also observed reduction in the growth rate, survival percentage and feed conversion efficiency in *L. rohita* fingerlings reared at higher temperature. Akhtar *et al.* (2011) also reported similar findings. Reduced growth rate and food conversion efficiency have been reported in fish at combined stress of higher temperature and salinity (Likongwe *et al.*, 1996). On the contrary, the higher growth rate, survival percentage and feed conversion efficiency were observed at 27 and 30^0C. The higher weight gain may be attributed to improved dietary protein utilization at ambient temperature. Das *et al*. (2005) and Akhtar *et al*. (2011) also reported higher growth rate and feed conversion efficiency at ambient temperature.

5.1.3 Body Indices

In the present study, HSI decreases significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. The reduced values in the fishes exposed to higher temperature may be due to thermal stress. However, GSI did not vary significantly among the different treatment. Ciji *et al*. (2011) also reported higher HSI values in *Labeo rohita* fingerlings reared at ambient water temperatures than the higher temperature.

5.1.4 DNA, RNA and RNA/DNA Ratio

In the present study, RNA and RNA/DNA ratio decreases significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. The reduced values in the fishes exposed to higher temperature can be correlated with the lower growth rate incurred due to temperature stress. The ratios between these two nucleic acids were reported to reduce, when the fishes were exposed to high temperatures (Spigarelli and Smith, 1976) or heavy metal salts (Kearns and Atchison, 1979). On the contrary, higher RNA and RNA/DNA ratios were observed at 27 and 30^0C. The higher RNA and RNA/DNA ratio can be correlated with higher growth rate observed during the study. A strong positive correlation has been reported between nucleic acid concentration and specific growth rate (Bulow, 1970; Bulow *et al*., 1981; Haines, 1973; Fukuda *et al*., 2001). As the RNA content is related to rate of protein synthesis, the RNA/DNA ratio can be used as an index of growth (Smith and Buckley, 2003; Akhtar *et al*., 2012a). We did not observe any significant change in DNA levels due to temperature change, and our findings are in support of Leslie (1955) who found that DNA content per somatic cell is constant within a species.

5.1.5 Tissue Glycogen

Glycogen breakdown takes place due to effect of catecholamine epinephrine/ nor epinephrine. Catecholamine epinephrine / nor epinephrine induce glycogenolysis through beta- adrenoreceptors (Sheridan and Muir, 1988). Several researchers have studied glycogenolysis in response to various stressors. Increase in glucose and concomitant decrease in glycogen on exposure to various environmental toxicants has been reported (Singh and Aggarwal, 1986). Nakano and Tomlinson *et al.* (1967) observed that all types of stress elevated the secretion of catecholamine which in turn increased the breakdown of glycogen and elevated blood glucose level. In the present study, glycogen content in liver and muscle tissues decreased significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. The glycogen content was of liver was found to be higher than the muscle tissue. The increase in glucose and concomitant depletion in glycogen shows that the glycogen was utilized for energy to cope with the temperature induced stress. The depletion of glycogen was lowest observed at 27 and 30^0C.

5.1.6 Ascorbic Acid

Vitamin C is a major water soluble antioxidant and is capable of maintaining sulphydryl compounds in a reduced state, particularly in several redox reactions (Deshpande *et al.*, 2002). Teleost do not have an active L-gulonolactone oxidase, the terminal enzyme in the ascorbic acid synthesis pathway (Dabrowski, 1990a). Thus, fishes are sensitive to a threshold level of vitamin C as they are unable to synthesize it *de novo* (Dabrowski, 1990b). Vitamin C is considered to play an important role in animal health as antioxidants, inactivating damage of free radicals produced during normal cellular activity from various stressors (Chew, 1995) have been reported, confirming protective roles of vitamin C. Cruz de Menezes *et al.* (2006) and Norouzitallab *et al.* (2008) also reported that vitamin C is well known for its anti-oxidative property and has also been used as an immuno-modulator in aquaculture. In the present study, ascorbic acid content in muscle tissue decreased significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. The decrease in ascorbic acid content in tissue at higher temperature groups could be attributed to its maximum utilization to reduce free radicals produced during temperature stress. Das *et al.* (2009) also reported decrease in ascorbic acid content at higher temperature. Similar results were also found in thornfish, *Therapon jarbua* (Forskal) when exposed to thermal stress (Chien *et al.*, 1999).

5.1.7 Enzymatic Activity in Tissue

5.1.7.1 Enzymes of protein metabolism (AST/GOT and ALT/GPT)

Transaminases are the enzymes that redistribute amino nitrogen among amino acids, forming new amino acids with the amino group from the pre-existing ones. Non-essential amino acids required for growth and other metabolic processes are produced by this manner. Amino acids are the major source of energy in teleost (Belinski, 1974). Fishes utilize proteins and lipids prior to carbohydrates (Demeal, 1978). Stress hormone cortisol induces gluconeogenesis, which mobilize the protein source and lipid source for glucose synthesis. The most important aminotransferases are aspartate amino transferases (AST) and alanine amino transferases (ALT) (also called glutamate oxalo acetate transaminase or GOT and glutamate pyruvate transaminase or GPT). AST transfers the amino group of aspartic acid to α-keto glutarate and converts to glutamic acid forming oxalo acetate. ALT transfers the amino group from alanine to α-keto glutarate forming glutamic acid pyruvate. The pyruvate and oxalo-acetate thus formed are responsible for the synthesis of non-essential amino acids like alanine, aspargine, glutamine, etc. which in turn helps in protein synthesis and hence growth of the animal. Apart from this the amino acids are deaminated or transaminated to produce TCA cycle intermediates also helping in energy production in the form of ATP (De Silva and Anderson, 1995).

In the present study, AST and ALT activity in liver and muscle tissues increased significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. The higher activity of both the transferase creating higher free amino acid mobilization to produce higher glucose to cope up with the stress incurred due to higher temperature. Similar observation was made by Verma *et al.* (2006) in *Cyprinus carpio* at higher temperatures. The increase in activity indicates greater mobilization of amino acid for glucose production via gluconeogenesis for glucose production to cope with stress (Chatterjee *et al.*, 2006). Knox and Greenland (1965) also reported that elevated levels of transaminase activity during stress would lead to increased feeding of ketoacids in to TCA cycle, there by affecting oxidative metabolism.

5.1.7.2 Enzymes of carbohydrate metabolism (LDH and MDH)

Lactate dehydrogenase is the terminal enzyme of the glycolysis (Embden-Meyerhoff pathway) pathway, which converts lactate to pyruvate in the presence of coenzyme NADH which is converted to NAD^+. Thus, lactate dehydrogenage helps in maintaining the glycolysis cycle by supplying NAD^+. In presence of enough oxygen, pyruvate enters the Kreb's cycle but when there is an oxygen shortage in the tissue, pyruvate is converted to lactate (Murray *et al*., 2000). Generally LDH activity increases in stress *viz*. temperature stress (Grigo, 1975), starvation stress (Vijayaraghavan and Rao, 1986). The increase in LDH activity is attributed to the production of preferred substrate (Lactate) for gluconeogenesis under thermal stress (Chatterjee *et al.,* 2006). A significant increase in LDH activity was observed at higher acclimation temperature (Verma *et al.,* 2006) that may be due to higher production of lactate, indicating oxygen limited condition in the cell. In the present study, LDH activity in liver and muscle tissues increased significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature, which was further aggravated at 36^0C. The result suggest animals under thermal stress showed increased lactate production for gluconeogenesis, which is supported by Chatterjee *et al.* (2006) and Verma *et al.* (2006).

Malate Dehydrogenase (MDH) is an enzyme of TCA cycle, which oxidizes malate to oxaloacetate, using NAD^+ as the electron acceptor. The activity of MDH showed an increase during gluconeogenesis and oxaloacetate is formed from the deamination of aspartate and malate or phosphoenol pyruvate from pyruvate. The conversion of oxaloacetate to malate in the mitochondria and the reverse in cytoplasm is catalyzed by MDH (Das, 2002c). Verma *et al*. (2006) observed that there was a significant increase in MDH activity in *C. carpio* fingerlings acclimated at higher temperatures in order to use the product (oxaloacetate) due to the higher activity of AST for production of more energy (ATP), which may be utilized for other physiological activities. Glucose might have therefore mobilized through non-carbohydrate source; mainly by protein, as transaminase activities were more at higher temperatures. In the present study, MDH activity in liver and muscle tissues increased significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. The higher activity of MDH is indicative of greater activity of TCA cycle due to increased energy demands during stress. This is supported by the findings of Das *et al.* (2006), who found elevated MDH activity in fishes at higher temperature.

5.1.7.3 Enzyme of neurotransmission (AchE)

Acetyl choline esterase (AchE) is one of the most widely used enzymes as a biomarker for environmental pollution. Biomarkers are defined as biochemical, cellular or physiological variations which can be measured in an organism as response to an environmental perturbation or a contamination event (Livingstone *et al*., 1997). Among different biomarkers, a prominent position is occupied by the measure of acetylcholine esterase inhibition. Acetylcholine is widely distributed in nervous tissue, stored and released from the synaptic vesicles. It helps in the depolarization in an adjacent neuron and thus passes nerve impulse. Acetylcholine is rapidly destroyed by choline esterase, a group of related enzymes which are hydrolytic in action (Stowe, 1969). It is well known that some pesticides inhibit AchE which increases the acetylcholine and stimulates the medullary cells to release catecholamine (Stowe, 1969) and also increase in aberrant behaviour (Murthy *et al.,* 1984; Jones *et al.,* 1999). Increased catecholamine may affect the activity of enzymes involved in glycogen synthesis and glycogenolysis. Thus, temperature has an effect on physiological parameters as well as neurotransmission. In the present study, AchE activity in brain tissue decreased significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. This indicates that decreased activity of AchE enzyme was due to stress acquired at higher temperature. Similar observation was made by Verma *et al*. (2006) in *C. carpio* at higher temperature.

5.1.7.4 Enzymes of oxidative stress (Catalase SOD and GST)

Under normal physiological conditions animal cell produces reactive oxygen species (ROS) such as H_2O_2, O_2^-, OH^- that may damage most cellular components leading to cell death. When the rate of ROS generation exceeds that of their normal, oxidative stress occurs. Oxidative stress results when the antioxidant defenses are overcome by pro-oxidant forces and reactive oxygen species are not adequately removed (Sies *et al.*, 1986). Living organisms are protected from the danger of reactive oxygen species (ROS) by several defense mechanisms including antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, etc. Antioxidants also act in conjunction with these enzymatic defenses. Superoxide dismutase (SOD), a cytosolic enzyme that is specific for scavenging superoxide radicals (O_2^-) is involved in protective mechanisms within tissue injury following oxidative process and phagocytosis. Catalase (CAT) is another major primary antioxidant defense component that works primarily to catalyze the decomposition of H_2O_2 to H_2O, sharing this function with glutathione peroxidase (GPX). Therefore both these enzymes detoxify H_2O_2 derived from SOD activity. In the presence of low H_2O_2 levels, organic peroxides are the preferred substrate for GPX. However at high H_2O_2 concentrations they are metabolized by CAT (Yu, 1995). Changes in antioxidative enzymes can be used as stress indicators in fish (Akhtar *et al.*, 2010; Ciji *et al.*, 2012). In the present study, catalase, SOD and GST activity in liver and gill tissues increased significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. The increased activity indicates oxidative stress due to higher temperature.

5.1.7.5 Enzyme of phospho monoesterase (ALP)

Alkaline phosphatase is zinc containing metallo-enzyme, which plays an important role in metabolism of phosphorus in the body. It is involved in membrane transport, glycogen metabolism, protein synthesis, calcium absorption and many other physiological processes including secretary function. In the present study, ALP activity in liver and muscle tissues increased significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. The higher ALP activity may be due to hydrolysis of high- energy phosphate bonds to liberate phosphate ions to combat stressful condition or higher metabolic rate. Similar observation was made by Verma *et al.* (2006) in *C. carpio* fingerlings.

5.1.7.6 Enzyme of gluconeogenic pathways (FDPase)

In the present study, Fructose-1, 6-Diphosphatase activity in liver and muscle tissues increased significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. The increase in FDPase activity with increasing temperature indicates that gluconeogenic pathway gets activated to cope with the increasing metabolic demand at higher temperature. A consistent increase of transaminase might have provided the substrate for gluconeogenesis (Suarez *et al.,* 1987; Moon and Foster, 1995). Hence, higher temperature may increase energy demands in the *L. gonius* fingerlings by increasing FDPase activity.

5.1.7.7 Enzyme of pentose phosphate pathway (G6PDH)

Glucose-6-phosphate dehydrogenase is an enzyme of the pentose phosphate pathway or hexose monophosphate shunt. It mediates the conversion of glucose-6-phosphate to 6-phospho-gluconolactone using $NADP^+$ as a coenzyme and releases NADPH. This pathway activity increases when the requirement of NADPH increases especially during the synthesis of fatty acids, cholesterol and sphingolipids (lipid anabolism). NADPH produced is also required by NADPH oxidase in producing superoxide anions for destroying phagocytosized material. So the enzyme activity may increase with increase in phagocytosis (Das, 2002b). In the present study, G6PDH activity in liver and muscle tissues tissue decreased significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C.The enzyme activity was higher in liver compared to muscle. The lower G6PDH activity at higher temperature is also reported by Verma *et al.* (2006) in *C. carpio* fingerlings. Similar reduction in G6PDH activity with increased cortisol concentration was observed by Tripathi and Verma (2003) in fishes. Lower activity of enzyme at higher temperature might be due to higher cortisol level.

5.1.7.8 Enzyme of energy metabolism (ATPase)

It hydrolyzes a high-energy phosphate of ATP and utilizes that energy for the simultaneous transport of three Na^+ ions outward and two K^+ ion inward across the plasma membrane (Das, 2002a) i.e. ATPase hydrolyzes the high energy phosphate (ATP) and utilizes the energy to maintain ionic gradient across the plasma membrane. The activity of this enzyme is inhibited by the depletion of substrate ATP or due to some organochlorines. Stress leads to imbalance in osmoregulation. In freshwater Na^+ and Cl^- ions are lost and in marine water the ions are gained (Mazeaud *et al*. 1977). In the present study, ATpase activity in gill and liver tissues increased significantly ($p<0.05$) on exposure to extreme higher temperature 36^0C as compared to ambient temperature groups. However, we did not observed any significance difference at 27, 30 and 33^0C.The increase in ATpase activity may be attributed to osmoregulatory stress. Similar observation was made by Verma *et al*. (2006) in *C. carpio* fingerlings.

5.1.7.9 Digestive enzyme (Protease)

In the present study, protease enzyme activity in intestinal tissue decreased significantly ($p<0.05$) with increase in rearing temperatures. The lower activity was recorded at higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. The decrease in enzyme activity at higher temperature indicates inappropriate utilization of nutrients due to thermal stress. Prabhu (2012) also reported to decrease in protease activity on exposure to elevated temperature and endosulfan stress.

5.1.8 Haemato-immunological Parameters

5.1.8.1 Erythrocyte (RBC), haemoglobin (Hb) and haematocrit (Hct)

In the present study, RBC, Hb and Hct count decreased significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. This indicates reduced blood oxygen carrying capacity at higher temperatures. The decreased count could be due to the erythrocytic cell lysis of blood cells and an increased removal of RBCs from the circulation as suggested in carp (Jensen, 1990; Knudsen and Jensen 1997) or haemodilution (Wedemeyer *et al*., 1984). Reduction in serum protein content in in the present study also supports the view of erythrocytic cell lysis and haemodilution. Akhtar *et al*. (2011) also reported reduced RBC and Hb count in *L. rohita* fingerlings at 33^0C and higher count at ambient temperature.

5.1.8.2 Leucocyte (WBC) count

Leucocytes (WBC) play an important role for enhancing non-specific or innate immunity, which is considered as an indicator of the health status of fish (Roberts, 1978). The leukocytes are involved in regulation of immunological function in the organism (Santhakumar *et al.*, 1999). In the present study, WBC count decreased significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. This may be due to increased basal cortisol level, which reduces leucocyte count either due to increased apoptosis, reduction in leucocyte proliferation or leucocyte redistribution or weakening the leucopoiesis process. Verma *et al.* (2007) also reported reduction in leucocyte in *C. carpio* fingerlings on exposure to higher temperature, which was exacerbated on concurrent exposure of temperature and chlorine stress. Similarly, Akhtar *et al.* (2011) also reported reduced WBC count in *L. rohita* fingerlings at 33^0C and higher values at ambient temperature.

5.1.8.3 Nitroblue tetrazolium (NBT)

The phenomenon known as respiratory burst gives a measure of oxygen dependent defense mechanism in vertebrate phagocytic cells, wherein there is generation of reactive oxygen intermediates with powerful anti-microbial activity (Itou *et al.*, 1997). Increased respiratory burst activity can be correlated with increased bacterial pathogen killing activity of phagocytes (Sharp and Secombes, 1993) and hence a better immunity. The respiratory burst activity of phagocytes was measured by reduction of NBT by intracellular superoxide radicals produced by leucocytes.

In the present study, NBT activity decreased significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. Verma *et al.* (2007) also reported reduction in NBT activity on exposure to higher temperature, which was exacerbated on concurrent exposure of temperature and chlorine stress in *C. carpio* fingerlings. Similarly, Akhtar *et al.* (2011) also reported reduced NBT activity in *L. rohita* fingerlings at 33^0C and higher values at ambient temperature.

5.1.8.4 Blood glucose

Glucose is one of the most important sources of energy for the animals and glucose has been studied as an indicator of stress caused by physical factors (Manush *et al*., 2005). Nakano and Tomlinson (1967) observed that all types of stress elevated the secretion of catecholamine which in turn increased the breakdown of glycogen and enhanced blood sugar level. The elevation of blood sugar levels in fish by both corticosteroids and catecholamines makes it the ideal parameter to study the secondary stress response, on activation of direct sympathetic (Chromaffin tissue) as well as humoral (internal tissue) pathways (Wedemeyer and Mcleay, 1981). In the present study, blood glucose level increased significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. Das *et al*. (2009) also reported increase level of blood glucose at higher temperature and low level at ambient temperature. The reason for increase in blood glucose may be due to higher cortisol levels, which is a hyperglycemic hormone. Nakano and Tomlinson (1967) also observed that all types of stress elevated the secretion of catecholamine which in turn increased the breakdown of glycogen and enhanced blood sugar level. Another reason could be increased in glycolysis is also indicated by increased LDH activity in liver and muscle due to higher production of lactate. Similar observation was made by Suarez and Mommsen (1987), Moon and Foster (1995) and Verma *et al.* (2006). Positive correlation between glucose level and temperature acclimation is reported by Costas *et al*. (2012).

5.1.8.5 Albumin (A), globulin (G), A-G ratio and total serum protein

Proteins are the most important compounds in the serum, and its concentration is used as a basic index for the health status of fish (Buchanan *et al*., 1997). Among the serum protein, albumin and globulin are the major proteins, which play a significant role in the immune response (Kumar *et al*., 2007). The gamma globulin fraction is the source of almost all the immunological active protein of the blood. Globulins like gamma globulins are absolutely essential for maintaining a healthy immune system. Serum albumin and globulin values in the fishes treated with different immunostimulants were always found to be higher than control. An increase in the serum protein, albumin and globulin levels are thought to be associated with a stronger innate response in fishes (Wiegertjes *et al.*, 1996).

In the present study, serum total protein, albumin and globulin levels decreased significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C group. On the contrary, A-G ratio increases significantly. The serum protein reduction may be attributed to protein catabolism, the process converting blood and structural protein to energy, to meet the higher energy demand during the prevailing stress. Haemolysis of erythrocytes, already been observed in the present study, also might have caused dilution of the plasma volume contributing to some extent in such reduction of serum protein content (Das *et al.*, 2004). Verma *et al.* (2007) also reported reduced level of serum total protein, albumin and globulin on exposure to higher temperature. Akhtar *et al.* (2011) also reported reduced protein and albumin in *L. rohita* fingerlings at 33^0C and high values at ambient temperature.

5.1.8.6 Lysozyme activity

Lysozyme is known to act as an important innate defense mediator against parasitic, bacterial and viral infections, and in response to infection, its activity is found to increase in fish blood (Ingram, 1980). Lysozyme plays important role in innate immunity by lysis of bacterial cell wall and thus stimulates the phagocytosis of bacteria (Ellis, 1990). Lysozyme activity can be affected by stressors, infectious micro-organisms (Mock and peters 1990). Temperature also plays an important role in regulating lysozyme activity in fish (Ndong *et al.*, 2007). An increased level has been considered a natural protective mechanism in fish (Ingram, 1980). In the present study, lysozyme activity decreased significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. Dominguez *et al.* (2005) observed reduction in plasma lysozyme of Nile tilapia when exposed to 33^0C from 28^0C. Akhtar *et al.* (2011) also reported to reduced lysozyme activity in *L. rohita* fingerlings at 33^0C and high values at ambient temperature. Similar observation was made by Mock and Peters (1990), strong stressors reduced the lysozyme level in rainbow trout. Jeney *et al.* (1997) also reported reduced lysozyme activity in rainbow trout due to transportation stress. This may be due to higher cortisol levels, which protects these cells from apoptosis and stimulates non-specific immune system during stress condition.

5.1.9 Serum Enzyme Activity

5.1.9.1 Serum GOT/AST and GPT/ALT

In the present study, AST and ALT activity in serum increased significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. The higher activity of both the transferase creating higher free amino acid mobilization to produce higher glucose to cope up with the stress incurred due to higher temperature, this is supported by Verma *et al.* (2006) and Chatterjee *et al.* (2006).

5.1.9.2 Serum ALP and ACP

In the present study, ALP and ACP activity in serum increased significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. An increase in the activity of the two enzymes with increasing temperature is indicative cell damage in liver, intestine or kidney. These findings are supported by Vasudevan and Sreekumari (1998) and Kumar *et al*. (2006).

5.1.9.3 Serum LDH

In the present study, LDH activity in serum increased significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. The result suggests animals under thermal stress showed increased lactate production for gluconeogenesis, this is supported by Chatterjee *et al.* (2006) and Verma *et al.* (2006).

5.1.10 Serum Cortisol

Corticotropin-releasing hormone (CRH) or factor (CRF) released from hypothalamus of the brain stimulates corticotrophic cells of anterior pituitary (adenohypophysis) to secrete adrenocorticotropic hormone (ACTH), which stimulates interrenal cells (adrenal cortex homologue) to synthesize and release corticosteroids particularly cortisol (Balm *et al*., 1994). This is the principal corticosteroid in fish (Henderson and Garland, 1980; Pickering, 1993; Schreck, 1996). The steroid hormone cortisol is widely accepted as an indicator of stress in fish, generally increases after exposure to physical stressors (Schreck, 1981; Barton and Iwama, 1991; Wendelaar Bonga, 1997; Barton, 2002). Plasma cortisol level is a general indicator of stressful conditions in vertebrates, particularly in fish (Pickering *et al.,* 1989). Environmental parameters including temperature and other stressors have been shown to affect the cortisol response (Barton *et al.,* 1988; Lankford *et al.,* 2003; Davis, 2004; Tejpal *et al.,* 2009; Hoseini and Hosseini, 2010; Costas *et al*., 2012; Kumar *et al*., 2014). In the present study, serum cortisol level was significantly higher ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. The higher cortisol level indicates higher secretion of cortisol due to stress incurred by higher temperature, which lead to immunosuppression in *L. gonius* fingerlings.

5.1.11 Hsp-70 Expression

Members of heat shock protein 70 (Hsp-70) are highly conserved proteins of about 70 kDa and play important roles in protein folding. Levels of these proteins increase when cells are under stress. Environmental temperature influences both the basal and induced levels of HSPs (Das *et al*., 2005). The sensitivity, lack of stressor specificity and highly conserved nature characterize HSPs as potential environmental biomarkers (Sanders, 1993). The environmental temperature also strongly influences both the basal and induced levels of HSPs (Dietz and Somero 1992).The intensity of the HSP induction by chemical stressors is also dependent on the environmental temperature (Hatayama and Hayakawa, 1999). Under normal physiological conditions HSPs are not apparent in many organisms (Currie *et al*., 2000), but some show detectable levels (Dietz and Somero, 1992; Hardewig *et al*., 2000). Large amounts of constitutive HSP70 have been detected in salamanders, turtles and fish (Yu *et al*., 1994). Moreover, the same or related species of animals from different ecological habitats may show different degrees of heat shock HSP expression (Tomanek and Somero, 1999).

In the present study, quantitative expression of Hsp-70 was significantly higher ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. The higher quantitative expression of Hsp-70, suggests cross protection against chronic stress imparted due to higher elevated temperature. HSPs, temperature associated stress indicators, are expressed differently with varying temperature (Mohapatra *et al.*, 2013). Das *et al.* (2006) reported that an increase in temperature can linearly induce Hsp production in *L. rohita* fingerlings. Mohapatra *et al.* (2014) also reported higher quantitative expression of Hsp-70 at higher temperatures. The expression Hsp-70 in muscle tissues was significantly low as compared to others tissues.

5.1.12 Caspase-3 Activity

Caspases are aspartic acid proteases which takes part an important role in the apoptosis process. There are two groups of caspases taking part in apoptosis, as initiators and executioners. Caspases 2, 8, 9 and 10 are initiators and caspases 3 and 6 are executioners (Mary Lini *et al.*, 2013). In the present study, caspase-3 enzyme activity in serum of *L. gonius* fingerlings was studied and found to increased significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. The high level of caspase-3 enzyme activity may be related to the enhancement of programmed cell death due to the temperature stress. The apoptosis might be due to the failure of cellular defense mechanism under excessive stressful condition (Li *et al*, 2012). Our results are also supported by the finding of (Mosser *et al.*, 2000). A diverse range of stimuli including UV irradiation, cytotoxic drugs, growth factor deficiency, heat shock and viral infection have been shown to induce apoptosis in various cell types (Vaux, 1993). During apoptosis, members of the caspase protease family are activated, and the cells undergo rapid death (Yuan *et al.*, 1993; Henkart, 1996). Induction of the stress responsive heat shock protein (Hsp-70) observed during the present study may also accelerate apoptosis in various fish tissues (Stensløkken *et al.*, 2010; Yamashita *et al.,* 2010).

5.1.13 Thyroid Hormones (T3 and T4)

The thyroid hormones thyroxine (T4) and tri-iodothyronine (T3) are known to play an important role in fish growth (Miwa and Inui, 1985) and early development (Brown, 1997). Arjona *et al.* (2008) also reported that the thyroid hormones are known to play key roles on growth, development, and reproduction and stress responses in fishes. A decrease in T3 and T4 levels was reported in fish under stress (Klaren *et al.*, 2007). When fish are exposed to stressors the levels of thyroid hormones have been demonstrated to be decreased (Deane *et al.*, 2001). Specific metabolic functions of thyroid hormones have been documented in a number of studies, which correlate with its growth-promoting activities (Gorg, 2007). In the present study, thyroid hormone levels in serum decreased significantly ($p<0.05$) on exposure to higher temperature 33^0C as compared to ambient temperature groups, which was further aggravated at 36^0C. The reduced growth rate in the fishes exposed to higher temperature can be correlated with the low level thyroid hormone levels and *vice versa*.

5.1.14 Thermal Tolerance Study

5.1.14.1 Thermal tolerance limit and polygon

Thermal tolerance limit of the *L. gonius* fingerlings is widely influenced by the acclimated temperatures. With increasing acclimation temperatures mean values of CT_{max}, CT_{min}, LT_{max} and LT_{min} of *L. gonius* fingerlings increased significantly ($p<0.05$). It is obvious from the results that the thermal tolerance of fishes is dependent on the thermal exposure history before the experiment, which is regarded as acclimation. The strong relationship between the acclimation temperatures and the thermal tolerance level of *L. gonius* fingerlings supports evidence that temperature adaptation is an essential physiological phenomenon in fishes and is dependent on the acclimation temperature (Beitinger and Bennetta, 2000). These results are in agreement with the findings of our earlier investigations on Indian major carps (*L. rohita, C. catla and C. mrigala*) advance fingerlings (Das *et al.*, 2004), Rohu (*L. rohita*) fry (Das *et al.*, 2005), Rohu (*L. rohita*) and common carp (*Cyprinus carpio*) early fingerlings (Chatterjee *et al.*, 2004), yellowtail catfish (*Pangasius pangasius*) fingerlings (Debnath *et al.*, 2006) and climbing perch (*Anabas testudineus*) (Sarma *et al.*, 2010). Fish have specific behaviour in response to thermal acclimation, beyond which breakdown occurs, leading to stress and production losses due to anorexia, disease outbreak and ultimately death. Debnath *et al.* (2006) reported that the thermal tolerance study is considered as a useful approach in assessing the impact of climatic change and culture potential of any candidate fish species. From this preliminary study, it is evident that thermal tolerance limit of *L. gonius* is comparable to Indian major carps, indicating their potential for diversifying freshwater aquaculture.

The area of the tolerance zone is a useful index of thermal tolerance (Elliott, 1981). Thermal tolerance polygon of *L. gonius* fingerlings over the range of acclimated temperatures (27-36^{0}C) was calculated as 256.24^{0}C^2. There is no parallel report on CTM test in this species. Indian carps are eurythermal in nature due to their capacity to tolerate wider range of temperature (Kasim, 2002). Data extracted from earlier investigation of Das *et al*. (2004) in Indian major carps (*L. rohita*, *C. catla*, *C. mrigala*) and rohu (*L. rohita*) early fingerlings (Chatterjee *et al*., 2004), reveals the similar zone of thermal tolerance polygon. Results of the present findings also indicated that the thermal tolerance zone of *L. gonius* fingerlings is within the range of Indian major carps, which support its tolerance capacity to temperature variations.

5.1.14.2 Oxygen consumption rates

Rate of oxygen consumption increased significantly with increasing acclimation temperatures ($p<0.05$). Oxygen consumption is an index of metabolism in freshwater fish (Kutty and Peer Mohamed, 1975) and is dependent on the acclimation temperatures (Kita *et al*., 1996). In fishes, the metabolic responses that are quantified in terms of oxygen consumption show a linear correlation to temperature due to its direct effect on the kinetics of the enzyme reactions involved (Hazel and Prosser, 1974; Hochachka and Somero, 1971). Absolute values of oxygen consumption rate were comparable to our earlier investigation in Indian major carps (Das *et al*., 2004), early fingerlings of *L. rohita* and *C. carpio* (Chatterjee *et al*., 2004) and *P. pangasius* (Debnath *et al*., 2006).

5.1.14.3 Temperature quotient (Q_{10}) value

Temperature quotient (Q_{10}) was found to be 1.72 for acclimated fish and 2.23 for non-acclimated fish over the range of acclimation temperatures (27-36°C), which indicates that acclimation procedure has played vital role to maintain the homeostasis of *L. gonius* over the test temperatures. Final preferred temperature estimated from the Q_{10} value was between 27-30°C (1.865) and 30-

33°C (2.209). So, culturing the species <33°C and > 27°C will be an ideal condition for better growth and maximization of the species. The point where the Q_{10} for oxygen consumption starts to decrease with increasing acclimation temperatures also corresponds to the optimal temperature for growth (Kita *et al.*, 1996). Thus the final preferred temperature may be estimated indirectly based on the relationship between oxygen consumption and acclimation temperature (Kita *et al.*, 1996). Tsuchida (1995) and Kita *et al.* (1996) have reported that the preferred temperature coincides with the optimum temperature for growth (Brett, 1971; Kellog and Gift, 1983). Das *et al.* (2004) reported that final preference temperature for Indian Major Carps in the range of 31-33^{0}C, which is comparable to the *L. gonius* species. They also hypothesized that the point at which Q_{10} diminishes related to acclimation temperatures corresponds to the optimal temperature for growth, because the decrease in Q_{10} indicates that the metabolism of the species has decreased and that more energy for growth is available, similar to that obtained for *L. gonius* exposed to different acclimation temperatures.

5.1.15 Histological Study

In the present study, histological study was carried out at the end of the experiment in gill, liver and intestinal tissue of *L. gonius* fingerlings exposed to four different temperatures. Histological study confirms that structural changes in gill, liver and intestinal tissues of *L. gonius* fingerlings increased significantly ($p<0.05$) on exposure to higher temperature 33^{0}C, which was further aggravated at 36^{0}C. However, there were no changes were observed at 27 and 30^{0}C.

The gills, which participate in many important functions in the fish, such as respiration, osmoregulation and excretion, remain in close contact with the external environment and particularly sensitive to changes in the quality of the water are considered the primary target of the contaminants (Camargo *et al.*, 2007; Fernandes *et al.*, 2003). Kaoud and El-Dahshan (2010) also reported the histological alteration due to prolonged exposure to heavy metal resulted in respiratory, osmoregulatory and circulatory impairment. Dash *et al.* (2011) reported damaged gill lamella at higher acclimated temperature. These reports support the present finding on the alteration of the gill structure of *L. gonius* fingerlings 33^{0}C at become severe at 36^{0}C.

The most common alterations in the liver of the fingerlings in the present study at higher temperatures are vacuolation of the hepatocytes. The vacuolation of the hepatocytes might indicate an imbalance between the rates of synthesis of substances in parenchymal cells and rate of their release into circular system (Gernhofer *et al.*, 2001). Liver of fish is sensitive to environmental contaminants because many contaminants tend to accumulate in the liver and exposing it to a much higher levels than in the environment, or in other organs (Heath, 1995). Dash *et al.* (2011) also reported vacuolation of haepatocyte at higher acclimated temperature.

According to Bhatnagar *et al.* (2007), the observed irritation and destruction of the mucosa membrane of the intestine, hampers absorption. The histological alterations in the intestine of the studied fish are in agreement with those observed by many investigators about the effects of different toxicants on fish intestine (Hanna *et al.*, 2005; Cengiz *et al.*, 2006). Epithelial degeneration, inflammatory cells infiltration in the sumucosa as well as submucosal edema was seen in the intestine of tilapia fish exposed to carbofuran (Soufy *et al.*, 2007).

Result of the histological study confirmed that there are no changes or damage in tissues at 27 and 30^{0}C, whereas changes begin at 33^{0}C and become severe at 36^{0}C. So, rearing the species 27-30^{0}C will be an ideal condition not only for better physiological activity but also better growth and survival of the species. Otherwise, we have to find an alternative stress mitigation measure. The histological alterations in all the tissues are due to the prolonged exposure of the fingerlings to higher elevated temperatures. However, the rate of changes depends on the amount and time of exposure. The changes at higher temperatures are a clear indication of stress which may be due to increase in metabolic activity, accumulation of toxic metabolites, oxygen deprivation, etc.

5.1.16 Scanning Electron Microscopy (SEM) of Gill Tissue

Gills are vital structures for fish, since they are the main site for gaseous exchange (Hughes, 1966) as well as partially responsible for osmoregulation (Gonzales and McDonald, 1992), acid base balance (Goss *et al.*, 1992), excretion of nitrogenous compounds (Sayer and Davenport, 1987) and taste (Rios and Fanta, 1998) through surrounding environment to the body. Hence, gill plays a very important physiological function in case of fish. Fish gills can be used as model for studies on environmental impact (McKim and Erickson, 1991). Laurent and Perry (1991) consider the morphologic changes in gills, as a consequence of environmental changes, as adaptive attempts in conserving some physiological functions. It has been shown in animal that extreme temperature changes induce stress in organism and that is characterized by changes at the physiological (Trot, 2011) as well as the cellular architectural levels (Egginton and Sidell, 1989).

SEM study confirmed the results of the histological study. No ultrastructural changes in gill tissue of *L. gonius* fingerlings at 27 and 30^0C, whereas changes begin at 33^0C and become severe at 36^0C. Fish gills were taken into consideration for present investigation as they were in direct contact with environment and therefore, gills could be good indicators of water quality. McKim and Erickson (1991) reported that fish gills can be used as model for studies on environmental impact. Conduction of heat between the environment and fish is mediated through the gills; hence, gills are regarded as the heat exchanger. Therefore, gills are considered to be the most appropriate organ for indicating thermal pollution (Alazemi *et al*., 1996) and consequently in all the pollution studies in gill tissues of fish should be invariably become an organ of paramount importance to assess the magnitude of damage. Fish exposed to high temperatures may suffer respiratory stress and nervous disorders. Increasing water temperature exerts a dual effect on their oxygen demand-supply ratio. Warmer waters carry less dissolved oxygen, yet the oxygen uptake by aquatic organisms is increased due to their higher metabolic rate. Earlier investigations on various fishes indicated that metabolic activity increased with increasing acclimation temperatures (Manush *et al*., 2004; Debnath *et al*., 2006). Electron microscopy study of the gill tissue of freshwater prawn tissue showed damaged at higher acclimation temperature (Manush *et al*., 2007). Das *et al*. (2014) also observed damaged lamellae at temperature extremes in electron microscopy study. Laurent and Perry (1991) consider the morphologic changes in gills, as a consequence of environmental changes, as adaptive attempts in conserving some physiological functions. From our results, it is evident that long-term exposure to increasing temperatures may cause respiratory stress, which ultimately may leads to death of the species.

5.2 Experiment 2

During the culture period, fishes are exposed to several stressors, such as faulty practices and environmental changes. These frequently cause various degree of trauma (stress) in aquatic organisms resulting in detrimental consequences. In fish, the stressful condition is not due to a single stressor but in fact, it is a combined effect of many stressors, which leads to reduction in growth rate, decrease in disease resistance, immunosuppression and even death of the fish. Understanding the interaction of multiple stressors is the biggest challenge to the researchers which subsequently help to develop mitigation strategies during aquaculture practices. Conversely, information on the combined effect of different stressors on growth and physiological responses of fishes is limited. In this backdrop, the present experiment was designed to elucidate the growth, biochemical and haemato-immunological responses of *L. gonius* fingerlings exposed to multiple stressors (temperature and pH).

5.2.1 Physico-chemical Properties of Water

In our experiment, we maintained a low stocking density and continuous aeration in order to avoid any confinement stress and ammonia accumulation in rearing tanks. Along with these, regular exchange of water in the rearing tank helps us to maintain the physico-chemical parameters of water such as dissolved oxygen, free carbon dioxide, ammonia, nitrite-N and nitrate-N within the optimum range of requirements for the fish as suggested by many authors.

Temperature plays an important role in regulating the metabolism of animals; therefore an optimum range of temperature is required for optimum metabolic activity, which in turn gives maximum yield. In the present study, water temperature was maintained at 27^0C as an ambient temperature in T_1, T_2 and T_3 experimental group, which is comparable to the optimum temperature (28^0C) suggested by Chakraborty *et al.* (1976) for carp culture, while the temperature in other experimental groups (T_4, T_5 and T_6) kept steady at 33°C to ensure chronic thermal stress to the animal during the entire experimental period of 60 days. The chronic thermal stress was decided based on the findings of the 1st experiment. Das *et al.* (2005) also reported that the chronic exposure of *L. rohita* fingerlings to 33^0C has imparted thermal stress fingerlings.

The water pH was maintained at 7.5 as an ambient pH in T_1 and T_4 experimental group, which is within optimum range (7.5-8.5) as suggested by Banerjea (1967) for carp culture, while the pH in other experimental groups kept steady at 5.5 (in T_2 and T_5) and 9.0 (in T_3 and T_6) to ensure chronic pH stress to the animal during the entire experimental period of 60 days. The pH range (5.5 and 9.0) was selected based on the findings of Acharya *et al.* (2005) and Das *et al.* (2006) on fingerlings of carp species. They have reported that the chronic exposure to acidic pH (5.5) and alkaline pH (9.0) imparted stress to the fingerlings.

The dissolved oxygen level in water varies with a large number of factors, such as water temperature, metabolic rate, biomass density, etc. In the present study, dissolved oxygen level was recorded within the range of 5.85-7.21 mgL^{-1}, which is within the optimum range 6-7 mg/L for cyprinids suggested by Huet (1975). From the above result, it is assumed that dissolved oxygen was optimum throughout the experimental period, which might be due to efficient aeration. Toxic concentrations of ammonia for short-term exposure are between 0.6 and 2 mg/L (EIFAC, 1973). The suggested value of ammonia in water ranges from 0 to 0.1 mg/L (Jhingran, 1991), which is in agreement with the recorded range (0.15- 0.29 mg/L) in the present study. Nitrite-N concentration was recorded in the range of 0.002 - 0.012 mg/L, which is well within the permissible range for pond aquaculture (Boyd and Trucker, 1998). Nitrate-N level in a productive pond can be within 0.1-5.0 mg/L (Boyd and Trucker, 1998). In the present study, nitrate-N (0.04 to 0.15 mg/L) was below the toxic level. Nitrite-N and nitrate-N levels were within the permissible limits for warm water fish (Boyd, 1982).

5.2.2 Growth and Survival

In the present study, weight gain %, SGR, FER, PER and survival % decreases, whereas FCR values increases significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). The decrease in growth rate upon exposure to temperature and/or pH induced stress can be attributed to re-allocation of energy towards maintenance and repair under stress (Kumaraguru and Beamish, 1986). Das *et al.* (2005) and

Akhtar *et al*. (2011) also observed reduction in the growth rate, survival % and low feed conversion efficiency in *L. rohita* fingerlings reared at higher temperature. Reduced growth rate and food conversion efficiency have been reported in fish at combined stress higher temperature and salinity (Likongwe *et al*., 1996). On the contrary, the higher growth rate, survival percentage and feed conversion efficiency was observed ambient temperature and pH group. The higher weight gain may be attributed to improved dietary protein utilization at ambient temperature and pH. Das *et al*. (2005) and Akhtar *et al*. (2011) also reported similar finding in *L. rohita* fingerlings reared at ambient temperature.

5.2.3 DNA, RNA and DNA/RNA Ratio

In the present study, RNA and RNA/DNA ratio decreases significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). The reduced values in the fishes exposed to temperature and/or pH induced stress can be linked with the lower growth rate incurred due to stress. The ratios between these two nucleic acids were reported to reduce, when the fishes were exposed to high temperatures (Spigarelli and Smith, 1976) or heavy metal salts (Kearns and Atchison, 1979). On the contrary, the higher RNA and RNA/DNA ratio was observed ambient temperature and pH group, which can be correlated with higher the growth indices observed at ambient temperature and pH. A strong positive correlation has been reported between nucleic acid concentration and specific growth rate (Bulow, 1970; Bulow *et al*., 1981; Haines, 1973; Fukuda *et al*., 2001). As the RNA content is related to rate of protein synthesis, the RNA/DNA ratio can be used as an index of growth (Smith and Buckley, 2003; Akhtar *et al*., 2012a). We did not observe any significant change in DNA levels in both the tissue, and our findings are in support of Leslie (1955) who found that DNA content per somatic cell is constant within a species.

5.2.4 Body Indices

In the present study, HSI decreases significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature

(33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic pH (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). The reduced values in the fishes may be due to temperature and/or pH stress. However, GSI did not vary significantly among the different treatment. Ciji *et al*. (2011) also reported higher HSI values in *Labeo rohita* fingerlings reared at ambient water temperatures than the higher temperature.

5.2.5 Tissue Glycogen

Glycogen breakdown takes place due to effect of catecholamine epinephrine/ nor epinephrine. Catecholamine epinephrine/ nor epinephrine induce glycogenolysis through beta- adrenoreceptors (Sheridan and Muir, 1988). Several researchers have studied glycogenolysis in response to various stressors. Increase in glucose and concomitant decrease in glycogen on exposure to various environmental toxicants has been reported (Singh and Aggarwal, 1986). Nakano and Tomlinson *et al*. (1967) observed that all types of stress elevated the secretion of catecholamine which in turn increased the breakdown of glycogen and elevated blood glucose level. In the present study, glycogen content in liver and muscle tissues decreased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). The increase in glucose and concomitant depletion in glycogen shows that the glycogen was utilised for energy to cope with the temperature and/or pH induced stress. The depletion of glycogen was lowest in ambient temperature and pH group.

5.2.6 Ascorbic Acid

Vitamin C is a major water soluble antioxidant and is capable of maintaining sulphydryl compounds in a reduced state, particularly in several redox reactions (Deshpande *et al*., 2002). Teleosts do not have an active L-gulonolactone oxidase, the terminal enzyme in the ascorbic acid synthesis pathway (Dabrowski, 1990a). Thus, fishes are sensitive to a threshold level of vitamin C as they are unable to synthesize it de novo (Dabrowski, 1990b). Vitamin C is considered to play an important role in animal health as antioxidants, inactivating damage of free radicals produced during normal cellular activity from various stressors (Chew, 1995) have been reported, confirming protective roles of vitamin C. Cruz de Menezes *et al.* (2006) and Norouzitallab *et al.* (2008) also reported that vitamin C is well known for its anti-oxidative property and has also been used as an immuno-modulator in aquaculture. In the present study, ascorbic acid content in muscle tissue decreased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). The decrease in ascorbic acid content could be attributed to its maximum utilization during temperature and/or pH induced stress. Das *et al.* (2009) also reported decrease in ascorbic acid content at higher temperature. Similar results were also found in thornfish, *Therapon jarbua* when exposed to thermal stress (Chien *et al.*, 1999).

5.2.7 Enzymatic Activity in Tissue

5.2.7.1 Enzymes of protein metabolism (AST/GOT and ALT/GPT)

Transaminases are the enzymes that redistribute amino nitrogen among amino acids, forming new amino acid with the amino group from the pre-existing ones. Non-essential amino acids required for growth and other metabolic processes are produced by this manner. Amino acids are the major source of energy in teleost (Belinski, 1974). Fishes utilize proteins and lipids prior to carbohydrates (Demeal, 1978). Stress hormone cortisol induces gluconeogenesis, which mobilize the protein source and lipid source for glucose synthesis. The most important aminotransferases are aspartate amino transferases (AST) and alanine amino transferases (ALT) (also called glutamate oxalo acetate transaminase or GOT and glutamate pyruvate transaminase or GPT). AST transfers the amino group of aspartic acid to α-keto glutarate and converts to glutamic acid forming oxalo acetate. ALT transfers the amino group from alanine to α-keto glutarate forming glutamic acid pyruvate. The pyruvate and oxalo-acetate thus formed are responsible for the synthesis of non-essential amino acids like alanine, aspargine, glutamine, etc. which in turn helps in protein synthesis and hence growth of the animal. Apart from this the amino acids are deaminated or transaminated to produce TCA cycle intermediates also helping in energy production in the form of ATP (De Silva and Anderson, 1995).

In the present study, AST and ALT activity in liver and muscle tissue of *L. gonius* fingerlings increased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). The higher activity of both the transferase with temperature and/or pH stress, creating higher free amino acid mobilization to produce higher glucose to cope up with stress. Similar observation was made by Verma *et al.* (2006) in *C. carpio* with temperature stress and in Tilapia with confinement stress (Vijayan *et al.,* 1997). The increase in activity indicates greater mobilization of amino acid for glucose production via gluconeogenesis for glucose production to cope with stress (Chatterjee *et al.*, 2006). Knox and Greenland (1965) also reported that elevated levels of transaminase activity during stress would lead to increased feeding of ketoacids in to TCA cycle, there by affecting oxidative metabolism.

5.2.7.2 Enzymes of carbohydrate metabolism

Lactate dehydrogenase is the terminal enzyme of the glycolysis (Embden-Meyerhoff pathway) pathway, which converts lactate to pyruvate in the presence of coenzyme NADH which is converted to NAD^+. Thus, lactate dehydrogenage helps in maintaining the glycolysis cycle by supplying NAD^+. In the presence of enough oxygen pyruvate enters the Kreb's cycle but when there is an oxygen shortage in the tissue, pyruvate is converted to lactate (Murray *et al.*, 2000). Generally LDH activity increases in stress *viz*. temperature stress (Grigo, 1975) and starvation stress (Vijayaraghavan and Rao, 1986). The increase in LDH activity attributed to the production of preferred substrate (Lactate) for gluconeogenesis under thermal stress (Chatterjee *et al.,* 2006). A significant increase in LDH activity was observed at higher acclimation temperature (Verma *et al.,* 2006) that may be due to higher production of lactate, indicating oxygen limited condition in the cell. In the present study, LDH activity in liver and muscle tissue of *L. gonius* fingerlings increased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). The LDH activity in different treatment groups suggesting that the animals under stress showed increased lactate production for gluconeogenesis, this is supported by Chatterjee *et al.* (2006) and Verma *et al.* (2006).

Malate Dehydrogenase (MDH) is an enzyme of TCA cycle, which oxidizes malate to oxaloacetate, using NAD^+ as the electron acceptor. The activity of MDH showed an increase during gluconeogenesis and oxaloacetate is formed from the deamination of aspartate and malate or phosphoenol pyruvate from pyruvate. The conversion of oxaloacetate to malate in the mitochondria and the reverse in cytoplasm is catalyzed by MDH (Das, 2002c). Verma *et al*. (2006) observed that there was a significant increase in MDH activity in *C. carpio* fingerlings acclimated at higher temperatures in order to use the product (oxaloacetate) due to the higher activity of AST for production of more energy (ATP), which may be utilized for other physiological activities. Glucose might have therefore mobilized through non-carbohydrate source; mainly by protein, as transaminase activities were more at higher temperatures. Result also strengthens the fact that higher temperature induces amino acid mobilization (alanine, aspartate). In the present study, MDH activity in liver and muscle tissue of *L. gonius* fingerlings increased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). The higher activity of MDH is indicative of greater activity of TCA cycle due to increased energy demands during temperature and/or pH stress. This is supported by the findings of Das *et al.* (2006).

5.2.7.3 Enzyme of neurotransmission (AchE)

Acetyl choline esterase (AchE) is one of the most widely used enzymes as a biomarker for environmental pollution. Biomarkers are defined as biochemical, cellular or physiological variations which can be measured in an organism as response to an environmental perturbation or a contamination event (Livingstone *et al.*, 1997). Among different biomarkers, a prominent position is occupied by the measure of acetylcholine esterase inhibition. Acetylcholine is widely distributed in nervous tissue, stored and released from the synaptic vesicles. It helps in the depolarization in an adjacent neuron and thus passes nerve impulse. Acetylcholine is rapidly destroyed by choline esterase, a group of related enzymes which are hydrolytic in action (Stowe, 1969). It is well known that some pesticides inhibit AchE which increases the acetylcholine and stimulates the medullary cells to release catecholamine (Stowe, 1969) and also increase in aberrant behaviour (Murthy *et al.,* 1984; Jones *et al.,* 1999). Increased catecholamine may affect the activity of enzymes involved in glycogen synthesis and glycogenolysis. Thus, stress has an effect on physiological parameters as well as neurotransmission. In the present study, AchE activity in brain tissue of *L. gonius* fingerlings decreased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). Similar observation was made by Verma *et al*. (2006) in *C. carpio* fingerlings at higher temperature.

5.2.7.4 Enzymes of oxidative stress (Catalase, SOD and GST)

Under normal physiological conditions animal cell produces reactive oxygen species (ROS) such as H_2O_2, O_2^-, OH^- that may damage most cellular components leading to cell death. When the rate of ROS generation exceeds that of their normal, oxidative stress occurs. Oxidative stress results when the antioxidant defenses are overcome by pro-oxidant forces and reactive oxygen species are not adequately removed (Sies *et al*., 1986). Living organisms are protected from the danger of reactive oxygen species (ROS) by several defense mechanisms including antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, etc. Antioxidants also act in conjunction with these enzymatic defenses. Superoxide dismutase (SOD), a cytosolic enzyme that is specific for scavenging superoxide radicals (O_2^-) is involved in protective mechanisms within tissue injury following oxidative process and phagocytosis. Catalase (CAT) is another major primary antioxidant defense component that works primarily to catalyze the decomposition of H_2O_2 to H_2O, sharing this function with glutathione peroxidase (GPX). Therefore both these enzymes detoxify H_2O_2 derived from SOD activity. In the presence of low H_2O_2

levels, organic peroxides are the preferred substrate for GPX. However at high H_2O_2 concentrations they are metabolized by CAT (Yu, 1995). Changes in antioxidative enzymes can be used as stress indicators in fish (Akhtar *et al.*, 2010; Ciji *et al.*, 2012). In the present study, catalase, SOD and GST activity in liver and gill tissues of *L. gonius* fingerlings increased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0).

5.2.7.5 Enzyme of phospho monoesterase (ALP)

Alkaline phosphatase is zinc containing metallo-enzyme, which plays an important role in metabolism of phosphorus in the body. It is involved in membrane transport, glycogen metabolism, protein synthesis, calcium absorption and many other physiological processes including secretary function. In the present study, ALP activity in liver, gill and muscle tissues of *L. gonius* fingerlings increased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). The higher ALP activity with temperature and/or pH stress may be due to hydrolysis of high- energy phosphate bonds to liberate phosphate ions to combat stressful condition or higher metabolic rate. Similar observation was made by Verma *et al.* (2006) in *C. carpio* fingerlings.

5.2.7.6 Enzyme of gluconeogenic pathways (FDpase)

In the present study, FDpase activity in liver and gill tissues of *L. gonius* fingerlings increased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). The increase in FDPase activity with temperature and/or pH stress indicates that gluconeogenic pathway gets activated to cope with the increasing metabolic demand at temperature and/or pH stress. A consistent increase of transmaninase might have provided the substrate for gluconeogenesis (Suarez *et al.,* 1987; Moon and Foster, 1995). Hence, temperature and/or pH stress may increase energy demands in the *L. gonius* fingerlings by increasing FDPase activity.

5.2.7.7 Enzyme of pentose phosphate pathway (G6PDH)

G6PDH is an enzyme of the pentose phosphate pathway or hexose monophosphate shunt. It mediates the conversion of glucose-6-phosphate to 6-phospho-gluconolactone using $NADP^+$ as a coenzyme and releases NADPH. This pathway activity increases when the requirement of NADPH increases especially during the synthesis of fatty acids, cholesterol and sphingolipids (lipid anabolism). NADPH produced is also required by NADPH oxidase in producing superoxide anions for destroying phagocytosized material. So, the enzyme activity may increase with increase in phagocytosis (Das, 2002b).

In the present study, G6PDH activity in liver and gill tissues of *L. gonius* fingerlings decreased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). The lower activity of enzyme at temperature and/or pH stress might be due to higher cortisol level. The lower activity of G6PDH at higher temperature is reported by Verma *et al.* (2006) in *C. carpio* fingerlings. Reduction of G6PDH activity with increased cortisol concentration was reported by Tripathi and Verma (2003) in fishes.

5.2.7.8 Enzyme of energy metabolism (ATpase)

It hydrolyzes a high-energy phosphate of ATP and utilizes that energy for the simultaneous transport of three Na^+ ions outward and two K^+ ion inward across the plasma membrane (Das, 2002a) *i.e.* ATPase hydrolyzes the high energy phosphate (ATP) and utilizes the energy to maintain ionic gradient across the plasma membrane. The activity of this enzyme is inhibited by the depletion of substrate ATP or due to some organochlorines. Stress leads to imbalance in osmoregulation. In freshwater, Na^+ and Cl^- ions are lost and in marine water the ions are gained (Mazeaud *et al.*, 1977).

In the present study, ATpase activity in gill and liver tissues of *L. gonius* fingerlings decreased significantly ($p<0.05$) on exposure to low acidic pH (5.5) and high alkaline pH (9.0) stress as compared to ambient temperature and pH group, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). However, there was no significance difference among ambient temperature and 33^0C group.

5.2.7.9 Digestive enzyme (Protease)

In the present study, protease activity in intestinal tissue of *L. gonius* fingerlings decreased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). The decrease in enzyme activity with temperature and/or pH stress indicates is due to inappropriate utilization of nutrients. Prabhu (2012) also reported to decrease in protease activity on exposure to elevated temperature and endosulfan stress.

5.2.8 Haemato-immunological Parameters

5.2.8.1 Erythrocyte (RBC), haemoglobin (Hb) and haematocrit (Hct)

In the present study, RBC, Hb and Hct count of *L. gonius* fingerlings decreased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). This indicates reduced blood oxygen carrying capacity in both the stressed groups. The decreased value could be due to the erythrocytic cell lysis of blood cells and an increased removal of RBCs from the circulation as suggested in carp (Jensen, 1990; Knudsen and Jensen, 1997) or haemodilution (Wedemeyer *et al*., 1984). Reduction in serum protein content in in the present study also supports the view of erythrocytic cell lysis and haemodilution. Akhtar *et al*. (2011) also reported reduced RBC, Hb and Hct count in *L. rohita* fingerlings at 33^0C and higher count at 26^0C. Das *et al*. (2006) also reported significant reduction in RBC and Hb count at acidic pH 5.5 and alkaline pH 9.0 in Indian major carps.

5.2.8.2 Leucocyte (WBC) count

Leucocytes (WBC) play an important role for enhancing non-specific or innate immunity, which is considered as an indicator of the health status of fish (Roberts, 1978). The leukocytes are involved in regulation of immunological function in the organism (Santhakumar *et al.*, 1999). A reduced white blood cell count is usually used a biochemical indicator of immunosuppression caused by various stressors (Kopp *et al*. 2010).

In the present study, WBC count of *L. gonius* fingerlings decreased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). This may be due to increased basal cortisol level, which reduces leucocyte count either due to increased apoptosis, reduction in leucocyte proliferation or leucocyte redistribution or weakening of the leucopoiesis process. Verma *et al*. (2007) also reported reduction in leucocyte in *C. carpio* fingerlings on exposure to higher temperature, which was exacerbated on concurrent exposure of temperature and chlorine stress. Similarly, Akhtar *et al*. (2011) also reported reduced WBC count in *L. rohita* fingerlings at 33^0C and higher values at ambient temperature. Das *et al*. (2006) also reported reduction in leucocyte count at acidic pH 5.5 and alkaline pH 9.0 in *Catla catla* and *Cirrhinus mrigala* fingerlings.

5.2.8.3 Nitroblue tetrazolium (NBT)

The phenomenon known as respiratory burst gives a measure of oxygen dependent defense mechanism in vertebrate phagocytic cells, wherein there is generation of reactive oxygen intermediates with powerful anti-microbial activity (Itou *et al.*, 1997). Increased respiratory burst activity can be correlated with increased bacterial pathogen killing activity of phagocytes (Sharp and Secombes, 1993) and hence a better immunity. The respiratory burst activity of phagocytes was measured by reduction of NBT by intracellular superoxide radicals produced by leucocytes.

In the present study, NBT activity of *L. gonius* fingerlings decreased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). Verma *et al.* (2007) also reported reduction in NBT activity on exposure to higher temperature, which was exacerbated on concurrent exposure of temperature and chlorine stress in *C. carpio* fingerlings. Similarly, Akhtar *et al.* (2011) also reported reduced NBT activity in *L. rohita* fingerlings at 33^0C and higher values at ambient temperature.

5.2.8.4 Blood glucose

Glucose is one of the most important sources of energy for the animals and glucose has been studied as an indicator of stress caused by physical factors (Manush *et al.*, 2005). Nakano and Tomlinson (1967) observed that all types of stress elevated the secretion of catecholamine which in turn increased the breakdown of glycogen and enhanced blood sugar level. The elevation of blood sugar levels in fish by both corticosteroids and catecholamines makes it the ideal parameter to study the secondary stress response, on activation of direct sympathetic (Chromaffin tissue) as well as humoral (internal tissue) pathways (Wedemeyer and Mcleay, 1981).

In the present study, blood glucose level of *L. gonius* fingerlings increased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). Das *et al.* (2009) also reported increase level of blood glucose at higher temperature and low level at ambient temperature. Das *et al.* (2006) observed higher blood glucose at acidic pH 5.5 and alkaline pH 9.0 in *C. catla* and *C. mrigala* fingerlings. The reason for increase in blood glucose may be due to higher cortisol levels, which is a hyperglycemic hormone. Nakano and Tomlinson (1967) also observed that all types of stress elevated the secretion of catecholamine which in turn increased the breakdown of glycogen and enhanced blood sugar level. Another reason could be increased in glycolysis is also indicated by increased LDH activity in liver and muscle due to higher production of lactate. Similar observation was made by Suarez and Mommsen (1987), Moon and Foster (1995) and Verma *et al.* (2006).

5.2.8.5 Albumin (A), globulin (G), A-G ratio and total serum protein

Proteins are the most important compounds in the serum, and their concentration is used as a basic index for the health status of fish (Buchanan *et al.*, 1997). Among the serum protein, albumin and globulin are the major proteins, which play a significant role in the immune response (Kumar *et al.*, 2007). The gamma globulin fraction is the source of almost all the immunological active protein of the blood. Globulins like gamma globulins are absolutely essential for maintaining a healthy immune system. Serum albumin and globulin values in the fishes treated with different immunostimulants were always found to be higher than control. An increase in the serum protein, albumin and globulin levels are thought to be associated with a stronger innate response in fishes (Wiegertjes *et al.*, 1996).

In the present study, serum total protein, albumin and globulin levels decreased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). On the contrary, A-G ratio increases significantly. The serum protein reduction may be attributed to protein catabolism, the process converting blood and structural protein to energy, to meet the higher energy demand during the prevailing stress. Das *et al.* (2006) also reported reduction in total serum protein at low acid pH 5.5 and high alkaline pH 9.0 in *C. catla* and *C. mrigala* fingerlings. Haemolysis of erythrocytes, already been implicated in this species in the present study, also might have caused dilution of the plasma volume contributing to some extent in such reduction of serum protein content (Das *et al.*, 2004). Verma *et al.* (2007) also reported reduced level of serum total protein, albumin and globulin on exposure to higher temperature, which was exacerbated on concurrent exposure of temperature and chlorine stress. Akhtar *et al.* (2011) also reported reduced albumin in *L. rohita* fingerlings at 33^0C and high values at ambient temperature.

5.2.8.6 Lysozyme activity

Lysozyme is known to act as an important innate defense mediator against parasitic, bacterial and viral infections, and in response to infection, its activity is found to increase in fish blood (Ingram, 1980). Lysozyme plays important role in innate immunity by lysis of bacterial cell wall and thus stimulates the phagocytosis of bacteria (Ellis, 1990). Lysozyme activity can be affected by stressors, infectious micro-organisms (Mock and Peters, 1990). Temperature also plays an important role in regulating lysozyme activity in fish (Ndong *et al.*, 2007). An increased level has been considered a natural protective mechanism in fish (Ingram, 1980).

In the present study, lysozyme activity decreased of *L. gonius* fingerlings increased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). Dominguez *et al.* (2005), who observed a reduction in plasma lysozyme of Nile tilapia when exposed to 33^0C from 28^0C. Akhtar *et al.* (2011) also reported to reduced lysozyme activity in *L. rohita* fingerlings at 33^0C and high values at ambient temperature. Similar observation was made by Mock and Peters (1990), strong stressors such as transportation or acute water pollution reduced the lysozyme level in rainbow trout. Jeney *et al.* (1997) also reported reduced lysozyme activity in rainbow trout due to transportation stress. This may be due to higher cortisol levels, which protects these cells from apoptosis and stimulates non-specific immune system during stress condition.

5.2.9 Serum Enzyme Activity

5.2.9.1 Serum GOT/AST and GPT/ALT

In the present study, amino transferase (AST and ALT) activity in serum of *L. gonius* fingerlings increased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). The higher activity of both the transferase with temperature and/or pH stress, creates higher free amino acid mobilization to produce higher glucose to cope up with stress, this is supported by Verma *et al.* (2006) and Chatterjee *et al.* (2006).

5.2.9.2 Serum ALP and ACP

In the present study, ALP and ACP activity in serum of *L. gonius* fingerlings increased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). An increase in the activity of the two enzymes with temperature and/or pH stress is an indicative of cell damage in liver, intestine or kidney. These findings are supported by Vasudevan and Sreekumari (1998) and Kumar *et al.* (2006).

5.2.9.3 Serum LDH

In the present study, LDH activity in serum of *L. gonius* fingerlings increased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). This suggests animals under chronic temperature and/or pH stress showed increased lactate production for gluconeogenesis; this is supported by Chatterjee *et al.* (2006) and Verma *et al.* (2006).

5.2.10 Serum Cortisol

Corticotropin-releasing hormone (CRH) or factor (CRF) released from hypothalamus of the brain stimulates corticotrophic cells of anterior pituitary (adenohypophysis) to secrete adrenocorticotropic hormone (ACTH), which stimulates interrenal cells (adrenal cortex homologue) to synthesize and release corticosteroids particularly cortisol (Balm *et al.*, 1994). This is the principal corticosteroid in fish (Henderson and Garland, 1980; Pickering, 1993; Schreck, 1996). The steroid hormone cortisol is widely accepted as an indicator of stress in fish, generally increases after exposure to physical stressors (Schreck, 1981; Barton and Iwama, 1991; Wendelaar Bonga, 1997; Barton, 2002). Plasma cortisol level is a general indicator of stressful conditions in vertebrates, particularly in fish (Pickering *et al.,* 1989). Environmental parameters including temperature and other stressors have been shown to affect the cortisol response (Barton *et al.,* 1988;

Lankford *et al.,* 2003; Davis, 2004; Tejpal *et al.,* 2009; Hoseini and Hosseini, 2010; Costas *et al*., 2012; Akhtar *et al*., 2012b).

In the present study, cortisol level in serum of *L. gonius* fingerlings increased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). The high level of serum cortisol indicates secretion of cortisol due to chronic temperature and/or pH stress, which leads to immunosuppression in *L. gonius* fingerlings. However, the intensity varies with the amount of exposure.

5.2.11 Hsp-70 Expression

Members of heat shock protein 70 (Hsp-70) are highly conserved proteins of about 70 kDa and play important roles in protein folding. Levels of these proteins increase when cells are under stress. Environmental temperature influences both the basal and induced levels of HSPs (Das *et al*., 2005). Besides by classical heat shocks, HSP synthesis is induced by a wide range of stressors (Das *et al*., 2005). Heat shock protein synthesis has also been reported during oxidative stress, patho-physiological conditions (Hendrick and Hartl 1993; Yu, 1995). The sensitivity, lack of stressor specificity and highly conserved nature characterize HSPs as potential environmental biomarkers (Sanders, 1993). Under normal physiological conditions HSPs are not apparent in many organisms (Currie *et al*., 2000), but some show detectable levels (Dietz and Somero, 1992). Large amounts of constitutive HSP70 have been detected in salamanders, turtles and fish (Yu *et al*., 1994). Moreover, the same or related species of animals from different ecological habitats may show different degrees of heat shock HSP expression (Tomanek and Somero, 1999).

In the present study, Hsp-70 expression in serum and tissues of *L. gonius* fingerlings increased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). The expression Hsp-70 in muscle tissues was significantly low as compared to others tissues and serum in all treatments groups. The high levels of Hsp-70, suggests cross protection against the chronic temperature and/or pH stress. However, expression varies based on the amount of exposure. Das *et al.* (2006) reported that higher temperature can linearly induce Hsp production in *L. rohita* fingerlings. HSPs, temperature associated stress indicators, are expressed differently with varying temperature (Mohapatra *et al.*, 2013). Mohapatra *et al.* (2014) also reported quantitative expression of Hsp-70 at higher temperatures. HSPs are also considered to be the key markers for fluctuating temperature and it has been shown that the protective mechanism of an animal (Hartl, 1996).

5.2.12 Caspase-3 Activity

Caspases are aspartic acid proteases which takes part an important role in the apoptosis process. There are two groups of caspases taking part in apoptosis, as initiators and executioners. Caspases 2, 8, 9 and 10 are initiators and caspases 3 and 6 are executioners (Mary Lini *et al.*, 2013).

In the present study, caspase-3 activity in serum of *L. gonius* fingerlings was studied and found to increased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). The high level of caspase-3 activity may be related to the enhancement of programmed cell death due to temperature and/or pH stress. The apoptosis might be due to the failure of cellular defense mechanism under excessive stressful condition (Li *et al.*, 2012). However, the activity varies based on amount stress incurred to the organism. Our results are also supported by the finding of (Mosser *et al.*, 2000). A diverse range of stimuli including UV irradiation, cytotoxic drugs, growth factor deficiency, heat shock and viral infection have been shown to induce apoptosis in various cell types (Vaux, 1993). During apoptosis, members of the caspase protease family are activated, and the cells undergo rapid death (Yuan *et al.*, 1993; Henkart, 1996). Induction of the stress responsive heat shock protein (Hsp-70) observed during the present study may also accelerate apoptosis in various fish tissues (Stensløkken *et al.*, 2010; Yamashita *et al.,* 2010).

5.2.13 Thyroid Hormones (T3 and T4)

The thyroid hormones thyroxine (T4) and tri-iodothyronine (T3) are known to play an important role in fish growth (Miwa and Inui, 1985) and early development (Brown, 1997). Arjona *et al.* (2008) also reported that the thyroid hormones are known to play key roles on growth, development, and reproduction and stress responses in fishes. A decrease in T3 and T4 levels was reported in fish under stress (Klaren *et al.*, 2007). When fish are exposed to stressors the levels of thyroid hormones have been demonstrated to be decreased (Deane *et al.*, 2001) Specific metabolic functions of thyroid hormones have been documented in a number of studies, which correlate with its growth-promoting activities (Gorg, 2007).

In the present study, thyroid hormone levels in serum of *L. gonius* fingerlings decreased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). The reduction in T4 hormone level was much higher than the T3 hormone. The reduced growth rate in the fishes exposed to chronic temperature and/or pH stress can be linked with the reduced level of thyroid hormone levels and vice versa. During the study, a linear relation between weight gain % and thyroid hormone was observed.

5.2.14 Serum Electrolytes (Na^+, K^+ and Cl^-)

Water pH plays an important role in maintenance of the homeostasis in aquatic animals and changes in pH are reported to cause disturbances in acid-base and ion regulation and ammonia excretion (Wood *et al.*, 1989; Wilkie and Wood, 1991; Wilkie *et al.*, 1993; Jensen and Brahm, 1995). The fish gill is highly permeable to hydrogen ions. The significant influx of H^+ at low environmental pH has also marked effect upon the respiratory homeostasis and osmoregulatory process. The effect of low environmental pH on fish includes increased branchial loss of ions, reductions in plasma pH, bicarbonate, Na^+ and Cl^- concentrations, slowing of apical H^+/Na^+ exchanger process (Ultsch *et al.*, 1981), increased urinary excretion of surplus H^+ ions for compensation of the net base loss at branchial epithelium (McDonald and Millican, 1981). Besides increases in blood pH and plasma ammonia concentrations, exposure to high water pH also reduces plasma electrolytes such as Na^+ and Cl^-, reduction in the blood protein and haemoglobin concentration (Wood *et al.*, 1989; Wilkie and Wood, 1991; Wilkie *et al.*, 1993).

In the present study, Na^+ and Cl^- in serum of *L. gonius* fingerlings decreased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) stress as compared to ambient temperature and pH group, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0) group. On the contrary, K^+ follows an opposite trend on exposure to temperature and/ or pH stress.

The fish gill is highly permeable to hydrogen ions. The main structural target of H^+ is likely to be the gills and the physiological target is likely to be ion regulation (McDonald, 1983). The variations in water pH are reported to cause disturbances in acid-base and ion regulation and ammonia excretion (Wood *et al.*, 1989; Wilkie and Wood, 1991; Wilkie *et al.*, 1993; Jensen and Brahm, 1995). Numerous researchers have reported decrease in Na^+ and Cl^- on exposure to low acidic and high alkaline pH in fishes (Ultsch *et al.*, 1981; Wood *et al.*, 1989; Wilkie and Wood, 1991; Wilkie *et al.*, 1993). The rise in serum K^+ and decline in Na^+ level in the present study can also be correlated with the inhibition of Na^+/ K^+ - ATPase enzyme activity observed during the present study. Jensen *et al.* (1987) also explained that the inhibition of Na^+/K^+- ATPase enzyme results in tissue K^+ efflux and Na^+ uptake in fish.

5.2.15 Serum Osmolality

In the present study, osmolality in serum of *L. gonius* fingerlings decreased significantly ($p<0.05$) on exposure to low acidic pH (5.5) and high alkaline pH (9.0) stress as compared to ambient and higher temperature (33 ^{0}C) group, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH (33^0C x pH 9.0) stress. The reduction of serum osmolality can be correlated mainly with the reduction of serum Na^+ and Cl^- levels observed during the present study. McDonald and Millican (1992) also mentioned that the sodium is the major cation in the blood of fishes and together with chloride contributes most to the serum osmolality. McDonald and Millican (1992) have argued that electrolyte measurements are more reliable than measurement of serum osmolality and osmotic effects can be monitored by Na^+ and Cl^- measurements.

5.2.16 Histological Study

In the present study, histological study was carried out at the end of the experiment in gill, liver and intestinal tissue of *L. gonius* fingerlings exposed to multiple stressors. Histological study confirms that structural changes in gill, liver and intestinal tissues of *L. gonius* fingerlings increased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress as compared to ambient temperature and pH, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) or alkaline pH stress (33^0C x pH 9.0). However, there were no changes at ambient temperature 27^0C and pH 7.5 group.

The gills, which participate in many important functions in the fish, such as respiration, osmoregulation and excretion, remain in close contact with the external environment and particularly sensitive to changes in the quality of the water are considered the primary target of the contaminants (Camargo *et al.*, 2007; Fernandes *et al.*, 2003). Kaoud and El-Dahshan (2010) also reported the histopathological alteration due to prolonged exposure to heavy metal resulted in respiratory, osmoregulatory and circulatory impairment. Dash *et al.* (2011) reported damaged gill lamella at higher acclimated temperature. These reports support the present finding on the alteration of the gill structure of *L. gonius* fingerlings exposed to temperature and/or pH stress.

The most common alterations in the liver of the fingerlings in the present study at temperature and/or pH stress are vacuolation of the hepatocytes. The vacuolation of the hepatocytes might indicate an imbalance between the rates of synthesis of substances in parenchymal cells and rate of their release into circular system (Gernhofer *et al.*, 2001). Liver of fish is sensitive to environmental contaminants because many contaminants tend to accumulate in the liver and exposing it to a much higher levels than in the environment, or in other organs (Heath, 1995). Dash *et al.* (2011) also reported vacuolation of haepatocyte at higher acclimated temperature.

According to Bhatnagar *et al*. (2007), the observed irritation and destruction of the mucosa membrane of the intestine, hampers absorption. The pathological alterations in the intestine of the studied fish are in agreement with those observed by many investigators about the effects of different toxicants on fish intestine (Hanna *et al*., 2005; Cengiz *et al*., 2006). Epithelial degeneration, inflammatory cells infiltration in the sumucosa as well as submucosal edema was seen in the intestine of tilapia fish exposed to carbofuran (Soufy *et al*., 2007).

Results of the histological study confirmed that there are no changes or damaged in histoarchitecture of tissues at ambient temperature 27^0C and pH 7.5, whereas changes occur at pH (5.0 and 9.0) and temperature (33^0C) stress, which become severe on exposure to their combined effect. So, rearing the species at ambient temperature and pH will be an ideal condition not only for better physiological activity but also better growth and survival of the species. Otherwise, we have to find a stress mitigation measure to overcome the problems incurred due to temperature and/or pH stress. Histological alterations in all the tissues are due to the prolonged exposure of the fingerlings to temperature and/or pH stress. However, the rate of changes depends on the amount and time of exposure. The changes are a clear indication of stress which may be due to increase in metabolic activity, accumulation of toxic metabolites, oxygen deprivation, etc.

5.2.17 Scanning Electron Microscopy (SEM) of Gill Tissue

Gills are vital structures for fish, since they are the main site for gaseous exchange (Hughes, 1966) as well as partially responsible for osmoregulation (Gonzales and McDonald, 1992), acid base balance (Goss *et al*., 1992), excretion of nitrogenous compounds (Sayer and Davenport, 1987) and taste (Rios and Fanta, 1998) through surrounding environment to the body. Hence, gill plays a very important physiological function in case of fish. Fish gills can be used as model for studies on environmental impact (McKim and Erickson, 1991). Laurent and Perry (1991) consider the morphologic changes in gills, as a consequence of environmental changes, as adaptive attempts in conserving some physiological functions. It has been shown in animal that extreme temperature changes induce stress in organism and that is characterized by changes at the physiological (Trot, 2011) as well as the cellular architectural levels (Egginton and Sidell, 1989).

In the present study, SEM study confirms the results of the histological study. Ultra-structural changes in gill tissue of *L. gonius* fingerlings increased significantly ($p<0.05$) on exposure to low acidic pH (5.5), high alkaline pH (9.0) and higher temperature (33^0C) stress, as compared to ambient temperature and pH group, which was further aggravated by combined effect of temperature and acidic (33^0C x pH 5.5) and alkaline pH stress (33^0C x pH 9.0).

Fish gills were taken into consideration for present investigation as they were in direct contact with environment and therefore, gills could be good indicators of water quality. McKim and Erickson (1991) reported that fish gills can be used as model for studies on environmental impact. Conduction of heat between the environment and fish is mediated through the gills; hence, gills are regarded as the heat exchanger. Therefore, gills are considered to be the most appropriate organ for indicating thermal pollution (Alazemi *et al.*, 1996) and consequently in all the pollution studies in gill tissues of fish should be invariably become an organ of paramount importance to assess the magnitude of damage. Acharya *et al.* (2005) reported that exposure of *L. rohita* fingerlings to to acidic pH 5.5 and alkaline pH 9.0 affects physiological process and structural changes in gill tissue.

Fish exposed to temperature and/or pH stress may suffer respiratory stress and nervous disorders. Increasing stress level exerts a dual effect on their oxygen demand-supply ratio. Warmer waters carry less dissolved oxygen, yet the oxygen uptake by aquatic organisms is increased due to their higher metabolic rate. Earlier investigations on various fishes indicated that metabolic activity increased with increasing acclimation temperatures (Manush *et al.*, 2004; Debnath *et al.*, 2006). Electron Microscopy study of the gill tissue of freshwater prawn tissue also showed damaged at higher acclimatization temperature (Manush *et al.*, 2007). Das *et al.* (2014) also observed damaged lamellae at temperature extremes in electron microscopy study. Laurent and Perry (1991) consider the morphologic changes in gills, as a consequence of environmental changes, as adaptive attempts in conserving some physiological functions. From the results of the study, it is evident that long-term exposure to temperature and/or pH stress may cause respiratory stress, which ultimately may leads to death of the species.

5.3 Experiment 3

In aquaculture system, application of dietary supplements is found to be beneficial as growth promoter, immunomodulator and also as a stress mitigator. A number of natural and synthetic compounds have been applied as in many fish species such as vitamin C, vitamin E, beta glucan, pyridoxine, methyl donor, microbial levan, etc. These have been proven to be beneficial for their multiple functions. Tryptophan is an essential amino acid, which cannot be synthesized in the body and thus must be obtained from food or supplements. In addition to being a constituent of protein, it acts as a precursor of neurotransmitter serotonin and nicotinic acid. However, there is no information on the application L-TRP as dietary supplement on *Labeo gonius* species. Hence, the present experiment was intended to evaluate the growth and immunmodulatory responses of *L. gonius* fingerlings to dietary L-TRP supplementation.

5.3.1 Physico-chemical Properties of Water

In our experiment, we maintained a low stocking density and continuous aeration in order to avoid any confinement stress and ammonia accumulation in rearing tanks. Along with these, regular exchange of water in the rearing tanks helps us to maintain the physico-chemical parameters of water such as temperature, pH, dissolved oxygen, free carbon dioxide, ammonia, nitrite-N and nitrate-N within the optimum range of requirements for the fish as suggested by many authors.

Temperature plays an important role in regulating the metabolism of animals; therefore an optimum range of temperature is required for optimum metabolic activity, which in turn gives maximum yield. The water temperature was maintained within the range of 27.0-27.8^{0}C as an ambient temperature in all the experimental groups, which is comparable to the optimum temperature (28^{0}C) suggested by Chakraborty *et al.* (1976) for carp culture. Water pH is considered as one of the important parameters for successful aquaculture production. The optimum pH range for culture of carps is 7.5-8.5 (Banerjea, 1967) and the majority of the Indian carp culture ponds have a pH in this range. In our experiment also, water pH was within the range of 7.43-7.69 in all the experimental groups, which is quite favorable for culture. The dissolved oxygen concentration in water varies with a large number of factors such as water temperature, metabolic rate, biomass density, etc. In the present study, dissolved oxygen content in all the experimental groups was recorded to be within the range of 6.18- 7.31 mg/L, which is within the optimum range 6-7 mg/L for cyprinids suggested by Huet (1975). The higher dissolved oxygen values might be due to efficient aeration.

Toxic concentrations of ammonia for short-term exposure are between 0.6 and 2 mg/L (EIFAC, 1973). The suggested value of ammonia in water ranges from 0 to 0.1 mg/L (Jhingran, 1991), which is in agreement with the recorded range (0.12 - 0.19 mg/L) in the present study. Nitrite-N concentration was recorded in the range of 0.001- 0.006 mg/L, which is well within the permissible range for pond aquaculture (Boyd and Trucker, 1998). Nitrate-N level in a productive pond can be within 0.1- 5.0 mg/L (Boyd and Trucker, 1998). In the present study, nitrate-N (0.03 to 0.07 mg/L) was below toxic level. Nitrite-N and nitrate-N levels were within the permissible limits for the warm water fishes (Boyd, 1982).

5.3.2 Growth and Survival

In the present study, weight gain %, SGR, FER and PER values increased and FCR decreased significantly ($p<0.05$) with increase in dietary L-TRP supplementation. The augmented growth indices of *L. gonius* fingerlings with L-TRP supplementation might be owing to the growth promoting functions of L-TRP. Walton *et al.* (1984) recorded increase growth rate with increase in dietary TRP supplementation in rainbow trout. Tejpal *et al*. (2009) also reported increase WG %, SGR % and PER in *C. mrigala* fingerlings supplemented with L-TRP in diet. The higher growth rate in L-TRP supplemented groups is also supported by the finding of Kim Kyu *et al*. (1987). Tryptophan is needed not only for body protein deposition but also for synthesis of a neurotransmitter serotonin, involved in regulation of feed intake, resistance to stress and animal behaviour (S‘eve, 1999). The improved FCR observed in L-TRP supplemented groups suggests that the level of tryptophan incorporated in the diet was neither deficient nor excess. Our findings are also in agreement with the findings of Tejpal *et al*. (2009) in *C. mrigala* fingerlings. We also observed 100% survival in all the experimental groups, which suggest no negative effect of dietary L-TRP on survival rate. Laranja *et al.* (2010) also reported improved survival of juvenile mud crab fed with L-TRP in diet.

5.3.3 DNA, RNA and RNA/DNA Ratio

In the present study, RNA and RNA/DNA ratio increases significantly ($p<0.05$) with increase in dietary L-tryptophan supplementation. The increased RNA and RNA/DNA ratio can be correlated with higher growth indices observed during the study in *L. gonius* fingerlings. During the study, a linear relationship between weight gain % and RNA & RNA/DNA ratio was observed. A strong positive relation has been reported between nucleic acid concentration and specific growth rate (Bulow, 1970; Bulow *et al.*, 1981; Haines, 1973; Fukuda *et al.*, 2001). As the RNA content is related to rate of protein synthesis, the RNA/DNA ratio can be used as an index of growth (Smith and Buckley, 2003; Akhtar *et al.*, 2012a). We did not observe any significant change in DNA levels due to dietary L-TRP supplementation, and our findings are in support of Leslie (1955) who found that DNA content per somatic cell is constant within a species.

5.3.4 Body Indices

In the present study, HSI and GSI does not show any significant ($p>0.05$) difference among the treatments. Kim *et a*l. (1987) also reported that HSI was not influenced by dietary tryptophan supplementation in rainbow trout. Similar finding are also reported by Ciji (2011) in *L. rohita* juveniles. However, GSI shows higher values in the group supplemented with L-TRP in diet of the fingerlings. The higher values may be correlated with the improved growth performance in L-TRP supplemented group as compared to non L-TRP fed counterpart.

4.3.5 Tissue Glycogen

In the present study, glycogen in liver tissues increases significantly ($p<0.05$) with increase in L-TRP supplementation. However, there was no significant change in glycogen content of the muscle tissue. Several researchers have studied glycogenolysis in response to various stressors. Increase in glucose and concomitant decrease in glycogen on exposure to various environmental toxicants has been reported (Singh and Aggarwal, 1986). Nakano and Tomlinson *et* *al*. (1967) observed that all types of stress elevated the secretion of catecholamine which in turn increased the breakdown of glycogen and elevated blood glucose level. Hence, the higher glycogen content in L-TRP supplemented groups indicates normal deposition glycogen reserves and organism are in unstressed condition.

4.3.6 Ascorbic Acid

Vitamin C is a major water soluble antioxidant and is capable of maintaining sulphydryl compounds in a reduced state, particularly in several redox reactions (Deshpande *et al*., 2002). Teleosts do not have an active L-gulonolactone oxidase, the terminal enzyme in the ascorbic acid synthesis pathway (Dabrowski, 1990a). Thus, fishes are sensitive to a threshold level of vitamin C as they are unable to synthesize it *de novo* (Dabrowski, 1990b). Vitamin C is considered to play an important role in animal health as antioxidants, inactivating damage of free radicals produced during normal cellular activity from various stressors (Chew, 1995) have been reported, confirming protective roles of vitamin C. Cruz de Menezes *et al*. (2006) and Norouzitallab *et al*. (2008) also reported that vitamin C is well known for its anti-oxidative property and has also been used as an immuno-modulator in aquaculture.

In the present study, ascorbic acid in muscle tissue increases significantly ($p<0.05$) with increase in L-TRP supplementation, suggest better immunity of the fingerlings. The present findings are in congruence with the observations of Kumar (2008) in *L. rohita* fingerling. Vitamin C was demonstrated to increase in immunoresistance (Li and Lovell, 1985; Navarre and Halver, 1989; Hardie *et al,* 1991; Orbach and Laurencin, 1992).

5.3.7 Enzymatic Activity in Tissue

5.3.7.1 Enzymes of protein metabolism (AST and ALT)

Transaminases are the enzymes that redistribute amino nitrogen among amino acids, forming new amino acid with the amino group from the pre-existing ones. Non-essential amino acids required for growth and other metabolic processes are produced by this manner. Amino acids are the major source of energy in teleost (Belinski, 1974). Fishes utilize proteins and lipids prior to carbohydrates (Demeal, 1978). Stress hormone cortisol induces gluconeogenesis, which mobilize the protein source and lipid source for glucose synthesis. The most important aminotransferases are aspartate amino transferases (AST) and alanine amino transferases (ALT) (also called glutamate oxalo acetate transaminase or GOT and glutamate pyruvate transaminase or GPT). AST transfers the amino group of aspartic acid to α-keto glutarate and converts to glutamic acid forming oxalo acetate. ALT transfers the amino group from alanine to α-keto glutarate forming glutamic acid pyruvate. The pyruvate and oxalo-acetate thus formed are responsible for the synthesis of non-essential amino acids like alanine, aspargine, glutamine, etc. which in turn helps in protein synthesis and hence growth of the animal. Apart from this the amino acids are deaminated or transaminated to produce TCA cycle intermediates also helping in energy production in the form of ATP (De Silva and Anderson, 1995).

In the present study, AST and ALT activity in liver and muscle tissues decrease significantly ($p<0.05$) with increase in L-TRP supplementation, which can be correlated with the lesser amino acids mobilization for energy production could be the reason. The present findings are in congruence with the observation of Kumar (2008) in *L. rohita* fingerling, Tejpal *et al*. (2009) in *C. mrigala* fingerling and Ciji (2011) in *L. rohita* juveniles.

5.3.7.2 Enzymes of carbohydrate metabolism (LDH and MDH)

Lactate dehydrogenase is the terminal enzyme of the glycolysis (Embden-Meyerhoff pathway) pathway, which converts lactate to pyruvate in the presence of coenzyme NADH which is converted to NAD^+. Thus, lactate dehydrogenase helps in maintaining the glycolysis cycle by supplying NAD^+. In the presence of enough oxygen pyruvate enters the Kreb's cycle but when there is an oxygen shortage in the tissue, pyruvate is converted to lactate (Murray *et al*., 2000). In the present study, LDH activity in liver and muscle tissues decrease significantly ($p<0.05$) with increase in L-TRP supplementation, which can be correlated with reduction of energy demands due to L-TRP in diet. The present findings are in congruence with the observation of Kumar (2008) in *L. rohita* fingerling, Tejpal *et al*. (2009) in *C. mrigala* fingerling and Ciji (2011) in *L. rohita* juveniles.

Malate Dehydrogenase (MDH) is an enzyme of TCA cycle, which oxidizes malate to oxaloacetate, using NAD^+ as the electron acceptor. The activity of MDH showed an increase during gluconeogenesis and oxaloacetate is formed from the deamination of aspartate and malate or phosphoenol pyruvate from pyruvate. The conversion of oxaloacetate to malate in the mitochondria and the reverse in cytoplasm is catalyzed by MDH (Das, 2002c). In the present study, MDH activity in liver and muscle tissues decrease significantly ($p<0.05$) with increase in L-TRP supplementation, which can be correlated with the reduction of energy demands due to L-TRP in diet. The present findings are in congruence with the observation of Kumar (2008) in *L. rohita* fingerling, Tejpal *et al*. (2009) in *C. mrigala* fingerling and Ciji (2011) in *L. rohita* juveniles.

5.3.7.3 Enzyme of neurotransmission (AchE)

Acetyl choline esterase (AchE) is one of the most widely used enzymes as a biomarker for environmental pollution. Biomarkers are defined as biochemical, cellular or physiological variations which can be measured in an organism as response to an environmental perturbation or a contamination event (Livingstone *et al*., 1997). Among different biomarkers, a prominent position is occupied by the measure of acetylcholine esterase inhibition. Acetylcholine is widely distributed in nervous tissue, stored and released from the synaptic vesicles. It helps in the depolarization in an adjacent neuron and thus passes nerve impulse. Acetylcholine is rapidly destroyed by choline esterase, a group of related enzymes which are hydrolytic in action (Stowe, 1969). It is well known that some pesticides (organophosphates and carbamates) inhibit AchE which increases the acetylcholine and stimulates the medullary cells to release catecholamine (Stowe, 1969) and also increase in aberrant behaviour (Murthy *et al.,* 1984; Jones *et al.,* 1999). Increased catecholamine may affect the activity of enzymes involved in glycogen synthesis and glycogenolysis. In the present study, AchE activity in brain tissue increased significantly ($p<0.05$) with increase in L-TRP supplementation. The present findings are in congruence with the observation of Kumar (2008) in *L. rohita* and Tejpal *et al*. (2009) in *C. mrigala* fingerling and Ciji (2011) in *L. rohita* juveniles.

5.3.7.4 Enzymes of oxidative stress (Catalase, SOD and GST)

Under normal physiological conditions animal cell produces reactive oxygen species (ROS) such as H_2O_2, O_2^-, OH^- that may damage most cellular components leading to cell death. When the rate of ROS generation exceeds that of their normal, oxidative stress occurs. Oxidative stress results when the antioxidant defenses are overcome by pro-oxidant forces and reactive oxygen species are not adequately removed (Sies *et al*., 1986). Living organisms are protected from the danger of reactive oxygen species (ROS) by several defense mechanisms including antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase etc. Antioxidants also act in conjunction with these enzymatic defenses. Superoxide dismutase (SOD), a cytosolic enzyme that is specific for scavenging superoxide radicals (O_2^-) is involved in protective mechanisms within tissue injury following oxidative process and phagocytosis. Catalase (CAT) is another major primary antioxidant defense component that works primarily to catalyze the decomposition of H_2O_2 to H_2O, sharing this function with glutathione peroxidase (GPX). Therefore both these enzymes detoxify H_2O_2 derived from SOD activity. In the presence of low H_2O_2 levels, organic peroxides are the preferred substrate for GPX. However at high H_2O_2 concentrations they are metabolized by CAT (Yu, 1995). In the present study, Catalase, SOD and GST activity in liver and gill tissues decrease significantly ($p<0.05$) with increase in L-TRP supplementation, which indicate improvement of health status even in unstressed condition by reducing oxidative changes. The present findings are in congruence with the observation of Kumar (2008) in *L. rohita* fingerling and Ciji (2011) in *L. rohita* juveniles.

5.3.7.5 Digestive enzymes (Protease, amylase and lipase)

Digestive enzyme activity can be used as an indicator of food acceptance and to some extent can serve as an indicator for digestive capacity in relation to the type of feed offered (Suzer *et al*., 2007). Breakdown of large nutrient into small absorbable subunits in the digestive tract of the animal depends largely on available nutrients (Cho, 1987).

In the present study, protease and amylase enzyme activities in intestinal tissue increase significantly ($p<0.05$) with increase in L-TRP supplementation. However, dietary L-TRP did not influence the lipase activity. The higher protease activity can be correlated with the efficient utilization of nutrients for the specified purpose and also with higher growth rate observed during the study. Growth rate of fish depends on digestive capacity or metabolic activity required for protein synthesis (Blier *et al*. (1997). There is no available literature to support the increase amylase activity due to dietary L-TRP supplementation.

5.3.8 Haemato-immunological Parameters

5.3.8.1 Erythrocyte (RBC), haemoglobin (Hb) and haematocrit (Hct)

In the present study, RBC, haemoglobin and haematocrit count showed higher values in the groups supplemented with L-TRP in diet, which can be correlated with immune enhance properties of L-TRP through stimulation of erythropoiesis process. The present findings are in congruence with the observation of Ciji (2011) and Ciji *et al*. (2013b) in *L. rohita* juveniles.

During post-challenge period, RBC, haemoglobin and hematocrit count of *L. gonius* fingerlings was significantly lower in the experimental groups fed without L-TRP supplemented diet, which might be due to bacterial stress. Foda (1973) also reported decreased Hb content in Atlantic salmon due to furunculosis caused by *Aeromonas salmonicida*. A decreased Hb content was also observed in *L. rohita* fingerlings after challenge with *A. hydrophila* (Misra *et al*., 2006). However, RBC, Hb and Hct count in L-TRP supplemented groups remains high, though overall lower values were observed as compared to pre-challenge. This suggests immune enhance properties of L-TRP through stimulation erythropoiesis process. The present findings are in congruence with the observation of Ciji (2011) and Ciji *et al*. (2013b) in *L. rohita* juveniles.

5.3.8.2 Leucocyte (WBC) count

Leucocytes (WBC) play an important role for enhancing non-specific or innate immunity, which is considered as an indicator of the health status of fish (Roberts, 1978). The leukocytes are involved in regulation of immunological function in the organism (Santhakumar *et al*., 1999).

In the present study, WBC counts increases significantly ($p<0.05$) with increase in L-TRP supplementation in *L. gonius* fingerlings, suggests immune enhance properties of L-TRP through stimulation of leucopoiesis process. The present findings are in congruence with the observation of Kumar (2008) in *L. rohita* fingerling, Ciji (2011) in *L. rohita* juveniles and Ciji *et al*. (2013b) in *L. rohita* juveniles. Hoseini *et al*. (2012) also reported that dietary supplementation of L-tryptophan can increased immune response in fish.

During post- challenge period, overall higher values were recorded as compared to pre-challenge. This suggests immune enhance properties of L-TRP through stimulating leucopoiesis process. The present findings are in congruent with the observation of Kumar (2008) in *L. rohita* fingerling and Ciji (2011) in *L. rohita* juveniles. Ciji *et al*. (2013b) also reported enhanced non-specific resistance factor (WBC count) in vitamin E and L-TRP diet fed groups compared with control upon challenge with *A. hydrophila*.

5.3.8.3 Nitroblue tetrazolium (NBT)

The phenomenon known as respiratory burst gives a measure of oxygen dependent defence mechanism in vertebrate phagocytic cells, wherein there is generation of reactive oxygen which intermediates with powerful anti-microbial activity (Itou *et al*., 1997). Increased respiratory burst activity can be correlated with increased bacterial pathogen killing activity of phagocytes (Sharp and Secombes, 1993) and hence a better immunity. The respiratory burst activity of phagocytes was measured by reduction of NBT by intracellular superoxide radicals produced by leucocytes.

In the present study, NBT values increased significantly ($p<0.05$) with increase in L-TRP supplementation suggesting increased bacterial pathogen killing activity of phagocytes and hence, better immune status of *L. gonius* fingerlings. The present findings are in congruence with the observation of Ciji (2011) and Ciji *et al*. (2013b) in *L. rohita* juveniles. Hoseini *et al*. (2012) also reported that dietary supplementation of L-tryptophan can increased immune response in fish.

During post- challenge period, overall higher values were recorded as compared to pre-challenge. This suggests immunostimulating properties of L-TRP. It has been shown that tryptophan can reduce the immunosuppressive effect of live hot virus and amplify specific protective immune response against herpes simplex virus (Adams *et al*., 2004). Emadi *et al*. (2010) also showed that dietary tryptophan increased the serum levels of interferons and immunoglobulins in chickens of Infectious Bursal Diseases Virus (IBDV) challenged broiler chickens. Ciji *et al*. (2013b) also reported enhanced non-specific resistance factor (NBT) in vitamin E and L-TRP diet fed groups compared with control upon challenge with *A. hydrophila*.

5.3.8.4 Blood glucose

Glucose is one of the most important sources of energy for the animals and glucose has been studied as an indicator of stress caused by physical factors (Manush *et al*., 2005). Nakano and Tomlinson (1967) observed that all types of stress elevated the secretion of catecholamine which in turn increased the breakdown of glycogen and enhanced blood sugar level. The elevation of blood sugar levels in fish by both corticosteroids and catecholamines makes it the ideal parameter to study the secondary stress response, on activation of direct sympathetic (Chromaffin tissue) as well as humoral (internal tissue) pathways (Wedemeyer and Mcleay, 1981).

In the present study, blood glucose level decreases significantly ($p<0.05$) with increase in L-TRP supplementation in *L. gonius* fingerlings, which can be correlated with inhibitory role of L-TRP on release of cortisol, hence reducing glucose levels. Another possible reason might be of efficient utilization of glucose from the blood due to L-TRP in diet. The present findings are in congruence with the observation of Kumar (2008) in *L. rohita* fingerling. However, Ciji (2011) did not observe any significant changes in *L. rohita* juveniles with dietary tryptophan.

During post-challenge period, blood glucose level of *L. gonius* fingerlings was significantly higher in the groups fed without L-TRP supplemented diet, which might be due to bacterial stress. However, the blood glucose level in L-TRP supplemented groups remains low, though overall higher values were recorded as compared to pre-challenge. This suggests immunostimulating properties of L-TRP in *L. gonius* fingerlings.

5.3.8.5 Albumin (A), globulin (G), A-G ratio and total serum protein

Proteins are the most important compounds in the serum, and its concentration is used as a basic index for the health status of fish (Buchanan *et al*., 1997). Among the serum protein, albumin and globulin are the major proteins, which play a significant role in the immune response (Kumar *et al*., 2007). The gamma globulin fraction is the source of almost all the immunological active protein of the blood. Globulins like gamma globulins are absolutely essential for maintaining a healthy immune system. Serum albumin and globulin values in the fishes treated with different immunostimulants were always found to be higher than control. An increase in the serum protein, albumin and globulin levels are thought to be associated with a stronger innate response in fishes (Wiegertjes *et al.*, 1996).

In the present study, the serum total protein, albumin and globulin levels increased and A-G ratio decreases significantly ($p<0.05$) with increase in L-TRP supplementation in *L. gonius* fingerlings, suggesting to stronger innate response in *L. gonius* fingerlings. Wiegertjes *et al.* (1996) substantiate supported our findings. The present findings are in congruence with the observation of Ciji (2011) and Ciji *et al.* (2013b) in *L. rohita* juveniles.

During post-challenge period, serum total protein, albumin and globulin levels of *L. gonius* fingerlings was significantly decreased and A-G ratio increases in the experimental groups fed without L-TRP supplemented diet. This may be due to vascular leaking of serum proteins because of increased permeability due to bacterial stress. Similar observation was made by Misara *et al.* (2006) in *L. rohita* juveniles. However, serum total protein, albumin, globulin levels remains high and low A-G ratio in L-TRP supplemented groups though overall variations were recorded as compared to pre-challenge. This suggests immune-stimulating properties of L-TRP in *L. gonius* fingerlings. Tryptophan has been shown to reduce the immunosuppressive effects of challenge virus on immunity by increasing the production of interferons and immune-globulins in chickens (Emadi *et al.*, 2010).

5.3.8.6 Lysozyme activity

Lysozyme is known to act as an important innate defence mediator against parasitic, bacterial and viral infections, and in response to infection, its activity is found to increase in fish blood (Ingram, 1980). Lysozyme plays important role in innate immunity by lysis of bacterial cell wall and thus stimulates the phagocytosis of bacteria (Ellis, 1990). Lysozyme activity can be affected by stressors, infectious micro-organisms (Mock and Peters, 1990). Temperature also plays an important role in regulating lysozyme activity in fish (Ndong *et al.*, 2007).

An increased level has been considered a natural protective mechanism in fish (Ingram, 1980).

In the present study, lysozyme activity increases significantly ($p<0.05$) with increase in L-TRP supplementation in *L. gonius* fingerlings, suggests immune-stimulating properties of L-TRP. It has been reported that oral administration of 300 mg of tryptophan to rats enhanced phagocytosis by macrophages and the innate immune response (Esteban *et al*., 2004). The present findings are in congruence with the observation of Ciji (2011) and Ciji *et al*. (2013b) in *L. rohita* juveniles. Hoseini *et al*. (2012) also reported that dietary supplementation of L-tryptophan can increased immune response in fish.

During post-challenge period, overall higher values were recorded as compared to pre-challenge. Among the entire group, serum lysozyme activity was lower in the group supplemented without L-TRP in diet, which might be due to bacterial stress. Mock and peters (1990) also reported reduced lysozyme activity in fish affected by infectious micro-organisms. The higher lysozyme activity in L-TRP supplemented groups suggests immune-stimulating properties of L-TRP in *L. gonius* fingerlings. Watson and Petro (1984) also reported that the dietary supplementation of 0.22% L-TRP increased the resistance to bacterial and parasitic infections in rats. Ciji *et al*. (2013b) also reported enhanced non-specific resistance factor lysozyme activity in vitamin E and L-TRP diet fed groups compared with control upon challenge with *A. hydrophila*.

5.3.9 Serum Enzyme Activity

In the present study, AST, ALT and LDH activity in serum decrease significantly ($p<0.05$) with increase in L-TRP supplementation. The reduced activity of AST and ALT can be correlated with lesser amino acids mobilization for energy production. The lower LDH activity in L-TRP supplemented group suggests reduction of energy demands due to L-TRP in diet. The present findings are in congruence with the observation of Kumar (2008) in *L. rohita* fingerling, Tejpal *et al*. (2009) in *C. mrigala* fingerling, Ciji (2011) in *L. rohita* juveniles, Akhtar *et al*. (2014) in *L. rohita* juveniles and Kumar *et al*. (2014) in *L. rohita* fingerlings in tissue LDH.

During post-challenge period, serum enzyme activity of *L. gonius* fingerlings was significantly higher in the experimental groups fed with L-TRP supplemented diet, which might be due to bacterial stress. However, the value in L-TRP supplemented groups remains low, though overall higher value observed as compared to pre-challenge. This suggests immune-stimulating properties of L-TRP in *L. gonius* fingerlings.

5.3.10 Serum Cortisol

The serum cortisol level observed in the group fed without L-TRP decreased significantly with increase in L-TRP supplementation, which indicates proper utilization of dietary L-TRP for the reduction of cortisol even in unstressed condition and resulted better immunity. The present findings are also in congruence with the observation of Tejpal *et al*. (2009) in *C. mrigala* fingerling and Ciji (2011) in *L. rohita* juveniles. Martins *et al*. (2013) also observed significant reduction in cortisol level in unstressed fish fed with TRP-supplemented diet.

During post-challenge period, cortisol level of *L. gonius* fingerlings was significantly higher in the experimental groups fed with % L-TRP supplemented diet, which might be due to bacterial stress. However, the value in L-TRP supplemented groups remains low, though overall higher value observed as compared to pre-challenge. This suggests immune-stimulating properties of L-TRP in *L. gonius* fingerlings.

5.3.11 Thyroid Hormones

The thyroid hormones thyroxine (T4) and tri-iodothyronine (T3) are known to play an important role in fish growth (Miwa and Inui, 1985) and early development (Brown, 1997). Arjona *et al*. (2008) also reported that the thyroid hormones are known to play key roles on growth, development, and reproduction and stress responses in fishes. Specific metabolic functions of thyroid hormones have been documented in a number of studies, which correlate with its growth-promoting activities (Gorg, 2007).

In the present study, thyroid hormone levels in serum increased significantly ($p<0.05$) with increase in L-TRP supplementation. The higher growth rate in the fishes fed with dietary L-TRP can be related with the thyroid hormone levels as the groups with lowest growth rate showed the lowest T4 and T3 levels and *vice versa*. During the study, a linear relation between weight gain % and thyroid hormone was observed. Ciji *et al*. (2013c) and Akhtar *et al*. (2013) also reported that dietary supplementation of L-tryptophan increased thyroid hormone level in *L. rohita* juveniles.

5.3.12 Survival % in Challenge Study

In India, *A. hydrophila* is one of the major bacterial pathogen, known to cause a variety of diseases in fish such as hemorrhagic septicemia, infectious dropsy, tropical ulcerative disease and fin rot leading to heavy mortality in aquaculture farms (Kumar and Dey, 1988; Karunasagar *et al*., 1997). In the present study, highest survival % was recorded in the groups supplemented with L-TRP in diet, which indicates positive influence of L-TRP on the survival of *L. gonius* fingerlings by resisting the *A. hydrophila* infection.

The higher survival could be an enhancement of macrophagocytic activity, as indicated by the respiratory burst activity as well as serum lysozyme activity in L-TRP supplemented groups. The low survival in the group supplemented without L-TRP in diet can be correlated with the higher level of induced cortisol observed during the study. Increased in cortisol level reduces the leucocytes count either due to increased apoptosis, reduction in leucocytes proliferation or leucocytes redistribution. Cortisol has been shown to suppress immunoglobulin production by lymphocytes and reduce the production of various intercellular mediators, such as prostaglandin, thereby reducing disease resistance resulting from stressful environmental condition (Wedemeyer *et.al*., 1996; Reddy and Leatherland, 1998).

5.4 Experiment 4

During the culture period, fishes are exposed to environmental changes. These frequently cause various degrees of trauma (stress) in aquatic organisms resulting in detrimental consequences. In fish, the stressful condition is not due to a single stressor but is a combined effect of multiple stressors. These stressors may lead to reduction in growth rate, decrease in disease resistance, immune-suppression and even death of fishes, thereby affecting both economic and socio-economic status of the aquaculturist. To overcome this, numbers of natural/ synthetic compounds have been found to be beneficial as stress mitigator in many fish species. In this context, considering L-tryptophan function as a cortisol blocker, it can be evaluated to use as a dietary supplement to mitigate combined effect of multiple stressors and to improve the protein utilization efficiency in fish and hence aquaculture production. In this backdrop, the fourth experiment was designed to study the possible role of dietary L-TRP on stress mitigation of *Labeo gonius* fingerlings to multiple stressors (temperature and pH stress).

5.4.1 Physico-chemical Properties of water

In our experiment, we maintained a low stocking density and continuous aeration in order to avoid any confinement stress and ammonia accumulation in rearing tanks. Along with these, regular exchange of water in the rearing tanks helped us to maintain the physico-chemical parameters of water such as dissolved oxygen, free carbon dioxide, ammonia, nitrite-N and nitrate-N within the optimum range of requirements for the fish as suggested by many authors.

Temperature plays an important role in regulating the metabolism of animals; therefore an optimum range of temperature is required for optimum metabolic activity, which in turn gives maximum yield. The water temperature was maintained at 27^0C as an ambient temperature in T_1 experimental group, which is comparable to the optimum temperature (28^0C) suggested by Chakraborty *et al.* (1976) for carp culture, while the temperature in other experimental groups (T_2 to T_9) was kept steady at 33^oC to ensure chronic thermal stress to the animal during the entire experimental period of 60 days. The chronic thermal stress was decided based on the findings of the 1st experiment. Das *et al.* (2005) also reported stress to *L. rohita* fingerlings on chronic exposure to 33^0C.

The pH 7.5 was maintained as an ambient pH in T_1 experimental group, which is within optimum range (7.5-8.5) as suggested by Banerjea (1967) for carp culture, while the pH in other experimental groups kept steady at 9.0 (in T_2, T_3, T_4 and T_5) and 5.5 (in T_6, T_7, T_8 and T_9) to ensure chronic pH stress to the *L. gonius* fingerlings during the entire experimental period of 60 days. The pH range (5.5 and 9.0) was selected based on the findings of Acharya *et al.* (2005) and Das *et al.* (2006) on fingerlings of carp species. They have reported that the chronic exposure to acidic pH (5.5) and alkaline pH (9.0) imparted stress to the fingerlings.

The dissolved oxygen level in water varies with a large number of factors such as water temperature, metabolic rate, biomass density, etc. The dissolved oxygen level in different experimental tubs was recorded to be within the range of 5.81- 7.29 mg/L, which is within the optimum range 6- 7 mg/L for cyprinids suggested by Huet (1975). From the above result, it is assumed that dissolved oxygen was optimum throughout the experimental period, which might be due to efficient aeration. Toxic concentrations of ammonia for short-term exposure are between 0.6 and 2 mg/L (EIFAC, 1973). The suggested value of ammonia in water ranges from 0 to 0.1 mg/L (Jhingran, 1991), which is in agreement with the recorded range (0.14- 0.29 mg/L) in the present study. Nitrite-N concentration was recorded in the range of 0.002-0.013 mg/L, which is well within the permissible range for pond aquaculture (Boyd and Trucker, 1998). Nitrate-N level in a productive pond can be within 0.1- 5.0 mg/L (Boyd and Trucker, 1998). In the present study, nitrate-N (0.03- 0.14 mg/L) was below the toxic level. Nitrite-N and nitrate-N levels were within the permissible limits for warm water fish (Boyd, 1982).

5.4.2 Growth and Survival

In the present study, weight gain %, SGR, FER, PER and survival % decreased, whereas FCR values increased significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. The decrease in growth rate upon exposure to multiple stressors can be attributed to re-allocation of energy towards maintenance and repair under stress (Kumaraguru and Beamish, 1986). Das *et al.* (2005) also observed reduction in growth rate and survival percentage in fishes exposed to higher temperature. Similar findings are also reported by Akhtar *et al.* (2011).

Reduced growth rate and food conversion efficiency have been reported in fish at combined stress of salinity and higher temperature (Likongwe *et al*., 1996).

However, weight gain %, SGR, FER, PER and survival % increased, whereas FCR decreased significantly ($p<0.05$) with increase in dietary L-tryptophan supplementation. Dietary supplementation of either 1.4 or 2.1% L-TRP improved growth indices in stressed groups. Walton *et al.* (1984) recorded improved growth performance with increase in dietary TRP supplementation in rainbow trout. The higher growth rate in L-TRP supplemented group is also supported by the finding of Kim Kyu *et al*. (1987). The augmented growth rate and improved FCR in stress exposed groups due to TRP supplementation might be owing to the stress-mitigating functions of TRP as reported by various authors (Lepage *et al*., 2002; Tejpal *et al*., 2009; Ciji *et al*., 2013a; Akhtar *et al*., 2013; Fatahi and Hoseini, 2013). Tryptophan is needed not only for body protein deposition but also for synthesis of a neurotransmitter serotonin, involved in regulation of feed intake, resistance to stress and animal behaviour (S‘eve, 1999). Laranja *et al*. (2010) also reported improved survival of juvenile mud crab fed with L-TRP in diet.

5.4.3 DNA, RNA and RNA/DNA Ratio

In the present study, RNA and RNA/DNA ratio decreased significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. The reduced values in the fishes exposed to multiple stressors can be correlated with the lower growth indices incurred due to stress. The ratios between these two nucleic acids were reported to reduce, when the fishes were exposed to high temperatures (Spigarelli and Smith, 1976) or heavy metal salts (Kearns and Atchison, 1979).

However, RNA and RNA/DNA ratios increased significantly ($p<0.05$) with increase in dietary L-tryptophan supplementation. The increased RNA and RNA/DNA ratio can be linked with higher growth indices observed during the study. During the study, a linear relation between weight gain % and RNA, RNA/DNA ratio was observed. A strong positive relation has been reported between nucleic acid concentration and specific growth rate (Bulow, 1970; Bulow *et al*., 1981; Haines, 1973; Fukuda *et al*., 2001; Akhtar *et al*., 2013). As the RNA content is related to rate of protein synthesis, the RNA/DNA ratio can be used as an index of growth

(Smith and Buckley, 2003; Akhtar *et al.*, 2012a; Akhtar *et al.*, 2013). We did not observe any significant change in DNA levels due to dietary tryptophan supplementation, and our findings are in support of Leslie (1955) who found that DNA content per somatic cell is constant within a species.

5.4.4 Tissue Glycogen

In the present study, glycogen content was assessed in liver and muscle of different experimental groups. The glycogen content decreased significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. The glycogen content of liver was found to be higher than the muscle tissue. In this study, the increase in glucose and concomitant depletion in glycogen shows that the glycogen was utilized for energy to cope with the stress. Several researchers have studied glycogenolysis in response to various stressors. Increase in glucose and concomitant decrease in glycogen on exposure to various environmental toxicants has been reported (Singh and Aggarwal, 1986). Nakano and Tomlinson *et al.* (1967) observed that all types of stress elevated the secretion of catecholamine which in turn increased the breakdown of glycogen and elevated blood glucose level. However, the glycogen content in both the tissue increased significantly ($p<0.005$) with increasing L-TRP supplementation, which could be attributed to lesser depletion of glycogen content. Hence, it may be inferred that supplementation of L-TRP in diet will reduce combined effect of temperature and pH stress in *L. gonius* fingerlings.

5.4.5 Ascorbic Acid

Vitamin C is a major water soluble antioxidant and is capable of maintaining sulphydryl compounds in a reduced state, particularly in several redox reactions (Deshpande *et al.*, 2002). Teleosts do not have an active L-gulonolactone oxidase, the terminal enzyme in the ascorbic acid synthesis pathway (Dabrowski, 1990a). Thus, fishes are sensitive to a threshold level of vitamin C as they are unable to synthesize it *de novo* (Dabrowski, 1990b). Vitamin C is considered to play an important role in animal health as antioxidants, inactivating damage of free radicals produced during normal cellular activity from various stressors (Chew,

1995) have been reported, confirming protective roles of Vitamin C. Cruz de Menezes *et al*. (2006) and Norouzitallab *et al*. (2008) also reported that vitamin C is well known for its anti-oxidative property and has also been used as an immuno-modulator in aquaculture.

In the present study, ascorbic acid content was assessed in muscle of different experimental groups. The ascorbic acid content decreased significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. The decrease in ascorbic acid content in tissue at higher stress groups could be attributed to its maximum utilization to reduce free radicals produced during stress. Das *et al.* (2009) also reported decrease in ascorbic acid content at higher temperature. Similar results were also found in thornfish *Therapon jarbua* (Forskal) when exposed to thermal stress (Chien *et al*., 1999). However, the ascorbic acid content in muscle tissue increased significantly ($p<0.005$) with increasing level of L-TRP in the diets, which could be due to stress mitigation role of L-TRP. Hence, it may be inferred that supplementation of L-TRP in diet will reduce combined of temperature and pH stress in *L. gonius* fingerlings. The present findings are in congruence with the observations of Kumar (2008) in *L. rohita* fingerling. Dietary high protein and vitamin C were supplemented for ameliorating stress (Manush *et al*., 2005) in *Macrobrachium rosenbergii*. Vitamin C was demonstrated to enhance tolerance to environmental stressors (Ishibashii *et al*., 1992) and increase in immunoresistance (Li and Lovell, 1985; Navarre and Halver, 1989; Hardie *et al*., 1991; Orbach and Laurencin, 1992).

5.4.6 Enzymatic Activity in Tissue

5.4.6.1 Enzymes of protein metabolism (AST and ALT)

Transaminases are the enzymes that redistribute amino nitrogen among amino acids, forming new amino acid with the amino group from the pre-existing ones. Non-essential amino acids required for growth and other metabolic processes are produced by this manner. Amino acids are the major source of energy in teleost (Belinski, 1974). Fishes utilize proteins and lipids prior to carbohydrates (Demeal, 1978). Stress hormone cortisol induces gluconeogenesis, which mobilize the protein source and lipid source for glucose synthesis. The most important aminotransferases are aspartate amino transferases (AST) and alanine amino transferases (ALT) (also called glutamate oxalo acetate transaminase or GOT and glutamate pyruvate transaminase or GPT). AST transfers the amino group of aspartic acid to α-keto glutarate and converts to glutamic acid forming oxalo acetate. ALT transfers the amino group from alanine to α-keto glutarate forming glutamic acid pyruvate. The pyruvate and oxalo-acetate thus formed are responsible for the synthesis of non-essential amino acids like alanine, aspargine, glutamine, etc. which in turn helps in protein synthesis and hence growth of the animal. Apart from this, the amino acids are deaminated or transaminated to produce TCA cycle intermediates also helping in energy production in the form of ATP (De Silva and Anderson, 1995).

In the present study, tissue specific AST, ALT activity was assessed in liver and muscle of different experimental groups. The activity of the enzyme increased significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. The activity of both the transferase increased with increasing stress, creating higher free amino acid mobilization to produce higher glucose to cope up with stress. Similar observation was made by Verma *et al.* (2006) in *C. carpio i.e*. the enzyme activity increases with increasing acclimation temperatures; in Tilapia after being exposed to confinement stress (Vijayan *et al.,* 1997). The increase in activity indicates greater mobilization of amino acid for glucose production via gluconeogenesis for glucose production to cope with stress (Chatterjee *et al.*, 2006). Knox and Greengard (1965) also reported that elevated levels of transaminase activity during stress would lead to increased feeding of ketoacids in to TCA cycle, there by affecting oxidative metabolism.

However, the activity of both the transferases decreased significantly ($p<0.05$) with increasing level of L-TRP in the diets. As there was less activity in the dietary L-TRP supplemented groups, which indicate lesser amino acids mobilization for energy production could be the reason. Supplementation of either 1.42 or 2.1% L-TRP caused maximum reduction in the activity of the enzyme both in muscle and liver. Hence, it may be inferred that supplementation of L-TRP in diet will reduce combined effect of temperature and pH stress in *L. gonius* fingerlings. The present findings are in congruence with the observation of Kumar (2008) in *L. rohita* fingerlings, Tejpal *et al*. (2009) in *C. mrigala* fingerlings, Ciji (2011) in *L. rohita* juveniles, Ciji *et al*. (2013a) in *L. rohita* juveniles, Akhtar *et al*. (2014) in *L. rohita* juveniles and Kumar *et al*., (2014) in *L. rohita* fingerling.

5.4.6.2 Enzymes of carbohydrate metabolism (LDH and MDH)

Lactate dehydrogenase is the terminal enzyme of the glycolysis (Embden-Meyerhoff pathway) pathway, which converts lactate to pyruvate in the presence of coenzyme NADH which is converted to NAD^+. Thus, lactate dehydrogenage helps in maintaining the glycolysis cycle by supplying NAD^+. In the presence of enough oxygen pyruvate enters the Kreb's cycle but when there is an oxygen shortage in the tissue, pyruvate is converted to lactate (Murray *et al*., 2000). Generally LDH activity increases in stress *viz*. temperature stress (Grigo, 1975), starvation stress (Vijayaraghavan and Rao, 1986), confinement stress (Chatterjee, 2003). The increase in LDH activity attributed to the production of preferred substrate (Lactate) for gluconeogenesis under thermal stress (Chatterjee *et al.,* 2006). A significant increase in LDH activity was observed at higher acclimation temperature (Verma *et al.,* 2006) that may be due to higher production of lactate, indicating oxygen limited condition in the cell. In the present study, tissue specific LDH activity was assessed in liver and muscle of different experimental groups. The activity of the enzyme increased significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. The higher enzyme activity suggesting the animals under stress showed increased lactate production for gluconeogenesis, this is supported by Chatterjee *et al.* (2006) and Verma *et al.* (2006). LDH activity decreased significantly ($p<0.05$) with increasing level of L-TRP in the diets indicating the stress mitigating properties of L-TRP. Supplementation of either 1.42 or 2.1% L-TRP caused maximum reduction in the activity of the enzyme both in muscle and liver. The present findings are in congruence with the observation of Kumar (2008) in *L. rohita* fingerlings, Tejpal *et al*. (2009) in *C. mrigala* fingerlings, Ciji (2011) in *L. rohita* juveniles, Ciji *et al*. (2013a) in *L. rohita* juveniles, Akhtar *et al*. (2014) in *L. rohita* juveniles and Kumar *et al*. (2014) in *L. rohita* fingerling.

Malate Dehydrogenase (MDH) is an enzyme of TCA cycle, which oxidizes malate to oxaloacetate, using NAD^+ as the electron acceptor. The activity of MDH showed an increase during gluconeogenesis and oxaloacetate is formed from the deamination of aspartate and malate or phosphoenol pyruvate from pyruvate. The conversion of oxaloacetate to malate in the mitochondria and the reverse in cytoplasm is catalyzed by MDH (Das, 2002c). Verma *et al*. (2006) observed that there was a significant increase in MDH activity in *C. carpio* fingerlings acclimated at higher temperatures in order to use the product (oxaloacetate) due to the higher activity of AST for production of more energy (ATP), which may be utilized for other physiological activities. Glucose might have therefore mobilized through non-carbohydrate source; mainly by protein, as transaminase activities were more at higher temperatures. Result also strengthens the fact that higher acclimation temperature induces amino acid mobilization (alanine, aspartate) in *C. carpio* fingerlings. In the present study, tissue specific MDH activity was assessed in liver and muscle of different experimental groups. The activity of the enzyme increased significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. Higher activity of MDH is an indicative of greater activity of TCA cycle due to increased energy demands during stress. This is supported by the findings of Das *et al.* (2006), who found elevated MDH activity in fishes at higher temperature. The dietary supplementation of L-TRP significantly ($p<0.05$) decreased the MDH activity both in liver and muscle indicating the stress mitigating effect of L-TRP. Higher activity of MDH indicates greater activity of TCA cycle due to increased energy demands. Lower activity of MDH in the stressed group fed with TRP supplemented diet suggested that the supplementation of dietary TRP may help in reducing energy demands in the *L. gonius* fingerlings. Supplementation of either 1.4 or 2.1% L-TRP caused maximum reduction in the activity of the enzyme both in muscle and liver. The present findings are in congruence with the observation of Kumar (2008) in *L. rohita* fingerling, Tejpal *et al*. (2009) in *C. mrigala* fingerling, Ciji (2011) in *L. rohita* juveniles, Ciji *et al*. (2013a) in *L. rohita* juveniles and Kumar *et al*., (2014) in *L. rohita* fingerling.

5.4.6.3 Enzyme of neurotransmission (AchE)

AchE is one of the most widely used enzymes as a biomarker for environmental pollution. Biomarkers are defined as biochemical, cellular or physiological variations which can be measured in an organism as response to an environmental perturbation or a contamination event (Livingstone *et al.*, 1997). Among different biomarkers, a prominent position is occupied by the measure of acetylcholine esterase inhibition. Acetylcholine is widely distributed in nervous tissue, stored and released from the synaptic vesicles. It helps in the depolarization in an adjacent neuron and thus passes nerve impulse. Acetylcholine is rapidly destroyed by choline esterase, a group of related enzymes which are hydrolytic in action (Stowe, 1969). It is well known that some pesticides (organophosphates and carbamates) inhibit AchE which increases the acetylcholine and stimulates the medullary cells to release catecholamine (Stowe, 1969) and also increase in aberrant behaviour (Murthy *et al.,* 1984; Jones *et al.,* 1999).

In the present study, AchE activity was assessed in brain of different experimental groups. The activity of the enzyme reduced significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. The reduced activity of acetylcholine esterase activity indicates stress to the animals induced by combined effect of temperature and pH stress. Similar observation was made by Verma *et al.* (2006) in *C. carpio* at higher temperature. In L-TRP treated groups the enzyme activity is higher and the minimum reduction was found in the group fed with diet containing either 1.4 or 2.1% L-TRP. This indicates the stress mitigating property of L-TRP in *L. gonius* fingerlings. The present findings are in congruence with the observation of Kumar (2008) in *L. rohita* fingerling, Tejpal *et al.* (2009) in *C. mrigala* fingerling, Ciji (2011) in *L. rohita* juveniles, Ciji *et al.* (2013a) in *L. rohita* juveniles and Kumar *et al.*, (2014) in *L. rohita* fingerling.

5.4.6.4 Enzymes of oxidative stress (Catalase, SOD and GST)

Under normal physiological conditions animal cell produces reactive oxygen species (ROS) such as H_2O_2, O_2^-, OH^- that may damage most cellular components leading to cell death. When the rate of ROS generation exceeds that of their normal, oxidative stress occurs. Oxidative stress results when the antioxidant defenses are overcome by pro-oxidant forces and reactive oxygen species are not adequately removed (Sies *et al.*, 1986). Living organisms are protected from the danger of reactive oxygen species (ROS) by several defense mechanisms including antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, etc. Antioxidants also act in conjunction with these enzymatic defenses. Superoxide dismutase (SOD), a cytosolic enzyme that is specific for scavenging superoxide radicals (O_2^-) is involved in protective mechanisms within tissue injury following oxidative process and phagocytosis. Catalase (CAT) is another major primary antioxidant defense component that works primarily to catalyze the decomposition of H_2O_2 to H_2O, sharing this function with glutathione peroxidase (GPX). Therefore both these enzymes detoxify H_2O_2 derived from SOD activity. In the presence of low H_2O_2 levels, organic peroxides are the preferred substrate for GPX. However at high H_2O_2 concentrations they are metabolized by CAT (Yu, 1995). Changes in antioxidative enzymes can be used as stress indicators in fish (Akhtar *et al.*, 2010; Ciji *et al.*, 2012; Ciji *et al.*, 2013a).

In the present study, catalase, SOD and GST activity was assessed in liver and gill tissue of different experimental groups. The activity of the enzyme increased significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. The higher activity of the enzymes decreases significantly with increasing L-TRP supplementation and the lowest activity was found in the group fed with either 1.4 or 2.1% L-TRP. This indicates that oxidative stress is lower in the L-TRP fed groups than the group fed without L-TRP. Thus, L-TRP is known to have anti-oxidative properties in mitigating oxidative stress brought about by combined effect of temperature and pH stress in *L. gonius* fingerlings. The present findings are in congruence with the observation of Kumar (2008) in *L. rohita* fingerlings, Tejpal *et al*. (2009) in *C. mrigala* fingerlings, Ciji (2011) in *L. rohita* juveniles, Akhtar *et al*. (2013) in *L. rohita* juveniles, Ciji *et al*. (2013a) in *L. rohita* juveniles and Kumar *et al*. (2014) in *L. rohita* fingerlings.

5.4.6.5 Enzyme of phospho monoesterase (ALP)

Alkaline phosphatase is zinc containing metallo-enzyme, which plays an important role in metabolism of phosphorus in the body. It is involved in membrane transport, glycogen metabolism, protein synthesis, calcium absorption and many other physiological processes including secretary function. In the present study, ALP activity was assessed in liver and muscle of different experimental groups. The activity of the enzyme increased significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. The higher enzyme activity may be due to hydrolysis of high- energy phosphate bonds to liberate phosphate ions to combat stressful condition or higher metabolic rate. Similar observation was made by Verma *et al*. (2006) in *C. carpio* fingerlings. However, L-TRP supplementation reduces the ALP activity in both liver and muscle. It indicates that combined effect of temperature and pH stress was decreased as L-TRP supplementation in feed increased. The present findings are in congruence with the observation of Kumar (2008) in *L. rohita* fingerling and Mohapatra *et al*. (2014) in *L. rohita* fingerling.

5.4.6.6 Enzyme of gluconeogenic pathways (FDpase)

In the present study, fructose 1, 6- diphophatase activity was assessed in liver and muscle of different experimental groups. The activity of the enzyme reduced significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. Lower activity of FDpase at higher temperature stress is reported by Verma *et al.* (2006) in *Cyprinus carpio* fingerlings. The higher in FDPase activity with multiple stressed without L-TRP supplementation group indicates that gluconeogenic pathway gets activated to cope with the increasing metabolic demand due to combined effect of temperature and pH stress. A consistent increase of transmaninase might have provided the substrate for gluconeogenesis (Suarez *et al.,* 1987; Moon and Foster, 1995). The significantly lower activity of this enzyme in the dietary L-TRP supplemented groups indicates that dietary L-TRP may lower gluconeogenic pathway activity by reducing the cortisol level. Hence, supplementation of dietary TRP may help in reducing energy demands in the *L. gonius* fingerlings by reducing FDPase activity. The present findings are in congruence with the observation of Kumar (2008) in *L. rohita* fingerlings.

5.4.6.7 Enzyme of pentose phosphate pathway (G6PDH)

G6PDH is an enzyme of the pentose phosphate pathway or hexose monophosphate shunt. It mediates the conversion of glucose-6-phosphate to 6-phospho-gluconolactone using $NADP^+$ as a coenzyme and releases NADPH. This pathway activity increases when the requirement of NADPH increases especially during the synthesis of fatty acids, cholesterol and sphingolipids (lipid anabolism). NADPH produced is also required by NADPH oxidase in producing superoxide anions for destroying phagocytosized material. So the enzyme activity may increase with increase in phagocytosis (Das, 2002b).

In the present study, glucose-6-phosphate dehydrogenase activity was assayed in liver and muscle of the fish of different experimental groups. The enzyme activity was higher in liver compared to muscle. The activity of the enzyme reduced significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. Lower activity of G6PDH at higher temperature is reported by Verma *et al.* (2006) in *Cyprinus carpio* fingerlings. Similar reduction in G6PDH activity with increased cortisol concentration was observed by Tripathi and Verma (2003) in fishes. Lower activity of enzyme due to combined effect of temperature and pH stress might be owed to higher cortisol level. The significantly higher activity of this enzyme in the dietary L-TRP supplemented groups indicates that dietary L-TRP may increase phagocytic activity by producing superoxide anions or have role in lipogenic activity. Pentose phosphate pathway also provides ribose-5-phosphate, which is used for the synthesis of nucleic acid. The higher nucleic acid synthesis has direct relation with growth of the fish. The higher growth in L-TRP fed groups can thus be related with higher G6PDH activity. The present findings are in congruence with the observation of Kumar (2008) in *L. rohita* fingerlings.

5.4.6.8 Enzyme of energy metabolism (ATpase)

It hydrolyzes a high-energy phosphate of ATP and utilizes that energy for the simultaneous transport of three Na^+ ions outward and two K^+ ions inward across the plasma membrane (Das, 2002a) *i.e.* ATPase hydrolyzes the high energy phosphate (ATP) and utilizes the energy to maintain ionic gradient across the plasma membrane. The activity of this enzyme is inhibited by the depletion of substrate ATP or due to some organochlorines. Stress leads to imbalance in osmoregulation. In freshwater Na^+ and Cl^- ions are lost and in marine water the ions are gained (Mazaeud *et al.*, 1977). In the present experiment, the activity of ATPase was assayed in gill and liver of *L. gonius* fingerlings in different experimental groups. The activity of the enzyme decreased significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. However, with increase in L-TRP supplementation the ATPase activity increases. This result suggests that the osmoregulation stress was higher on exposure to multiple stressors, which was improved by supplementation of L-TRP in diet. The presence of L-TRP in feed might have maintained the osmoregulation and prevented ion imbalance by reducing the stress level. The present findings are in congruence with the observation of Kumar (2008) in *L. rohita* fingerlings and Ciji (2011) in *L. rohita* juveniles and Akhtar *et al*. (2013) in *L. rohita* juveniles.

5.4.6.9 Digestive enzyme (Protease)

In the present experiment, the activity of protease was assayed in intestinal tissue of *L. gonius* fingerlings in different experimental groups. The activity of the enzyme reduced significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. Prabhu (2012) also reported to decrease in protease activity on exposure to elevated temperature and endosulfan stress. However, with increase in L-TRP supplementation increased protease activity in intestinal tissue. Digestive enzyme activity can be used as an indicator of food acceptance and to some extent can serve as an indicator for digestive capacity in relation to the type of feed offered (Suzer *et al*., 2007). The higher protease activity can be correlated with the efficient utilization of nutrients for the specified purpose and also with the higher growth rate observed during the study. Growth rate of fish depends on digestive capacity or metabolic activity required for protein synthesis (Blier *et al*., 1997). Breakdown of large nutrient into small absorbable subunits in the digestive tract of the animal depends largely on available nutrients (Cho, 1987).

5.4.7 Haemato-immunological Parameters

5.4.7.1 Erythrocyte (RBC), haemoglobin (Hb) and haematocrit (Hct)

In the present experiment, RBC, Hb and Hct count of *L. gonius* fingerlings in varied significantly ($p<0.05$) in different experimental groups. RBC, Hb and Hct counts decreased significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. This indicates reduced blood oxygen carrying capacity in both the stressed groups. The decreased value could be due to the erythrocytic cell lysis of blood cells and an increased removal of RBCs from the circulation as suggested in carp (Jensen 1990; Knudsen and Jensen, 1997) or haemodilution (Wedemeyer *et al*., 1984). Reduction in serum protein content in in the present study also supports the view of erythrocytic cell lysis and haemodilution. Das *et al*. (2006) also reported significant reduction in RBC and Hb count at acidic pH 5.5 and alkaline pH 9.0 in Indian major carps. Akhtar *et al*. (2011) also reported reduced level of RBC, Hb and Hct count in *L. rohita* fingerlings at 33^0C. The reduced blood oxygen carrying capacity can be compensated either through increasing oxygen affinity and capacity of Hb and/or increase in RBC production. Hence, with increase in L-TRP supplementation RBC, Hb and Hct count increases, suggesting immune enhancement properties of L-TRP through stimulation of erythropoiesis process. Hoseini *et al*. (2012) also reported that dietary supplementation of L-tryptophan can reduced the negative effects of stress and increased immune response in fish. The present findings are also in congruence with the observation of Ciji *et al*. (2013a) in *L. rohita* juveniles.

During post-challenge period, RBC, Hb and Hct count of *L. gonius* fingerlings significantly decreased in the groups fed with 0% L-TRP supplemented diet, which might be due to bacterial stress. Foda (1973) also reported decreased Hb content in Atlantic salmon due to furunculosis caused by *Aeromonas salmonicida*. A decreased Hb content was also observed in *L. rohita* fingerlings after challenge with *A. hydrophila* (Misra *et al*., 2006). However, RBC, Hb and Hct count in L-TRP supplemented groups remains high, though overall lower RBC, Hb and Hct value was observed as compared to pre-challenge. This suggests immune enhance properties of L-TRP through stimulation erythropoiesis process. The present findings are in congruence with the observation of Kumar (2008) in *L. rohita* fingerling and Ciji (2011) in *L. rohita* juveniles.

5.4.7.2 Leucocyte (WBC) count

Leucocytes (WBC) play an important role for enhancing non-specific or innate immunity, which is considered as an indicator of the health status of fish (Roberts, 1978). The leucocytes are involved in regulation of immunological function in the organism (Santhakumar *et al.*, 1999). In the present experiment, WBC count of *L. gonius* fingerlings varies significantly ($p<0.05$) in different experimental groups. WBC count decreased significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. This may be due to increased basal cortisol level, which reduces leucocyte count either due to increased apoptosis, reduction in leucocyte proliferation or leucocyte redistribution or weakening the leucopoiesis process. Verma *et al*. (2007) also reported reduction in leucocyte in *Cyprinus carpio* fingerlings on exposure to higher temperature, which was exacerbated on concurrent exposure of temperature and chlorine stress. Similarly, Akhtar *et al*. (2011) also reported reduced WBC count in *L. rohita* fingerlings at high temperature. Das *et al*. (2006) also reported reduction in leucocyte count at acidic pH 5.5 and alkaline pH 9.0 in *Catla catla* and *Cirrhinus mrigala* fingerlings. However, with increase in L-TRP supplementation WBC counts increases. This may be due to role of L-TRP on reduction of basal cortisol level and capacity to stimulate leucopoiesis process in *L. gonius* fingerlings. Hoseini *et al*. (2012) also reported that dietary supplementation of L-tryptophan can reduced the negative effects of stress and increased immune response in fish. The present findings are also in congruence with the observation of Ciji *et al*. (2013a) in *L. rohita* juveniles.

During post-challenge period, overall higher WBC value was observed as compared to pre-challenge. However, the values were not significant in 0% L-TRP supplemented group. The higher significant values in the L-TRP supplemented groups suggest immune enhancement properties of L-TRP through stimulating leucopoiesis process. The present findings are in congruent with the observation of Kumar (2008) in *L. rohita* fingerling and Ciji (2011) in *L. rohita* juveniles. Ciji *et al*. (2013b) also reported enhanced non-specific resistance factor (WBC count) in vitamin E and L-TRP diet fed groups compared with control upon challenge with *A. hydrophila*.

5.4.7.3 Nitroblue tetrazolium (NBT)

The phenomenon known as respiratory burst gives a measure of oxygen dependent defense mechanism in vertebrate phagocytic cells, wherein there is generation of reactive oxygen intermediates with powerful anti-microbial activity (Itou *et al*., 1997). Increased respiratory burst activity can be correlated with increased bacterial pathogen killing activity of phagocytes (Sharp and Secombes, 1993) and hence a better immunity. The respiratory burst activity of phagocytes was measured by reduction of NBT by intracellular superoxide radicals produced by leucocytes.

In the present experiment, NBT values of *L. gonius* fingerlings varied significantly ($p<0.05$) in different experimental groups. NBT value decreased significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. Verma *et al*. (2007) also reported reduction in NBT activity on exposure to higher temperature, which was exacerbated on concurrent exposure of temperature and chlorine stress in *Cyprinus carpio* fingerlings. Similarly, Akhtar *et al*. (2011) also reported reduced NBT activity in *L. rohita* fingerlings at high temperature. However, with increase in L-TRP supplementation NBT activity increases, suggesting increased bacterial pathogen killing activity of phagocytes and hence, better immune status of *L. gonius* fingerlings. The present findings are also in congruence with the observation of Ciji *et al*. (2013a) in *L. rohita* juveniles.

During post-challenge period, overall higher NBT values were observed as compared to pre-challenge. However, the values were not significant in 0% L-TRP supplemented groups. The higher values in the L-TRP supplemented groups suggest immunostimulating properties of L-TRP. It has been shown that L-TRP can reduce the immunosuppressive effect of live hot virus and amplify specific protective immune response against herpes simplex virus (Adams *et al*., 2004). Emadi *et al*. (2010) also showed that dietary tryptophan increased the serum levels of interferons and immunoglobulins in chickens of Infectious Bursal Diseases Virus (IBDV) challenged broiler chickens. Ciji *et al*. (2013b) also reported enhanced non-specific resistance factor (NBT) in vitamin E and L-TRP diet fed groups compared with control upon challenge with *A. hydrophila*.

5.4.7.4 Blood glucose

Glucose is one of the most important sources of energy for the animals and glucose has been studied as an indicator of stress caused by physical factors (Manush *et al*., 2005). Nakano and Tomlinson (1967) observed that all types of stress elevated the secretion of catecholamine which in turn increased the breakdown of glycogen and enhanced blood sugar level. The elevation of blood sugar levels in fish by both corticosteroids and catecholamines makes it the ideal parameter to study the secondary stress response, on activation of direct sympathetic (chromaffin tissue) as well as humoral (internal tissue) pathways (Wedemeyer and Mcleay, 1981). Ample of literature exists on the rise of glucose level on application of various stressors.

In the present experiment, blood glucose level of *L. gonius* fingerlings varied significantly ($p<0.05$) in different experimental groups. Blood glucose level increased significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. Das *et al.* (2009) also reported increase level of blood glucose at higher temperature. Das *et al.* (2006) observed higher blood glucose at acidic pH 5.5 and alkaline pH 9.0 in *Catla catla* and *Cirrhinus mrigala* fingerlings. The reason for increase in blood glucose may be due to higher cortisol levels, which is a hyperglycemic hormone. Nakano and Tomlinson (1967) also observed that all types of stress elevated the secretion of catecholamine which in turn increased the breakdown of glycogen and enhanced blood sugar level. Another reason could be increased in glycolysis is also indicated by increased LDH activity in liver and muscle due to higher production of lactate. Similar observation was made by Suarez and Mommsen (1987), Moon and Foster (1995) and Verma *et al.* (2006). However, with increase in L-TRP supplementation reduces the blood glucose levels. The decrease in blood glucose level in dietary L-TRP supplemented groups could be inhibitory role of L-TRP on release of cortisol, hence reducing glucose levels. Another possible reason might be of efficient utilization of glucose from the blood due to L-TRP in diet. The lower blood glucose content in L-TRP fed groups indicated stress-mitigating role of tryptophan (Lepage *et al.*, 2002; Tejpal *et al.*, 2009) is an effective mitigator of multiple stress in *L. gonius* fingerlings. Tejpal *et al.* (2009) have reported decrease in blood glucose levels in *C. mrigala* when fed with tryptophan-supplemented diets.

During post-challenge period, blood glucose level of *L. gonius* fingerlings was significantly higher in the groups fed with 0% L-TRP in diet, which might be due to bacterial stress. However, the blood glucose level in L-TRP supplemented groups remains low, though overall higher value was observed as compared to pre-challenge. This suggests immune-stimulating properties of L-TRP in *L. gonius* fingerlings. Hoseini *et al*. (2012) also reported that dietary supplementation of L-tryptophan can reduced the negative effects of stress and increased immune response in fish.

5.4.7.5 Albumin (A), globulin (G), A-G ratio and total serum protein

Proteins are the most important compounds in the serum, and its concentration is used as a basic index for the health status of fish (Buchanan *et al*., 1997). Among the serum protein, albumin and globulin are the major proteins, which play a significant role in the immune response (Kumar *et al*., 2007). The gamma globulin fraction is the source of almost all the immunological active protein of the blood. Globulins like gamma globulins are absolutely essential for maintaining a healthy immune system. Serum albumin and globulin values in the fishes treated with different immunostimulants were always found to be higher than control. An increase in the serum protein, albumin and globulin levels are thought to be associated with a stronger innate response in fishes (Wiegertjes *et al*., 1996).

In the present experiment, serum total protein, albumin and globulin levels of *L. gonius* fingerlings varied significantly ($p<0.05$) in different experimental groups. The values decreased significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. On the contrary, A-G ratio increases significantly. The serum protein reduction may be attributed to protein catabolism, the process converting blood and structural protein to energy, to meet the higher energy demand during the prevailing stress. Das *et al*. (2006) also reported reduction in total serum protein at low acid pH 5.5 and high alkaline pH 9.0 in *Catla catla* and *Cirrhinus mrigala* fingerlings. Haemolysis of erythrocytes, already been implicated in this species in the present study, also might have caused dilution of the plasma volume contributing to some extent in such reduction of serum protein content (Das *et al*., 2004). Verma *et al*. (2007) also reported reduced level of serum total protein, albumin and globulin on exposure to higher temperature, which was exacerbated on concurrent exposure of temperature and chlorine stress. Akhtar *et al*. (2011) also reported reduced protein and albumin levels in *L. rohita* fingerlings at high temperature. However, with increase in L-TRP supplementation, the serum total protein, albumin and globulin levels increases and A-G ratio decreases, suggesting to stronger innate response in *L. gonius* fingerlings. Wiegertjes *et al*. (1996) substantiate supported our findings. The present findings are also in congruence with the observation of Ciji *et al*. (2013a) in *L. rohita* juveniles.

During post-challenge period, serum total protein, albumin and globulin levels of *L. gonius* fingerlings significantly decreases and A-G ratio increases further in the experimental groups fed with 0 % L-TRP supplemented diet. This may be due to vascular leaking of serum proteins because of increased permeability. Similar observation was made by Misara *et al.* (2006) in *L. rohita* juveniles. However, serum total protein, albumin, globulin levels in L-TRP supplemented groups remains high, though overall lower value was observed as compared to pre-challenge. This suggests immunostimulating properties of L-TRP in *L. gonius* fingerlings. Tryptophan has been shown to reduce the immunosuppressive effects of challenge virus on immunity by increasing the production of interferons and immunoglobulins in chickens (Emadi *et al*., 2010).

5.4.7.6 Lysozyme activity

Lysozyme is known to act as an important innate defence mediator against parasitic, bacterial and viral infections, and in response to infection, its activity is found to increase in fish blood (Ingram, 1980). Lysozyme plays important role in innate immunity by lysis of bacterial cell wall and thus stimulates the phagocytosis of bacteria (Ellis, 1990). Lysozyme activity can be affected by stressors, infectious micro-organisms (Mock and peters 1990). Temperature also plays an important role in regulating lysozyme activity in fish (Ndong *et al*., 2007). An increased level has been considered a natural protective mechanism in fish (Ingram, 1980).

In the present experiment, serum lysozyme activity of *L. gonius* fingerlings varied significantly ($p<0.05$) in different experimental groups. The lysozyme activity decreased significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. Dominguez *et al*. (2005) observed a reduction in plasma lysozyme of Nile tilapia when exposed to 33^0C from 28^0C. Akhtar *et al*. (2011) also reported to reduced lysozyme activity in *L. rohita* fingerlings at 33^0C. Similar observation was made by Mock and Peters (1990), strong stressors such as transportation or acute water pollution reduced the lysozyme level in rainbow trout. Jeney *et al*. (1997) also reported reduced lysozyme activity in rainbow trout due to transportation stress. This may be due to higher cortisol levels, which protects these cells from apoptosis and stimulates non-specific immune system during stress condition. However, with increase in L-TRP supplementation increases the serum lysozyme activity, suggesting their immune-stimulating properties. It has been reported that oral administration of 300 mg of L-TRP to rats enhanced phagocytosis by macrophages and the innate immune response (Esteban *et al*., 2004). The present findings are also in congruence with the observation of Ciji *et al*. (2013a) in *L. rohita* juveniles.

During post-challenge period, overall higher lysozyme activity was observed as compared to pre-challenge. However, the values were not significant in the groups supplemented with 0% L-TRP in diet. The higher values in the L-TRP supplemented groups suggest immunostimulating properties of L-TRP. Dietary supplementation with 0.22% L-tryptophan reported to increase the resistance to bacterial and parasitic infections in rats (Watson and Petro, 1984). Ciji *et al*. (2013b) also reported enhanced non-specific resistance factor *viz*., lysozyme activity in vitamin E and L-TRP diet fed groups compared with control upon challenge with *A. hydrophila*.

5.4.8 Serum Enzyme Activity

5.4.8.1 Serum AST/GOT and ALT/GPT

In the present study, AST and ALT activity was assessed in serum of different experimental groups. The activity of the enzyme increased significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. The higher activity of both the transferase increased with temperature and/or pH stress, creating higher free amino acid mobilization to produce higher glucose to cope up with stress, this is supported by Verma *et al*. (2006) and Chatterjee *et al.* (2006). However, the activity of both the transferases decreased with increasing level of L-TRP in the diets. As there was less activity in the dietary L-TRP supplemented groups, which indicate lesser amino acids mobilization for energy production could be the reason. Hence, it may be inferred that supplementation of L-TRP in diet will reduce combined of temperature and pH stress in *L. gonius* fingerlings. Mohapatra *et al*. (2014) also reported the decreased AST and ALT activity in serum with supplementation of dietary probiotics in *L. rohita* fingerlings. The present findings are also in congruence with the observation of Kumar (2008) in *L. rohita* fingerlings, Tejpal *et al*. (2009) in *C. mrigala* fingerlings, Ciji (2011) in *L. rohita* juveniles, Ciji *et al*. (2013a) in *L. rohita* juveniles, Akhtar *et al*. (2014) in *L. rohita* juveniles and Kumar *et al*. (2014) in *L. rohita* fingerlings.

5.4.8.2 Serum ALP and ACP

In the present study, ALP and ACP activity was assessed in serum of different experimental groups. The activity of the enzyme increased significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. An increase in the activity of the two enzymes with temperature and/or pH stress is an indicative of cell damage in liver, intestine or kidney. These findings are supported by Vasudevan and Sreekumari (1998) and Kumar *et al*. (2006). However, L-TRP supplementation reduces the ALP and ACP activity in serum. It indicates that combined effect of temperature and pH stress was decreased as L-tryptophan supplementation in feed increased. Kumar *et al*. (2006) also reported reduced level of sALP and sACP in in *L. rohita* fingerlings when fed with probiotic enriched diet. Mohapatra *et al*. (2014) reported the decreased ALP and ACP activity in serum with supplementation of dietary probiotics in *L. rohita* fingerlings. The present findings are also in congruence with the observation of Kumar (2008) in *L. rohita* fingerlings.

5.4.8.3 Serum LDH

In the present study, LDH activity was assessed in serum of different experimental groups. The activity of the enzyme increased significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. The higher enzyme activity suggests the animals under stress showed increased lactate production for gluconeogenesis, this is supported by Chatterjee *et al.* (2006) and Verma *et al.* (2006). LDH activity decreased significantly ($p<0.05$) in the groups fed with diets supplemented with L-TRP indicating the stress mitigating properties of L-TRP. Supplementation of either 1.4 or 2.1% L-TRP caused maximum reduction in the activity of the enzyme. The present findings are in congruence with the observation of Kumar (2008) in *L. rohita* fingerlings, Tejpal *et al*. (2009) in *C. mrigala* fingerlings, Ciji (2011) in *L. rohita* juveniles, Ciji *et al*. (2013a) in *L. rohita* juveniles, Akhtar *et al*. (2014) in *L. rohita* juveniles and Kumar *et al*. (2014) in *L. rohita* fingerlings.

5.4.9 Serum Cortisol

Cortisol is also widely accepted as an indicator of stress in fish, generally increases after exposure to stressors (Wendelaar Bonga, 1997; Davis, 2004; Tejpal *et al*., 2009; Hoseini and Hosseini, 2010; Costas *et al*., 2012; Akhtar *et al*., 2012b). In the present study, the highest serum cortisol was found in T_6 and T_2 group fed without dietary L-TRP, indicates secretion of cortisol due to stress caused by multiple stressors. Gradual decrease in serum cortisol level in the stress groups fed with the increasing level of dietary L-TRP confers that dietary L-TRP mitigates the combined effect of temperature and pH stress in *L. gonius* fingerlings. Supplementation of either 1.4 or 2.1% L-TRP caused maximum reduction in the cortisol level. The present findings are in agreement with observations of Lepage *et al*. (2002) for *Oncorhnchus mykiss*, Tejpal *et al*. (2009) for *C. mrigala*, Hoseini and Hosseini (2010) for *C. carpio*, Ciji *et al*. (2013c) in *L. rohita* juveniles and Akhtar *et al*. (2013) in *L. rohita* juveniles. Hence, it can be concluded that increased cortisol levels normally led to immunosuppression in fish and therefore, dietary TRP supplementation may result in a positive immune-modulation at high stressful condition. Serotonin has been known to implicated in both epinephrine and cortisol regulation in fish during stress (Fritsche *et al*., 1993; Winberg and Nilsson, 1993; Winberg *et al*., 1997).

Similar trend of was observed for cortisol after challenge with *A. hydrophila* but the mean value was higher than the pre- challenge, this may be due to increased level of stress incurred due to bacterial challenge. Significant reduction of cortisol was observed in the L-TRP fed groups in comparison to non L-TRP fed counterparts. Stress reducing factor producing by L-TRP (Tejpal *et al*., 2009) might have lowered the cortisol levels in L-TRP fed fish during bacterial challenge and resulted better immunity.

5.4.10 Hsp-70 Expression

The Hsp-70 expression increased significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. The higher expression of Hsp-70 in serum and other tissues suggesting cross protection against the lethal stress incurred due to combined effect of temperature and pH stress. Das *et al*. (2006) reported that higher level of Hsp production in *L. rohita* fingerlings to combat thermal stress. Mohapatra *et al*. (2014) also reported quantitative expression of Hsp-70 at higher temperatures. In both the stressed group, gradual increase in dietary L-TRP reduces the HSP expression significantly. Stress reducing factor producing by L-TRP (Tejpal *et al*., 2009) might have lowered the Hsp levels in L-TRP fed fish exposed to combined effect of temperature and pH and resulted better growth and immunity. Among the TRP supplemented groups, maximum reduction of Hsp level was found with supplementation of either 1.4 or 2.1% L-TRP. Mohapatra *et al*. (2014) reported the dietary supplementation of probiotics reduces the quantitative expression of Hsp-70.

5.4.9 Thyroid Hormones

Thyroid hormones are known to play key roles on growth, development, reproduction and stress responses in fishes (Arjona *et al*., 2008). A decrease in T3 and T4 levels was reported in fish under stress (Klaren *et al*., 2007). Specific metabolic functions of thyroid hormones have been documented in a number of studies, which correlate with its growth-promoting activities (Gorg, 2007). In the present study, thyroid hormone levels (T3 and T4) in serum decreased significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) group without L-TRP supplementation. The reduction in T4 level was much higher than the T3 hormone. The reduced growth rate in the fishes exposed to multiple stressors can be correlated with the thyroid hormone levels as the groups with lowest growth rate showed the lowest T3 and T4 levels and vice versa. The low level of thyroid hormone recorded in stressed groups increase significantly ($p<0.05$) with increase in dietary L-tryptophan supplementation. During the study, a linear relation between weight gain % and thyroid hormone was observed. Dietary supplementation of either 1.4 or 2.1%

increased T3 and T4 hormone levels. Ciji *et al*. (2013c) and Akhtar *et al*. (2013) also reported that dietary supplementation of L-tryptophan improved thyroid hormone level by combating stress in *L. rohita* juveniles.

5.4.12 Serum Electrolytes

In the present study, Na^+ and Cl^- in serum of *L. gonius* fingerlings decreased significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. The fish gill is highly permeable to hydrogen ions. The main structural target of H^+ is likely to be the gills and the physiological target is likely to be ion regulation (McDonald, 1983). The variations in water pH are reported to cause disturbances in acid-base and ion regulation and ammonia excretion (Wood *et al*., 1989; Wilkie and Wood, 1991; Wilkie *et al*., 1993; Jensen and Brahm, 1995). Numerous of researchers have reported to decreased in Na^+ and Cl^- on exposure to low acidic and high alkaline pH in fishes (Ultsch *et al*., 1981; Wood *et al*., 1989; Wilkie and Wood, 1991; Wilkie *et al*., 1993). The rise in serum K^+ and decline in Na^+ in the present study can also be correlated with the inhibition of Na^+/K^+ ATPase enzyme activity observed during the present study. Jensen *et al*. (1987) also explained that the inhibition of Na^+/K^+ ATPase enzyme results in tissue K^+ efflux and Na^+ uptake in fish. However, supplementations of L-TRP in diet able prevent the loss of ions in *L. gonius* fingerlings on concurrent exposure to multiple stressors. Hoseini and Hosseini (2010) also reported that supplementation of L-TRP in diet of common carp able to withstand osmotic stress.The improved Na^+/K^+ ATPase enzyme activity observed during the present study in L-TRP supplemented group can also be correlated to maintenance of Na^+ and K^+ levels in serum.

5.4.13 Serum Osmolality

In the present study, osmolality in serum of *L. gonius* fingerlings decreased significantly ($p<0.05$) on exposure to multiple stressors in the T_6 group (33^0C x pH 5.5) followed by T_2 group (33^0C x pH 9.0) without L-TRP supplementation. The reduction of serum osmolality can be correlated mainly with the reduction of serum Na^+ and Cl^- levels. McDonald and Millican (1992) reported that the sodium is the major cation in the blood of fishes and together with chloride contributes most to the serum osmolarity. McDonald and Millican (1992) have argued that electrolyte measurements are more reliable than measurement of serum osmolarity and that osmotic effects can be monitored by Na^+ and Cl^- measurements. However, supplementations of L-TRP in diet able inhibit the loss of ions in *L. gonius* fingerlings on concurrent exposure to multiple stressors. The maintenance of Na^+ and Cl^- during the present study can be correlated with the normal osmolality value. Hoseini and Hosseini (2010) also reported that supplementation of L-TRP in diet of common carp able to withstand osmotic stress.

5.4.14 Survival % in Challenge Study

A. hydrophila, one of the major bacterial pathogens in India, is known to cause a variety of diseases in fish such as haemorrhagic septicaemia, infectious dropsy, tropical ulcerative disease and fin rot leading to heavy mortality in aquaculture farms (Kumar and Dey, 1988; Karunasagar *et al*., 1997). In the present study the highest percentage of survival was registered in the groups supplemented with L-tryptophan and no stress group. Present study shows that supplementation of dietary L-tryptophan had a positive influence on the survival of *L. gonius* fingerlings by resisting the *A. hydrophila* infection. The higher survival could be an enhancement of macrophagocytic activity, as indicated by the respiratory burst activity as well as serum lysozyme activity by dietary supplementation of L-TRP. The low survival in 0% dietary L-TRP supplemented group is due to more serum cortisol levels. The WBC count was lower in the stressed group fed with 0% dietary L-TRP supplemented diet; this may be due to increased basal cortisol level. Increase in cortisol level reduces leucocytes count either due to increased apoptosis, reduction in leucocytes proliferation or leucocytes redistribution. Cortisol has been shown to suppress immunoglobulin production by lymphocytes and reduce the production of various intercellular mediators, such as prostaglandin, therefore reduces disease resistance resulting from stressful environmental condition (Wedemeyer *et al*., 1996; Reddy and Leatherland, 1998).

Summary

6. SUMMARY

In today's climate change scenario, environmental degradation and disease outbreaks are the major constraints to aquaculture production. During the culture period, fishes are exposed to various environmental changes, which cause degree of trauma (stress) to the fish. The stressful condition in aquatic environment is not only due to a single stressor, but it is a combined effect of many stressors. They can adversely affect the survival, growth and reproduction of fish. Reducing the level and exposure time of stressors and minimizing the effect of stressors, lead to better survival, growth and production. Hence, understanding the interactions of multiple stressors is the biggest challenge to the researchers which can subsequently help to develop mitigation strategies during aquaculture practices. In this backdrop, the present study was conducted to evaluate the stress responses of *Labeo gonius* fingerlings exposed to multiple stressors (temperature and pH) and its mitigation through dietary L-tryptophan (L-TRP) in four different experiments. Considering its potential as an alternative candidate species in aquaculture, *L. gonius* was selected for the study.

First experiment was conducted to evaluate the physio-biochemical responses of *L. gonius* fingerlings exposed to four different temperatures (27, 30, 33 and 36^0C) for a period of 60 days. The results of the experiment showed normal physiological activities at 27 and 30^0C and causes stress at 33^0C and 36^0C, which in turn affect the growth and survival of the animal. Growth rate of the fingerlings was significantly higher at 27 and 30^0C ($p<0.05$), which decreased with increase in temperature. Reduction in weight gain % at higher temperatures also followed a linear relationship with RNA, RNA/DNA ratio and thyroid hormones thyroxine (T4) and tri-iodothyronine (T3) indicating depression of the growth indices due to stress. Higher temperatures significantly increased the cortisol level, blood glucose, SOD, catalase, GST, caspase-3 activity, AST, ALT, LDH, MDH, ALP, FDpase activity and reduction of AChE, G6PDH, liver glycogen, RBC, haemoglobin, haematocrit and WBC indicating stress to the fish. Reduction of NBT, lysozyme activity, serum protein, albumin globulin and increased in A/G ratio at higher temperatures indicated immune-depression of the species due to stress. However, quantitative expression of Hsp-70 at higher temperatures suggests the cross protection of the fish to thermal stress. Histological study of gill, liver and intestinal tissue and Scanning Electron Microscopy (SEM) studies of gill tissue ascribed the changes at higher temperatures. Thermal tolerance study carried out under the acclimation temperature 27, 30, 33 and 36^0C deliberates that acclimation temperature significantly influences the thermal tolerance limit and oxygen consumption rates of the *L. gonius* fingerlings ($p<0.05$).

Second experiment was conducted to elucidate the growth, biochemical and haemato-immunological responses of *L. gonius* fingerlings exposed to multiple stressors. Fishes were exposed to combined effect of temperature and pH *viz.*, T_1 (27^0C × pH 7.5), T_2 (27^0C × pH 5.5), T_3 (27^0C × pH 9.0), T_4 (33^0C × pH 7.5), T_5 (33^0C × pH 5.5) and T_6 (33^0C × pH 9.0) for a period of 60 days. Results of the interaction study revealed that the chronic exposure of *L. gonius* fingerlings to multiple stressors is more severe than the single stressor ($p<0.05$). However, combined effect of temperature and acidic pH stress was higher than the alkaline counterpart. Among the individual stressor, acidic pH stress was severe than the temperature or alkaline pH stress. It was found that temperature and/or pH stress had a significant adverse effect ($p<0.05$) on fingerlings. Growth rate and survival of the fingerlings were affected significantly ($p<0.05$) on exposure to temperature and/or pH stress. Reduction in weight gain % at temperature and/or pH stress showed a linear relationship with thyroid hormones thyroxine (T4) and tri-iodothyronine (T3) suggesting depression of growth indices. Increased levels of cortisol, blood glucose, caspase-3 enzyme, SOD, catalase, GST, AST, ALT, LDH, MDH, ALP, FDpase and reduction of AChE, G6PDH, RBC, haemoglobin, haematocrit and WBC indicates severe stress to the organisms. Reduction of NBT, lysozyme activity, total serum protein, albumin and globulin and increased in A/G ratio at temperature and/or pH stress indicated immune-depression of the species due to stress. However, higher quantitative expression of Hsp-70 in serum, gill and liver suggests the cross protection of the fingerlings against these stressors. Reduction of serum electrolytes (Na^+ and Cl^-) and osmolality and inhibition of Na^+/K^+ ATPase activity appeared to be one of the significant effects of pH fluctuations either alone or in combination with thermal stress. Histological study of gill, liver and intestinal tissue and Scanning Electron Microscopy (SEM) study of gill tissue ascribed the visual confirmation of the changes. However, rate of changes was different depending on the extent of exposure.

Third experiment was conducted to evaluate the growth and immunomodulatory responses of *L. gonius* fingerlings to dietary L-tryptophan supplementation. Fishes were fed with five isonitrogenous diets containing graded level of L-tryptophan (0, 0.35, 0.7, 1.4 and 2.1%) for a period of 60 days. Growth rate and immune response of the fingerlings were significantly ($p<0.05$) higher in treatment groups fed with L-TRP compared to control group. Higher weight gain % observed in L-TRP supplemented groups showed a linear relationship with RNA, RNA/DNA ratio and thyroid hormones thyroxine (T4) and tri-iodothyronine (T3) attributed growth promoting effect of L-tryptophan. Dietary supplementation L-TRP also significantly increased the lysozyme activity, NBT, albumin and globulin, specifying its immunomodulatory property. Challenge study confirmed that supplementation of L-TRP in diets of *L. gonius* fingerlings positively influence the survival rate by resisting the *Aeromonas hydrophila* infection and resulted better immunity.

Fourth experiment was conducted to elucidate the possible role of dietary L-tryptophan on stress mitigation of *L. gonius* fingerlings exposed to multiple stressors. Fishes were fed with iso-nitrogenous purified diets containing graded levels of L-TRP (0, 0.7, 1.4 and 2.1%) to mitigate effect of multiple stressors (33^0C x pH 5.5 or 33^0C x pH 9.0). Dietary supplementation of 0.7% L-TRP significantly reduced the stress incurred due to combined effect of temperature and pH by reducing the cortisol levels, blood glucose and oxidative enzymes and increasing the lysozyme activity, NBT, total serum protein, albumin and globulin. However, stress level reduced to the extent comparable with the control group (No stress) with 1.4% L-TRP supplementation in diet and improved further with 2.1% L-TRP indicating its stress mitigating property. Dietary supplementation of L-TRP enhanced the weight gain %, SGR and PER, suggesting improvement of growth indices through mitigating stress. Challenge study conducted under this experiment also confirmed that supplementation of L-TRP in diets of *L. gonius* fingerlings positively influence the survival rate by resisting the *A. hydrophila* infection and resulted better immunity. The results obtained in this experiment indicate that dietary L-TRP mitigates the effect multiple stressors (combined effect of temperature and pH) and enhances the growth of *L. gonius* fingerlings.

Following conclusions were made from the present study:

- ✓ The physiological activities of *L. gonius* fingerlings were normal at 27 and 30 ^{0}C. Higher temperatures cause stress, which ultimately affect the growth and survival of the animal. Hence, rearing the species within the range of 27 to 30^{0}C will be an ideal condition for normal physiological activity, better growth and survival.
- ✓ Acclimation temperature significantly influences the thermal tolerance limit and oxygen consumption rates of the *L. gonius* fingerlings ($p<0.05$).
- ✓ Temperature and/or pH stress had a significant adverse effect ($p<0.05$) on fingerlings. However, rate of changes depends on the extent of exposure.
- ✓ The stress incurred on chronic exposure to multiple stressors was more severe than the single stressor. However, combined effect of temperature and acidic pH stress was higher than the alkaline counterpart. Among individual stressor, effect of acidic pH stress appeared to be more severe than the temperature or alkaline pH stress.
- ✓ Dietary supplementations of L-TRP positively influence the survival rate of *L. gonius* fingerlings by resisting the *Aeromonas hydrophilla* infection and resulted better immunity.
- ✓ Dietary supplementation of L-TRP at a minimum level of 1.4% is useful to mitigate combined effect of multiple stressors (temperature and pH).

This basic information will be useful for researchers to understand the interactions of multiple stressors in today's climate change scenario, which can subsequently help to develop stress mitigation strategies. This will be a reference baseline data on stress mitigation of *L. gonius* and other tropical species having similar physiological background. However, further intensive study is required on molecular physiology for better understanding of the interactions of multiple stressors and also for stress mitigation. The findings of the present study will help to establish *L. gonius* as an alternative candidate species for diversification in aquaculture.

References

7. REFERENCES

Abidi, S. F. and Khan, M. A., 2010. Dietary tryptophan requirement of fingerling rohu, *Labeo rohita* (Hamilton), based on growth and body composition. *Journal of the World aquaculture Society*, 41 (5): 700-709.

Acharya, S., Dutta, T. and Das, M. K., 2005. Physiological and ultrastructural changes in *Labeo rohita* (Hamilton-Buchanan) fingerlings exposed to sub-lethal acidic and alkaline pH for long duration. *Asian Fisheries Science*, 18: 295-305.

Adams, O., Besken, K., Oberdorfer, C., Mackenzie, C. R., Takikawa, O. and Daubener, W., 2004. Role of indoleamine, 2-3 dioxygenase in alpha/beta and gamma interferone mediated anti-viral effects against herps simplex virus infections. *Journal of Virology*, 78: 2632-2636.

Ahmed, I. and Khan, M. A., 2005. Dietary tryptophan requirement of fingerling Indian major carp, *Cirhinus mrigala* (Hamilton). *Aquaculture Research*, 36(5): 687-695.

Akhtar, M. S., Pal, A. K., Sahu, N. P., Alexander, C., Gupta, S. K., Choudhary, A. K., Jha, A. K., and Rajan, M. G., 2010. Stress mitigating and immunomodulatory effect of dietary pyridoxine in *Labeo rohita* (Hamilton) fingerlings. *Aquaculture Research*, 41: 991-1002.

Akhtar, M. S., Pal, A. K., Sahu, N. P., Ciji, A. and Gupta, S. K., 2012a. Effects of dietary pyridoxine on growth and biochemical responses of *Labeo rohita* fingerlings exposed to endosulfan. *Pesticide Biochemistry and Physiology*, 103: 23-30.

Akhtar, M. S., Pal, A. K., Sahu, N. P., Ciji, A. and Kumar, N., 2011. Effects of dietary pyridoxine on haemato-immunological responses of *Labeo rohita* fingerlings reared at higher water temperature. *Journal of Animal Physiology and Animal Nutrition*, DOI: 10.1111/j.1439-0396.2011.01181.x.

Akhtar, M. S., Pal, A. K., Sahu, N. P., Ciji, A. and Meena, D. K., 2012b. Effects of dietary pyridoxine on growth and physiological responses of *Labeo rohita* fingerlings reared at higher water temperature. *Israeli Journal of Aquaculture-Bamidgeh*, 64: 1-6.

Akhtar, M. S., Pal, A. K., Sahu, N. P., Ciji, A., Gupta, S. K., Dasgupta, S. 2014. Serum electrolytes, osmolality and selected enzyme activities of *Labeo rohita* juveniles exposed to temperature and salinity stress: effect of dietary L-tryptophan. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*, 4: 1-8.

Akhtar, M. S., Pal, A. K., Sahu, N. P., Ciji, A., Meena, D.K. and Das, P., 2013. Physiological responses of dietary tryptophan fed *Labeo rohita* to temperature and salinity stress. *Journal of Animal Physiology and Animal Nutrition*, DOI: 10.1111/jpn.12017.

Alazemi, B. M., Lewis, J. W. and Andrews, E. B., 1996. Gill damage in the freshwater fish *Gnthonemus ptersii* (family: Mormyridae) exposed to selected pollutants: an ultrastructure study. *Environ. Technol*., 17: 225-238.

Aldegunde, M., Garcia, J., Soengas, J. L. and Rozas, G., 1998. Uptake of tryptophan into brain of rainbow trout. *J. Exp. Zool*., 282: 285-289.

Aldegunde, M., Soengas, J. L. and Rozas, G., 2000. Acute effects of L-tryptophan on tryptophan hydroxylation rate in brain regions (hypothalamus and medulla) of rainbow trout (*Oncorhynchus mykiss). J. Exp. Zool.,* 286: 131-135.

AOAC, 1995. Official Methods of Analysis of AOAC International, Vol. 1, 16th edn. (ed. Cunniff, P. A.). AOAC International, Arlington, USA.

APHA, 1998. Standard methods for the examination of water and wastewater, 20th edn. (eds. Clesceri, L. S., Greenberg, A. E. and Eaton, A. D.). American Public Health Association, American Water Works Association, Water Environment Federation, Washington DC.

Arjona, F. J., Vargas-Chacoff, L., Marticn del Rico, M. P., Flik, G., Mancera, J. M. and Klaren, P. H. M., 2008. The involvement of thyroid hormones and cortisol in the osmotic acclimation of *Solea senegalensis*. *General Comparative Endocrinology*, 155: 796-803.

Aston-jones, G., Akoka, H., Charlety, P. and Chouvet, G., 1991. Serotonin selectively attenuates glutamate evoked activation of noradrenergic locus coerulus neurons. *J. Neurosci.,* 11: 760-769.

Badaway, A. A. B. and Evans, M., 1975. The regulation of rat liver tryptophan pyrrolase by its cofactor haemexperiments with haematin and 5-aminolaevulinate and comparison with the substrate and hormonal mechanisms. *Biochemical Journal,* 150: 511-520.

Balm, P. H. M., Pepels, P., Helfrich, S., Hovens, M. L. M. and Wendelaar Bonga, S. E., 1994. Adrenocorticotropic hormone in relation to interrenal function during stress in tilapia (*Oreochromis mossambicus*). *Gen. Comp. Endocrinol*., 96:347-360.

Banerjea, S. M., 1967.Water quality and soil condition of fish ponds in some states of India in relation to fish production. *Indian J. Fish*., 14 (1&2): 115-144.

Barton, B. A. and Iwama, G. K., 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response in juvenile Chinook salmon (*Oncorhynchus tshawytscha*). *Aquaculture*, 62: 299-310.

Barton, B. A. and Peter, R. E., 1982. Plasma cortisol stress response in fingerling rainbow trout, *Salmo gairdneri*, Richardson, to various transport conditions anaesthesia and cold shock. *J. Fish. Biol.*, 20: 39-51.

Barton, B. A., 1997. Stress in finfish: Past, present and future-a historical perspective. *In*: Iwama, G. K., Pickering, A. D., Sumpter, J.P. and Schreck, C.B. (eds.), Fish stress and health in aquaculture. Soc. Exp. Biol. Sem. Ser. 62, Cambridge Univ. Press, Cambridge. U.K. pp. 1-33.

Barton, B. A., 2000. Stress. *In*: Robert Stickney (Ed.), Encyclopaedia of Aquaculture. John Wiley and sons, Inc. pp. 892-898.

Barton, B. A., 2002. Stress in fishes: a diversity of responses with particular reference to changes in circulation corticosteroids. *Integ. Comp. Biol.* 42, 517-525.

Barton, B. A., Schreck, C. B. and Fowler, L. G., 1988. Fasting and diet content affect stress- induced changes in plasma glucose and cortisol in juvenile chinook salmon. *The Prog. Fish-Cult.*, 50: 16-22.

Beitinger, T. L. and Bennetta, W. A., 2000. Quantification of the role of acclimation temperature in temperature tolerance of fishes. *Environmental Biology of Fishes*, 58: 277-288.

Belinski, E., 1974. Biochemical aspects of fish swimming. *In*: Malins, B. C. and Sargent, J. R. (eds.), Biochemical Perspectives in Marine Biology. Academic press. pp. 239 - 288.

Bender, D. A., 1978. Amino Acid Metabolism. Chichester: John Wiley & Sons. p. 221.

Bhatnagar, C., Bhatnagar, M. and Regar, B., 2007. Fluoride-induced histopathological changes in gill, kidney and intestine of freshwater teleost, *Labeo rohita*. *Res. Rep. Fluoride*, 40: 55-61.

Blaxhall, P. C. and Daisley, K. W., 1973. Routine haematological methods for use with fish blood. *J. Fish Bid.,* 5: 771-781.

Blier, P. U., Pelletier, D. and Dutil, J. D., 1997. Does aerobic capacity set a limit on fish growth rate? *Reviews in Fisheries Science*, 5: 323-340.

Boadle- Biber, M. C., 1993. Regulation of serotonin synthesis. *Prog. Biophys. Mol. Biol.*, 60: 1-15.

Boyd, C. E. and Tucker, C. S., 1998. Pond aquaculture water quality management. Kluwer Academic Publisher, Boston, pp. 87-152.

Boyd, C. E., 1982. Water quality management for pond fish culture. Elsevier science, UK, pp. 6-50.

Brett, J. R., 1971. Energetic responses of salmon to temperature. A study of thermal relations in the physiology and freshwater ecology of sockeye salmon (*Oncorhynchus nerka*). *Am. Zool.*, 11: 99-113.

Brett, J. R., 1979. Environmental factors and growth. *In*: Hoar, W.S., Randall, D. J. and Brett, J.R. (Eds.), Fish Physiology, vol. VIII. Academic Press, London, pp. 599-675.

Brown, J. N. and Dodgen, C. L., 1968. Fish liver tryptophan pyrrolase: the apparent absence of both hormonal regulation and substrate induction. *Biochemical Biophysica. Acta*., 165: 463-469.

Brown, R. M. S., 1997. US assessment of the Georges Bank haddock stock, The 24th Stock Assessment Workshop/SARC Working Paper Number C1. NEFSC, Woods Hole, MA, pp. 69-70.

Brown, S. B., Eales, J. G., Evans, R. E. and Hara, T. J., 1984. Interrenal thyroid and carbohydrate responses of rainbow trout (*Salmo gairdneri*) to environmental acidification. *Can. J. Fish. Aquat. Sci.,* 41: 36-45.

Buchanan, J., Sarac, H. Z., Poppy, D. and Cowan, R. T., 1997: Effects of enzyme addition to canola meal in prawn diets. *Aquaculture*, 151: 29-35.

Bulow, F. J., 1970. RNA-DNA ratios as indicators of recent growth rates of a fish. *Journal of the Fisheries Research Board of Canada*, 27: 2343-2349.

Bulow, F. J., Zeman, M. E., Winningham, J. R. and Hudson, W. F., 1981. Seasonal variations in RNA-DNA ratios and in indicators of feeding, reproduction, energy storage, and condition in a population of blue gill, *Lepomis macrochirus*, Rafinesque. *J. Fish Biol.*, 18: 237-244.

Burel, C., Ruyet, P. L., Gaumet, F., Roux, A. L., Severe, A. and Boeuf, G., 1996. Effects of temperature on growth and metabolism in juvenile turbot. *J. Fish Biol.*, 49, 678-692.

Camargo, M. M. and Martinez, C. B., 2007. Histopathology of gills, kidney and liver of a Neotropical fish caged in an urban stream. *Neotrop. Ichthyol.*, 5: 327-336.

Cech, J. J., 1990. Respirometry. *In*: Schreck, C.B. and Moyle, P.B. (eds.), Methods for Fish Biology. American Fisheries Society, Bethesda, MD, pp. 335-356.

Cengiz, E. and Unlu, E., 2006. Sublethal effects of commercial deltamethrin on the structure of the gill, liver and gut tissues of mosquitofish, *Gambusia affinis*: A microscopic study. *Environ. Toxicol. Pharmacol.*, 21: 246-253.

Chakraborty, R. D., Sen, P. R., Rao, N. G. S. and Ghosh, S. R., 1976. Intensive culture of Indian major carps. *In*: Pillay, T. V. R. and Dill, Wm. A. (eds.), Advances in Aquaculture. FAO Fishing News Books Ltd.

Chaouloff, F., 1993. Physio-pharmacological interactions between stress hormones and central serotonergic systems. *Brain Res. Rev.*, 18: 1-32.

Chatterjee, N., Pal, A. K., Das,T., Manush, S.M., Sarma, K., Venkateshwarlu, G. and Mukherjee, S. C., 2006. Secondary stress response in Indian major carps *Labeo rohita* (Ham), *Catla catla* (Ham) and *Cirrhinus mrigala* (Ham) fry to increasing packing densities. *Aqua. Research.,* 37: 472-476.

Chatterjee, N., Pal, A. K., Manush, S. M., Das, T. and Mukherjee, S. C., 2004. Thermal tolerance and oxygen consumption of *Labeo rohita* and *Cyprinus carpio* early fingerlings acclimated to three different temperatures. *J. Therm. Biol.*, 29, 265-270.

Chattterji, A., 1992. Age and growth of *Labeo gonius* (Cyprinidae) in Kali river, Uttar Pradesh, India. *Asian Fisheries Science*, 5: 257-260.

Chauloff, F., 1993. Physiopharmacological interactions between stress hormones and central serotonergic systems. *Brain Res. Rev.*, 18: 1-32.

Chen, S. M. and Chen, J. C., 2003. Effects of pH on survival, growth, moulting and feeding of giant freshwater prawn, *Macrobrachium rosenbergii*. *Aquaculture*, 218: 613-623.

Cherry, I. S. and Crandell, L. A. Jr., 1932. The specificity of pancreatic lipase: its appearance in blood after pancreatic injury. *Am. J. Physiol.*, 100: 266-273.

Chew, B. P., 1995. Antioxidant vitamins affect food animal immunity and health. *Journal of Nutrition*, 125: 1804S-1808S.

Chien, L. T., Hwang, D. F. and Jeng, S. S., 1999. Effect of thermal stress on dietary requirement of vitamin C in Thornfish, *Therapon jarbua*. *Fisheries Science*, 65: 731-735.

Cho, C. Y., 1987. La energia en la nutricion de los peces. De la Monteros ESpinosa nad Lanarta U. (ed.), Nutricion en Acuicultura, CAICYT, Madrid, Spain: 197-244.

Chung, M. K., Choi, J. H. Chung, Y. K. and Chee, K. M., 2005. Effects of dietary vitamins C and E on eggshell quality of broiler breeder hens exposed to heat stress. *Asian-Aust. J. Anim. Sci.*, 18 (4): 545-551.

Ciji, A., 2011. Physio-metabolic responses of *Labeo rohita* to nitrite stress and its possible mitigation through dietary vitamin E and tryptophan. Ph.D.Thesis, Central Institute of Fisheries Education, Mumbai. p. 166.

Ciji, A., Sahu, N. P., Pal, A. K. and Akhtar, M. S., 2011. Haemato-immunological and stress responses of *Labeo rohita* (Hamilton) fingerlings: effect of rearing temperature and dietary gelatinized carbohydrate. *Journal of Animal physiology and Animal Nutrition*, 95: 653-663.

Ciji, A., Sahu, N. P., Pal, A. K. and Akhtar, M. S., 2013a. Dietary L-tryptophan modulates growth and immuno-metabolic status of *Labeo rohita* juveniles exposed to nitrite. *Aquaculture Research*, DOI: 10.1111/are.12355.

Ciji, A., Sahu, N. P., Pal, A. K., Akhtar, M. S. and Meena, D. K., 2013b. Haemato-immunological responses of nitrite exposed *Labeo rohita* fed diets supplemented with vitamin E and L-tryptophan, challenged by *Aeromonas hydrophila*. *The Israeli Journal of Aquaculture - Bamidgeh*, IJA_64.2013.889.

Ciji, A., Sahu, N. P., Pal, A. K., Akhtar, M. S., 2013c. Nitrite-induced alterations in sex steroids and thyroid hormones of *Labeo rohita* juveniles: effects of dietary vitamin E and L-tryptophan. *Fish Physiol. Biochem*., 39:1297-1307.

Ciji, A., Sahu, N. P., Pal, A. K., Dasgupta, S., Akthar, M. S., 2012. Alterations in serum electrolytes, antioxidative enzymes and haematological parameters of *Labeo rohita* on short-term exposure to sub-lethal dose of nitrite. *Fish Physiol. Biochem*., 38:1355-1365.

Coloso, R. M., Tiro, L. B. and Benitez, L. V., 1992. Requirement for tryptophan by milkfish (*Chanos chanos* Forsskal) juveniles. *Fish Physiol. Biochem*., 10 (1): 35-41.

Costas, B., Aragao, C., Ruiz-Jarabo, I., Vargas-Chacoff, L., Arjona, F. J., Mancera, J. M., Dinis, M. T. and Conceicao, L. E. C., 2012. Different environmental temperatures affect amino acid metabolism in the eurytherm teleost Senegalese sole (*Solea senegalensis* Kaup, 1858) as indicated by changes in plasma metabolites. *Amino Acids*, 43:327-335.

Cox, D. K.,1974. Effects of three heating rates on the critical thermal maximum of bluegill. *In*: Gibbons, J. W. and Sharitz, R. R. (Eds.), Thermal Ecology. National Technical Information Service, CONF-730505, Springfield, Virginia, pp. 158-163.

Crain, C. M., Kroeker, K. and Halpern, B. S., 2008. Interactive and cumulative effects of multiple human stressors in marine systems. *Ecological Letters*, 11 (12): 1304-1315.

Cruz de Menezes, G., Tavares-Dias, M., Ono, E. A., Alves de Andrade, J. I., Brasil, E. M., Roubach, R., Urbinati, E. C., Marcon, J. L. and Afoonso, E. G., 2006. The influence of dietary vitamin C and E supplementation on the physiological response of pirarucu, *Arapaima gigas* in net culture. *Comparative Biochemistry and Physiology A*, 145: 274-279.

Cui, Y. and Wootton, R. J., 1988. Effects of ration, temperature and body size on the body composition and energy content of the minnow, *Phoxinus phoxinus* (L.). *J. Fish Biol*., 32, 749-764.

Currie, S., Moyes, C. D. and Tufts, B. L., 2000. The effects of heat shock and acclimation temperature on hsp70 and hsp30 mRNA expression in rainbow trout: *in vivo* and *in vitro* comparisons. *J. Fish Biol*., 56: 398-408.

Dabrowski, K., 1990a. Gulonolactone oxidase is missing in teleost fish - a direct spectrophotometric assay. *Biol. Chem. Hoppe-Seyler*, 371: 207-214.

Dabrowski, K., 1990b. Ascorbic acid status in the early life of whitefish (*Coregonus ivaretus*, L). *Aquaculture*, 84: 61-70.

Dalvi, R. S., Pal, A. K., Tiwari, L. R., Das, T. and Baruah, K., 2009. Thermal tolerance and oxygen consumption in *Horabagrus brachysoma* (Gunther) acclimated to different temperatures. *Aquaculture*, 295: 116-119.

Das, D. 2002a. Metabolism of proteins. *In*: Das, D. (ed.), Biochemistry, Academic Publishers. pp. 463-504.

Das, D. 2002c. Metabolism of carbohydrates. *In*: Das, D. (ed.), Biochemistry, Academic Publishers. pp. 419-462.

Das, D., 2002b. Metabolism of lipids. *In*: Das, D. (ed.), Biochemistry, Academic Publishers. pp. 505 - 566.

Das, P. C., Ayyappan, S. and Jena, J. K., 2006. Haematological changes in the three Indian major carps, *Catla catla* (Hamilton), *Labeo rohita* (Hamilton) and *Cirrhinus mrigala* (Hamilton) exposed to acidic and alkaline water pH. *Aquaculture*, 256: 80-87.

Das, P. C., Ayyappan, S., Jena, J. K. and Das, B. K., 2004. Acute toxicity of ammonia and its sub-lethal effects on selected haematological and enzymatic parameters of mrigal, *Cirrhinus mrigala* (Hamilton). *Aquacult. Res.*, 35, 134-143.

Das, P., Gupta, A. and Manna, S. K., 2005. Heat shock protein 70 expression in different tissues of *Cirrhinus mrigala* (Ham.) following heat stress. *Aquaculture Research*, 36: 525-529.

Das, T., Pal A. K., Chakraborty, S. K., Manush, S. M., Dalvi, R. S., Aptes, S. K., Sahu, N. P. and Baruah, K., 2009. Biochemical and stress responses of rohu *Labeo rohita* and *Cirrhinus mrigala* in relation to acclimation temperatures. *Journal of Fish Biology*, 74: 1487-1498.

Das, T., Pal, A. K., Chakraborty, S. K., Manush, S. M., Chatterjee, N. and Apte, S. K., 2006. Metabolic elasticity and induction of heat shock protein (hsp-70) in *Labeo rohita* acclimated to four temperatures. *Asian Australasian Journal of Animal Sciences,* 19(7): 1033-1039.

Das, T., Pal, A. K., Chakraborty, S. K., Manush, S. M., Chatterjee, N. and Mukherjee, S. C., 2004. Thermal tolerance and oxygen consumption of Indian Major Carps acclimated to four temperatures. *J. Therm. Biol.*, 29, 157-163.

Das, T., Pal, A. K., Chakraborty, S. K., Manush, S. M., Sahu, N. P. and Mukherjee, S.C., 2005. Thermal tolerance, growth and oxygen consumption of *Labeo* *rohita* fry (Hamilton, 1822) acclimated to four temperatures. *J. Therm. Biol*., 30: 378-383.

Das, T., Sahu, N. P., Chakraborty, S. K., Chatterjee, N., Mohammed, M. S., Dalvi, R. S., Baruah, K. and Pal, A. K., 2014. Ultrastructural alterations in the gill of *Labeo rohita* fingerlings exposed to thermal extremes. *Anatomia, Histologia, and Embryologia*, 43 (1): 75-80.

Dash, G., Yonzone, P., Chanda, M. and Paul, M., 2011. Histopathological changes in *Labeo rohita* (Hamilton) fingerlings to various acclimation temperatures. Chron*. Young Sci*., 2 (1): 29-36.

Davis, K. B., 2004. Temperature affects physiological stress responses to acute confinement in sunshine bass (*Morone chrysops* × *Morone saxatilis*). *Comp. Biochem. Physiol*. A, 139: 433-440.

Day, F., 1878. The fishes of India; being a natural history of the fishes known to inhabit the seas and fresh waters of India, Burma and Ceylon. Vols. I & II. Reprinted by Today and Tomorrow Book Agency, New Delhi, India.

De, B. C., Meena, D.K., Behera, B. K., Das, P., Mohapatra, D. P. K. and Sharma, A. P., 2013. Probiotics in fish and shellfish culture: immunomodulatory and ecophysiological responses. *Fish Physiol. Biochem*., DOI 10.1007/s10695-013-9897-0.

De Moss, R. D., 1955. Glucose-6-phosphate and 6-phosphogluconic dehydrogenase from *Leuconostoc mesenteroides*. *In*: Colowick, S. P. and Kalpan, N. O. (eds.), Methods in Enzymology, Vol. I, Academic Press Inc., New York, pp. 328-332.

De Silva, S. S. and Anderson, T. A., 1995. Metabolism. *In*: Fish Nutrition and Aquaculture. Chapman and Hall, 2-6 Boundary Row, London SE1 8HN. pp. 41-87.

Deakin, J. W. F. and Graeff, F. G., 1991. 5-HT mechanism of defense, *J. Psychopharmacolol*., 5: 305-315.

Deakin, J. W. F., 1991. Depression and 5-HT. *Int. Clin. Psychopharmacol*., 3: 23-28.

Deane, E. E., Li, J. and Woo, N. Y. S., 2001. Hormonal status and phagocytic activity in sea bream infected with vibriosis. *Comp. Biochem. Physiol. B*, 129: 687-693.

Debnath, D., Pal, A. K., Sahu, N. P., Baruah, K., Yengkokpam, S., Das, T. and Manush, S., 2006. Thermal tolerance and metabolic activity of yellowtail catfish *Pangasius pangasius* (Hamilton) advanced fingerlings with emphasis on their culture potential. *Aquaculture*, 258: 606-610.

Demeal, N. A., 1978. Some characteristics of carbohydrate metabolism in fish. *Oceanis. DOC. Oceanogr.*, 4: 35-365.

Deshpande, U. R., Joseph, L. J., Patwardhan, U. N. and Samuel, A. M., 2002. Effects of antioxidants (Vitamin C, E and turmeric extract) on methimazole induced hypothyroidism in rats. *Indian J. Exp. Biol.*, 40(6):735-739.

Dietz, T. J. and Somero, G. N., 1992. The threshold induction temperature of the 90-kDa heat shock protein is subject to acclimation in eurythermal goby fishes (genus *Gillichthys*). *Proceedings of the National Academy of Sciences, USA*, 89:3389-3393.

Dinan, T. G., 1996. Serotonin and the regulation of hypothalamic-pitutary-adrenal axis function. *Life Sci.*, *58*: 1683-1694.

Dockray, J. J., Morgan, I. J., Reid, S. D. and Wood, C. M., 1998. Responses of juvenile rainbow trout, under food limitation, to chronic low pH and elevated summer temperatures, alone and in combination. *J. Fish Biol.*, 52: 62-82.

Dominguez, M., Takemura, A. and Tsuchiya, M., 2005: Effects of changes in environmental factors on the non-specific immune response of Nile tilapia, *Oreochromis niloticus* L. *Aquaculture Research*, 36: 391-397.

Donaldson, E. M., 1981. The pituitary-interrenal axis as an indicator of stress in fish. *In*: Pickering, A. D. (ed.), Stress and Fish. Academic Press, London, pp.11-47.

Doumas, B. T., Watson, W. and Biggs, H. G., 1971. Albumin standards and measurement of serum albumin with bromocresol green. *Clin. Chim. Acta.*, 31:87-96.

Drapeau, G., 1974. Protease from *Staphylococcus aureus*. *In*: Methods in Enzymology (ed. Lorand B.L.) Academic Press, New York, USA, pp. 469.

Egginnton, S. and Sidell, B. D., 1989. Thermal acclimation induces changes in subcellular structure of fish skeletal muscle. *Amer. J. Physiol.*, 256: 1-9.

EIFAC, 1973. Water quality criteria for European fresh water fish. Report on ammonia and inland fisheries. *Water Res.*, 7: 1011-1122.

Elliott, J. M., 1981. Some aspects of thermal stress freshwater teleosts. *In*: Pickering, A.D. (ed.), Stress and Fish. Academic press, London, pp. 209-245.

Ellis, A. E., 1990. Immunity to bacteria in fish. *Fish Shellfish Immunol.*, 9: 291-308.

Emadi, M., Jahanshri, F., Kaveh, K., Hair-Bejo, M., Ideris, A. and Alimon, R., 2010. Tryptophan stimulates immune responses in broiler chickens challenged with infectious bursal disease vaccine. *Journal of Animal and Veterinary Advances*, 9(3): 610-616.

Esteban S., Nicolaus, C., Garmundi A. and Rial, R. E., 2004. Effect of orally administration tryptophan on serotonin, melatonin and the innate immune response in the rat. *Molecular and Cellular Biochemistry*, 267: 39-46.

Fatahi, S. and Hoseini, S. M., 2013. Effect of dietary tryptophan and betaine on tolerance of Caspian roach (*Rutilus rutilus* caspicus) to copper toxicity. *International Journal of Aquatic Biology*, (1(2): 76-81.

Feigelson, P. and Greengard, O., 1962. Immunochemical evidence for increased titers of liver tryptophan pyrrolase during substrate and hormonal enzyme induction. *Journal of Biological Chemistry,* 237: 3714-3717.

Fernandes, M. N. and Mazon, A. F., 2003. Environmental pollution and fish gill morphology. *In*: Val, A.L. and B.G. Kapoor (eds.). Fish adaptation. Enfield, Science Publishers, pp. 203-231.

Fernstrom, J. D. and Wurtman, R. J., 1972. Brain serotonin content: regulation by plasma neutral amino acids. *Science*, 178: 414-416.

Fernstrom, J. D., 1983. Role of precursor availability in control of monoamine biosynthesis in brain. *Physiol. Rev.*, 60: 484-546.

Fiske, C. H. and Subbarow, Y., 1925. The colorimetric determination of phosphorus. *J. Bio. Chem.*, 66: 375-400.

Floc'h, N. N., Otten, W. F. and Merlot, E., 2010. Tryptophan metabolism, from nutrition to potential therapeutic applications. *Amino Acids*, DOI 10.1007/s00726-010-0752-7.

Foda, A., 1973. Changes in hematocrit and haemoglobin in Atlantic salmon (*Salmo salar*) as a result of furunculosis disease. *Journal of Fisheries Research Board of Canada*, 30(3): 467-468.

Forsyth, R. B., Candido, E. P. M., Babich, S. L. and Iwama, G. K., 1997. Stress protein expression in coho salmon with bacterial kidney disease. *J. Aquat. Anim. Health,* 9:18-25.

Fraser, E. J., Bosma, P. T., Trudeau, V. L. and Docherty, K., 2002.The effect of the water temperature on the GABAergic and reproductive systems in female and male gold fish (*Carassius auratus*). *General and Comparative Endocrinology*, 125:163-175.

Freeland, R. A. and Harper, A. L., 1959. The study of metabolic pathway by means of adaptation. *J. Biol. Chem.*, 234: 1350-1354.

Fritsche, R., Reid, S. G., Thomas, S. and Perry, S. F., 1993. Serotonin mediated release of catecholamines in the rainbow trout, *Oncorhynchus mykiss.* J. *Exp. Biol.*, 178: 191-204.

Fry, F. E. J., 1971. The effect of environment factors on the physiology of fish. *In*: Hoar, W. S. and Randall, D. J. (eds.), Fish physiology: environment relation and behaviour. Academic press, New York, pp. 1-98.

Fukuda, M., Sako, H., Shigeta, T. and Shibata, R., 2001. Relationship between growth and biochemical indices in laboratory reared juvenile Japanese flounder (*Paralichthys olivaceus*) and its application to wild fish. *Marine Biology*, 138: 47-20.55.

Fuller, R. W. and Roush, B. W., 1973. Binding of tryptophan to plasma proteins in several species. *Comparative Biochemistry and Physiology-Part B: Biochemistry and Molecular Biology*, 46 (2): 273-276.

Garen, A. and Levinthal, C. A., 1960. Fine-structure genetic and chemical study of the enzyme Alkaline phosphatase of *E. coli.* I. Purification and characterization of Alkaline phosphatase. *Biochem. Biophys. Acta*., 38: 470.

Gernhofer, M., Pawet, M., Schramm, M., Muller, E. and Triebskorn, R., 2001. Ultrastructural biomarkers as tools to characterize the health status of fish in contaminated streams. *J. Aquat. Ecosys*., 8: 241-260.

Gonzales, R. J. and McDonald, D. G., 1992. The relationship between oxygen consuption and ion loss in a freshwater fish. *J. Exp. Biol.,* 163: 317-332.

Gorg, S. K., 2007. Effect of oral administration of L. thyroxine (T4) on growth performance, digestibility, and nutrient retention in *Channa punctatus* (Bloch) and *Heteropneustes fossilis* (Bloch). *Fish Physiology and Biochemistry*, 53(4): 347-358.

Goss, G. G., Perry, S. F., Wood, C. M. and Laurent, P., 1992. Mechanisms of ion and acid-base regulation at the gills of freshwater fish. *J. Exp. Zool*. 263: 143-159.

Grigo, F., 1975. How much is carp (*Cyprinus carpio*) stressed by temperature? Blood composition, with a special look at the serum sletrolytes. *Zool. Anz., Fena.,* 8: 215-330.

Habig, W. H., Pabst, M. J. and Jakoby, W. B., 1974. Glutathione S-transferases: The first enzymatic step in mercapturic acid formation. *J. Biol. Chem*., 249: 7130-7139.

Haines, T. A., 1973. An evaluation of RNA-DNA ratio as a measure of long term growth in fish populations. *Journal of the Fisheries Research Board of Canada*, 30: 195-199.

Halver, J. E., 1957. Nutrition of salmonid fishes III water soluble vitamin requirement Chinook salmon. *J. Nutr*., 62: 225-243.

Halver, J. E., 1965. Tryptophan requirement in chinook, sockeye and silver salmon. *Fedderation Proceedings*, 24, p.229 (abstract).

Hanna, M., Shaheed, I. and Elias, N., 2005. A contribution on chromium and lead toxicity in cultured *Oreochromis niloticus*. *Egypt. J. Aquat. Biol. Fish*., 9: 177-209.

Hardewig, I., van Dijk P. L. M., Leary, S. C. and Moyes, C. D., 2000. Temporal changes in enzyme activity and mRNA levels during thermal challenge in white sucker. *Journal of Fish Biology*, 56:196-207.

Hardie, I. J., Fletcher, T. C. and Secombes, C. J., 1991. The effect of dietary vitamin C on the immune response of Atlantic salmon (*Salmo salar* L.). *Aquaculture*, 95: 201-214.

Hartl, F. U., 1996. Molecular chaperones in protein folding. *Nature*, 381: 571–580.

Hassid, W. J. and Abraham, S., 1957. Chemical procedures for analysis of polysaccharides. *In*: Calowick, S. P. and Kaplan, N. O. (eds.), Methods in Enzymology, Vol. 3, New York, NY: Academic Press Inc. pp. 35-36.

Hatayama, T. and Hayakawa, M., 1999. Differential temperature dependency of chemical stressors in HSF1-mediated stress response in mammalian cells. *Biochemical and Biophysical Research Communications*, 265:763-769.

Hazel, J. R. and Prosser, C. L., 1974. Molecular mechanisms of temperature compensation in poikilotherms. *Physiol. Rev*., 54: 620-677.

Heath, A. C., 1995. Water pollution and fish physiology. 2nd edn. Lewis Publishers, Boca Raton. pp. 125-140.

Hecht, T. and Pinienaar, A. G., 1993. A review of cannibalism and its implications in fish larviculture. *J. World Aquac. Soc*., 24: 246-261.

Henderson, I. W. and Garland, H. O., 1980. The interrenal gland of pisces part II physiology. *In*: Jones, C. and Handerson I. W. (eds.), General cooperative and clinical endocrinology of the adrenal cortex, Vol. 3, Academic press, London. pp. 473-523.

Hendrick, J. P. and Hartl, F. U., 1993. Molecular chaperone functions of heat shock proteins. *Annual Reviews in Biochemistry*, 62: 349-384.

Henkart, P. A., 1996. Ice family proteases: mediators of all apoptotic cell death. *Immunity*, 4: 195-201.

Hering, D., Carvalho, L., Argillier, C., Beklioglu, M., Borja, A., Cardoso, C. A., Duel, H., Ferreira, T., Globevniki, L., Hanganu, J., Hellsten, S., Jeppesen, E., Kodeš, V., Solheim, A. L., Nõges, T., Ormerod, S., Panagopoulos, Y., Schmut, S., Venoh, M. and Birk, S., 2014. Managing aquatic ecosystems and water resources under multiple stress - an introduction to the MARS project. *Science of the Total Environment,* DOI: 10.1016/j.scitotenv. 2014.06.106.

Hestrin, 1949 (modified by Augustinsson, 1957). The reaction of acetyl choline esters and other carboxylic acid derivatives with hydroxylamine and its analytical application. *J. Biol. Chem.*, 180: 249-261.

Hilton, D. E., 1998. Multiple stressors in the Sacramento river watershed. *EXS*, 86: 303-17.

Hochachka, P. W. and Somero, G. N., 1971. Biochemical adaptation to the environment. *In*: Hoar, W.S., Randall, D.J. (eds.), Fish Physiology. Academic Press, New York and London, pp. 100-148.

Hoglund, E., Bakke, M.J., Øverli, Ø., Winberg, S. and Nilsson, G.E., 2005. Suppression of aggressive behaviour in juvenile Atlantic cod (*Gadus morhua*) by L-tryptophan supplementation. *Aquaculture*, 249: 525–531.

Hoglund, E., Balm, P. H. M., and Winberg, S., 2000. Skin darkening, a potential signal in subordinate artic charr *salvenius alpinus*: the regulation role of brain monoamines and pro opiomelanocortin-derived peptides. *J. Exp. Biol.,* 203: 1711-1721.

Hoguland, E., Kolam, N. and Winberg, S., 2001. Stressed-induced effects on brain serotonergic activity, plasma cortisol and aggressive behaviour in Arctic char (*Salvelinus alpinus*) is counteracted by L-dopa. *Physiol. Behav*. 74, 381-389.

Hoseini, S. M. and Hosseini, S. A., 2010. Effect of dietary L-tryptophan on osmotic stress tolerance in common carp, *Cyprinus carpio*, juveniles. *Fish Physiol. Biochem.*, 36: 1061-1067.

Hoseini, S. M., Hoseini, S. A and Soudagar, M., 2012. Dietary tryptophan changes serum stress markers, enzyme activity, and ions concentration of wild common carp *Cyprinus carpio* exposed to ambient copper. *Fish Physiol. Biochem*. DOI 10.1007/s10695-012-9629-x.

Hoseni, S. A. and Hoseini, S. M., 2013. Effect of dietary tryptophan on stress response of wild common carp *Cyprinus carpio* L. *World Journal of Fish and Marine Sciences*, 5 (1): 49-55.

Hseu, J. R., Lu, F. I., Su, H. M., Wang, L. S., Tsai, C. L. and Hwang, P. P., 2003. Effects of exogenous tryptophan on cannibalism, survival and growth in juvenile grouper, *Epinephelus coioides*. *Aquac Eng.*, 218:251-263.

Huet, M., 1975. *In:* Textbook of Fish Culture, Publ. By Fishing News Books Ltd., England, pp. 346.

Huether, G., 1996. The central adaptation syndrome: psychological stress as a trigger for adaptive modifications of brain structure and brain function. *Prog. Neurobiol.,* 48: 568-612.

Hughes, G. M., 1966. Species variation in gas exchange. *Proceeding of Royal Society of Medicine*, 59(6): 494-500.

Hussain, M. G. and Mazid, M. A., 2001. Genetic improvement and conservation of carp species in Bangladesh. Bangladesh Fisheries Research Institute and International Center for Living Aquatic Resources Management, pp.74.

ICLARM, 2001. Genetic improvement of carp species in Asia: Final Report. Asian Development Bank Regional Technical Assistance No. 5711, International Center for Living Aquatic Resources Management, Penang, Malaysia.

Ingram, G. A., 1980. Substances involved in the natural resistance of fish to infection- a review. *Journal of Fish Biology*, 16: 23-60.

IPCC (Intergovernmental Panel on Climate Change), 2012. Managing the risks of extreme events and disaster to advance climate change adaptation. Cambridge University Press, Cambridge, UK.

Ishiibashi, Y., Kato, K., Ikeda, S., Murata, O., Nasu, T. and Kumai, H., 1992. Effect of dietary ascorbic acid on tolerance to intermittent hypoxic stress in Japanese parrot fish. *Nippon Suisan Gakk*, 58: 2147-2152.

Itou, T., Lida, T. and Kawatsu, H., 1997: Kinetics of oxygen metabolism during respiratory burst in Japanese eel neutrophils. *Developmental and Comparative Immunology*, 20: 323-330.

Iwama, G. K,. MCGeer, J. C. and Bernier, N. J., 1992. The effects of stock and rearing density on the stress response in juvenile coho salmon (*Oncorhynchus kisutch*). *ICES Mar. Sci. Symp*., 194: 67-83.

Iwama, G. K., Vijayan, M. M., Forsyth, R. B. and Ackerman, P.A., 1999. Heat shock proteins and physiological stress in fish. *Am. Zool*. 39, 901-909.

Jacobson, I. and Sapulsky, R., 1991. The role of hippocampus in the feedback regulation of the hypothalamic pituitary adrenocortical axis. *Endocrinology Rev*.,12: 118-134.

Janssens, L., Van, K. D. and Stoks, R., 2014. Extreme temperatures in the adult stage shape delayed effects of larval pesticide stress: A comparison between latitudes. *Aquatic Toxicology*, 148: 74-82.

Jena, J. K. and Das, P. C., 2011. Grow-out performance of Kuria labeo, *Labeo gonius* (Hamilton), with major carps in carp polyculture system. *Aquaculture Research*, 42: 1332-1338.

Jena, J. K., Das, P. C., Mitra, G. Patro, B., Mohanta, D. and Mishra, B., 2011. Evaluation of growth performance of *Labeo fimbriatus* (Bloch), *Labeo gonius* (Hamilton) and *Puntius gonionotus* (Bleeker) in polyculture with *Labeo rohita* (Hamilton) during fingerlings rearing at varied densities. *Aquaculture*, 319: 493-496.

Jeney, G., Galeotti, M., Volpattil, D., Jeney, Z and Anderson, D. P., 1997. Prevention of stress in rainbow trout (*Oncorhynchus mykiss*) fed diets containing different doses of glucan. *Aquaculture*, 154:1-15.

Jensen, F. B. and Brahm, J., 1995. Kinetics of chloride transport across fish red blood cell membranes. *J. Exp. Biol.*, 198: 2237-2244.

Jensen, F. B., 1990. Sub-lethal physiological changes in freshwater crayfish, *Astacus astacus*, exposed to nitrite: haemolymph and muscle tissue electrolyte status, and haemolymph acid-base balance and gas transport. *Aquat. Toxicol.*, 18: 51-60.

Jentsch, A., Kreyling, J. and Beierkuhnlein, C., 2007. A new generation of climate change experiments: events, not trends. *Frontiers in Ecology and the Environment*, 5: 365-374.

Jhingran, V. G., 1975. Fish culture in freshwater ponds. *In*: Jhingran, V.G. (ed.), Fish and Fisheries of India, Hindustan Publishing Corp., New Delhi, p.276.

Jobling, M., Johnsen, H. K., Pettersen, G. W. and Henderson, R. J., 1995. Effect of temperature on reproductive development in Arctic charr, *Salvelinus alpinus* (L). *Journal of Thermal Biology*, 20: 157-165.

Johnston, W. L., Atkinson, J. L., Hilton, J. W. and Were, K. E., 1990. Effect of dietary tryptophan on plasma and brain tryptophan, brain serotonin and brain 5-hydroxyindoleacetic acid in rainbow trout. *J. Nutr. Biochem.*, 1: 49-54.

Jones, S. B., Beauvais, S. L., Brewer, S. K. and Little, E. E., 1999. Physiological and behavioural measures of neurotoxicity in rainbow trout. *In*: Kennedy, C. and Mackinlay, D. (eds.), Fish response to toxic environments. p. 2728.

Kalita, D., Dutta, M. and Islam, N. F., 2005. Few plants and animal based folk medicines from Dibrugarh district, Assam. *Indian J. Traditional knowledge*, 4(1):81-85.

Kaoud, H. A. and El-Dahshan, A. R., 2010. Bioaccumulation and histopathological alterations of the heavy metals in *Oreochromis niloticus* fish. *Nature and Science*, 8(4): 148-156.

Karunasagar, I., Ali, A., Otta, S. K. and Karunasagar, I., 1997. Immunization with bacterial antigen: Infection with motile *Aeromonas. In*: Gudding, R., Lillehaug, A. and Midtlyng, J. (eds.), Fish vaccinology. *Dev. Biol. Stand,* 90, 1-7.

Kasim, H. M., 2002. Thermal ecology: a vital prerequisite for aquaculture and related practices. *In*: Venkataramani, B. and Sukumarn, N. (eds.), Thermal Ecology. BRNS, DAE Mumbai Publishers, pp. 222-234.

Kearns, P. K. and Atchison, G. J., 1979. Effects of trace metals on growth of yellow perch (*Perca flavescens*) as measured by RNA-DNA ratios. *Environmental Biology of Fishes*, 4: 383-387.

Kellog, R. L. and Gift, J. J., 1983. Relationships between optimum temperatures for growth and preferred temperatures for the young of four fish species. *Trans. Am. Fish. Soc.*, 112: 424-430.

Khan, M. A. and Jafri, A. K., 1993. Quantitative dietary requirement for some indispensable amino acids in the Indian major carp, *Labeo rohita* (Hamilton) fingerling. *Journal of Aquaculture in the Tropics*, 8:67-80.

Kim Kyu, I. L., Terrence, B. K. and Clyde, H. A., 1987: Effects of dietary tryptophan level on growth, feed/gain, carcass composition and liver glutamate dehydrogenase activity in rainbow trout (*Salmo gairdenerii*). *Comparative Biochemistry and Physiology*, 88B: 737-741.

Kita, J., Tsuchida, S. and Setoguma, T., 1996.Temperature preference and tolerance and oxygen consumption of the marbled rock-fish, *Sebastiscus marmoratus*. *Mar. Biol.*, 125: 467-471.

Klaren, P. H. M., Guzmacn, J. M., Reutelingsperger, S. J., Mancera, J. M. and Flik, G., 2007. Low salinity acclimation and thyroid hormone metabolizing enzymes in gilthead seabream (*Sparus auratus*). *General and Comparative Endocrinology*, 152: 215-222.

Kloppel, T. M. and Post, G., 1975. Histological alteration in tryptophan deficient rainbow trout. *Journal of Nutrition*, 105: 861-866.

Knox, W. E. and Greengard, O., 1965. *In*: Weber, G. (ed.), An introduction to enzyme Physiology, Advan. Enzyme Regul. Pergamon Press, New York, London, 3: 247-248.

Knudsen, P. K. and Jensen, F. B., 1997. Recovery from nitrite induced methaemoglobinaemia and potassium balance disturbances in carp. *Fish Physiol. Biochem.*, 16: 1-10.

Kopp, R., Palı´kova´, M., Navra´til, S., Kubı´cˇek, Z., Zikova´, A., Jan Maresˇ, J., 2010. Modulation of biochemical and haematological indices of silver carp (*Hypophthalmichthys molitrix* Val.) exposed to toxic cyanobacterial water bloom. *Acta. Vet. Brno.*, 79:135-146.

Kumar, D. and Dey, R. K., 1988. Fish diseases in India. *In:* Sinha, V. R. and Shrivastava, H. C. (eds.), Proceedings of the Productivity. Calcutta, India. pp. 315-343.

Kumar, N., Jadhao, S. B., Chandan, N. K., Aklakur, M. and Rana, R. S., 2012. Methyl donors potentiates growth, metabolic status and neurotransmitter enzyme in *Labeo rohita* fingerlings exposed to endosulfan and temperature. *Fish Physiol. Biochem.*, 38 (5): 1343-53.

Kumar, P., 2008. Immuno-physiological responses to thermal stress in *Labeo rohita* and its mitigation. Ph. D. Thesis, CIFE, Mumbai, p. 400.

Kumar, P., Saurabh, S., Pal, A. K. Sahu, N. P. and Arasu, A. R. T., 2014. Stress mitigating and growth enhancing effect of dietary tryptophan in rohu (*Labeo rohita*, Hamilton, 1822) fingerlings. *Fish Physiol. Biochem*., DOI 10.1007/s10695-014-9927-6.

Kumar, R., Mukherjee, S. C., Pani Prasad, K. and Pal, A. K., 2006. Evaluation of *Bacillus subtilis* as a probiotics to Indian major carp, *Labeo rohita* (Ham.). *Aq. Res*., 37: 1215-1221.

Kumar, V., Sahu, N. P., Pal, A. K. and Kumar, S., 2007. Immunomodulation of *Labeo rohita* juveniles due to dietary gelatinized and non-gelatinized starch. *Fish and Shellfish Immunology*, 23: 341-353.

Kumaraguru, A. K. and Beamish, F. W. H.,1986. Effect of permethrin (NRDC-173) on the Bioenergetics of rainbow trout, *Salmo gairdneri*. *Aquatic Toxicology*, 9: 47-58.

Kutty, M. N. and Peer Mohamed, M., 1975. Metabolic adaptations of mullet, *Rhinomugil cersula* (Hamilton) with special reference to energy utilization. *Aquaculture*, 5: 253-270.

Lankford, S. E., Adams, T. E. and Cech Jr. J. J., 2003. Time of day and water temperature modify the physiological stress response in green sturgeon, *Acipenser medirostris*. *Comp. Biochem. Physiol*, *A Mol. Integr. Physiol*. ,135, 291-302.

Laranja Jr, J. L. Q., Quinitio, E. T., Catacutan, M. R. and Coloso, R. M., 2010. Effects of dietary L-tryptophan on the agonistic behavior, growth and survival of juvenile mud crab *Scylla serrata*. *Aquaculture*, 310: 84-90.

Laurent, P. and Perry, S. F., 1991. Environmental effects on fish gill morphology. *Physiological Zoology*, 64(1): 4-25.

Lepage, O., Larson, E. T., Mayer, I. and Winberg, S., 2002. Elevated dietary intake of l-tryptophan counteracts the stress-induced elevation of plasma cortisol in rainbow trout *(Oncorhnchus mykiss)*. *J. Exp. Biol*., 205: 3679-3687.

Lepage, O., Vilchez, I. M., Pottinger, T. G. and Winberg, S., 2003. Time course of the effect of dietary L-tryptophan on plasma cortisol levels in rainbow trout, *Oncorhynchus mykiss*. *J. Exp. Biol*., 206: 3589-3599.

Leslie, I., 1955. The nucleic acid content of tissues and cells. *In*: Chargaff, E. and Davidson, J. N. (eds.), The Nucleic Acids: Chemistry and Biology. Academic Press, New York, pp. 1-50.

Li, C. C. and Chen, J. C., 2008. The immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio alginolyticus* under low and high pH stress. *Fish Shelfish Immunol*. doi:10.1016/j.fsi.2008.01.007.

Li, S. Q., Li, R. F., Xi, S. M., Hu, S. and Jia, Z. Q., 2012. Systematical analysis of impacts of heat stress on the proliferation, apoptosis and metabolism of mouse hepatocyte. *Journal of Physiological Sciences*, 62: 29-43.

Li, Y. and Lovell, R. T., 1985. Elevated levels of dietary ascorbic acid increase the immune responses in channel catfish. *Journal of Nutrition*, 115: 123-131.

Likongwe, J. S., Stecko, T. D., Stauffer, J. R. Jr. and Carline, R. F., 1996. Combined effects of water temperature and salinity on growth and feed utilization of juvenile Nile tilapia *Oreochromis niloticus* Linnaeus. *Aquaculture*, 146: 37-46.

Liposists, Z., Phelix, C. and Paull, W. K., 1978. Synaptic interaction of serotonergic axons and corticotropin releasing factor (CRF) synthesizing neurons in the hypothalamic paraventricular nucleus of the rat. A light and electron microscopic immunocytochemical study, *Histochem.,* 86: 541-549.

Livingstone, D.R., Forlin, L. and George, G.,1997. Molecular biomarkers and toxic consequences of impact by organic pollution in aquatic organism. *In*: Sutcliffe D.W. (ed.), Water quality and stress indicators in marine and freshwater ecosystems. Linking levels of organization (individuals, populations, communities), pp.154-171.

Lowry, O. H., Ronebrough, N. J., Farr, A. L. and Randell, R. J., 1951. Protein measurement with folin phenol reagent. *J. Biol. Chem.,* 193: 265-276.

Maes, M. and Meltzer, H., 1995. The serotonin hypothesis of major depression. *In*: Bloom, F. E. and Kupfer, D. J., (eds.), Psycopharmacology: the fourth generation of progress. Raven press, Newyork. pp. 933-944.

Mandal, S., Mahapatra, B. K., Tripathi, A. K., Verma, M. R., Datta, K. K. and Ngachan, S. V., 2007. Agribusiness opportunities of ornamental fisheries in North-eastern region of India. *Agricultural Economics Research Review*, 20: 471-488.

Manush, S. M., Pal, A. K., Chatterjee, N., Das, T. and Mukherjee, S. C., 2004. Thermal tolerance and oxygen consumption of *Macrobrachium rosenbergii* acclimated to three different temperatures. *J Therm. Biol.*, 29:15-19.

Manush, S. M., Pal, A. K., Das, T., and Mukherjee, S. C., 2005. Dietary high protein and vitamin C mitigate stress due to chelate claw ablation in *Macrobrachium rosenbergii* males. *Comp. Biochem. and Physiology Part A*, 142: 10-18.

Manush, S. M., Pal, A. K., Das, T., Chatterjee, N., Sarma, K. and Mukherjee, S. C., 2007. Ultrastructural alterations in the gills of *Macrobrachium rosenebergii* *a*cclimated to three temperatures. *Asian Journal of Cell Biology*, 2 (1): 1-10.

Markus, R., Pahuysen, G., Tuiten, A. and Koppeschaar, H., 2000. Effects of food on cortisol and mood in vulnerable subjects under controllable and uncontrollable stress. *Physiol. Behav*., 70: 333-342.

Martins, C. I. M., Silva, P. I. M., Costas, B., Larsen, B. K., Santos, G. A., Conceicao, L. E. C., Dias, J., Overli, O., Hoglund, E. and Schrama, J. W., 2013. The effect of tryptophan supplemented diets on brain serotonergic activity and plasma cortisol under undisturbed and stressed conditions in grouped-housed Nile tilapia, *Oreochromis niloticus*. *Aquaculture*, 400 & 401:129-134.

Mary Lini, R., Kurcheti, P.P., Babu, G. and Purushothaman, C. S., 2013. Effect of *Aeromonas hydrophila* infection on caspase-3 expression and activity in rohu, *Labeo rohita*. *J. Aquac. Res. Development*, 4: 200 doi:10.4172/2155-9546.1000200.

Mazeaud, M. M., Mazeaud, F. and Donaldson, E. M., 1977. Primary and secondary effects of stress in fish: some new data with a general review. *Trans. Am. Fish. Soc.*, 106 (3): 201-212.

McDonald, D. G., 1983. The interaction of environmental calcium and low pH on the physiology of the rainbow trout, *Salmo gairdneri*. Branchial and renal net ion and H^+ fluxes. *J. Exp. Biol.,* 152: 123-140.

McDonald, D. G. and Millican, C. L., 1992. Chemical properties of the blood. *In*: Hoar, W. S., Randall, D. J. and Farrel, A. P. (eds.), Fish physiology, Vol 11, Part B. The cardiovascular system. Academic Press, San Diego, pp. 55-133.

McKim, J. M. and Erickson, R. J., 1991. Environmental impacts on the physiological mechanisms controlling xenobiotic transfer across fish gills. *Physiological Zoology*, 64(1): 39-67.

Meena, D. K., Das, P., Kumar, S., Mandal, S. C., Prusty, A.K., Singh, S. K., Akhtar, M. S., Behera, B. K., Kumar, K., Pal, A. K. and Mukherjee, S. C., 2012. Beta-Glucan: an ideal immunostimulant in aquaculture (a review). *Fish Physiology and Biochemistry*. DOI: 10.1007/s10695-012-9710-5

Misra, H. P. and Fridovich, T., 1972.The role of superoxide ion in the autooxidation of epinephrine and a simple assay for super oxide dismutase. *J. Biol. Chem*., 247: 3170-3175.

Misra, S., Sahu, N. P., Pal, A. K., Xavier, B., Kumar, S. and Mukherjee, S.C., 2006. Pre and post challenge immuno-haematological changes in *L. rohita* juveniles fed gelatinized or non-gelatinised carbohydrate. *Fish & shellfish Immunology*, 21: 346-356.

Miwa, S. and Inui, Y., 1985. Effects of l-thyroxine and bovine growth hormone on smoltification of amago salmon (*Oncorhynchus rhoduras*). *Gen. Comp. Endocrinol*., 58: 436-442.

Mock, A. and Peters G., 1990. Lysozyme activity in Rainbow trout, *O. mykiss*. Stress by handling, transport and water pollution. *J. Fish Biol.*, 37: 873-885.

Mohanta, K. N., Subramanian, S., Komarpant, N. and Saurabh, S., 2008. Alternate carp species for diversification in freshwater aquaculture in India. *Aquaculture Asia*, 13 (1): 11-15.

Mohapatra, M., Sahu, N. P. and Chaudhari, A., 2003. Utilization of gelatinized carbohydrate in diets in *Labeo rohita* fry. *Aquacult. Nutr.*, 8: 1-8.

Mohapatra, S., Chakraborty, T., Kumar, V., DeBoeck, G., Mohanta, K. N., 2013. Aquaculture and stress management: a review of probiotic intervention *Journal of Animal Physiology and Animal Nutrition*, 97(3): 405-430.

Mohapatra, S., Chakraborty, T., Prusty, A. K., Prasad, P. K. and Mohanta, K. N., 2014. Beneficial effects of dietary probiotics mixture on haemato-immunology and cell apoptosis of *Labeo rohita* fingerlings reared at higher water temperatures. *PLoS ONE*, 9(6): e100929. doi:10.1371/ journal. pone.0100929.

Moon, T. W. and Foster, G. D., 1995. Tissue carbohydrate metabolism, gluconeogenesis hormonal and environmental influences. *In*: Hochachka, P. W. and Mommsen, T. P. (eds.), Biochemistry and Molecular Biology of Fishes. Vol. 4, Metabolic biochemistry, Elsevier. pp. 33-65.

Morgan, I. J., D'Cruz, L. M., Dockray, J. J., Linton, T. K. and Wood, C. M., 1999.The effect of elevated summer temperature and sublethal pollutants (ammonia, Low pH) on protein turn over in the gill and liver of rainbow trout (*Onchorhynchus mykiss*) on a limited food ration. *Comparative Biochemistry and Physiology* A, 123: 653-654.

Mosser, D. D., Caron, A. W., Bourget, L., Meriin, A. B., Sherman, M. Y., Morimoto, R. I. and Massie, B., 2000. The chaperone function of hsp70 is required for protection against stress-induced apoptosis; *Mol. Cell Biol.*, 20: 7146-7159.

Mukhopadhyay, P. K., 1993. Lipid and fatty acid requirements of carp and catfish. *In*: Tripathi, S. D. (ed.), Summer Institute on Recent Advances in freshwater Aquaculture, Central Institute of Freshwater Aquaculture, Bhubaneswar, May 20-June 8, pp.75-79.

Munilkumar, S. and Nandeesha, M. C., 2007. Aquaculture practices in Northeast India: current status and future directions. *Fish Physiol. Biochem.*, 33: 399-412.

Murray, R. K., Granner, D. K., Mayes, P. A. and Rodwell, V. W., 2000. Glycogen metabolism. *In*: Harper's Biochemistry. 25th edition. pp. 199-207.

Murthy H. S. and Varghese, T. J., 1997. Dietary tryptophan requirement for the growth of rohu, *Labeo rohita*. *Journal of Applied Aquaculture*, 7(2): 71-79.

Murthy, V. K., Reddy, Y. D., Reddy, R. M., Bhaskar, K. M. and Govindappa, S., 1984. Changes in brain acetylenoline esterase activity in behaviour of freshwater fish in acid polluted environment. *Environ. Ecol*., 2(2): 79-82.

Naatjes, M., Htoo, J. K., Walter, K., Tolle, K. H. and Susenbeth, A., 2014. Effect of dietary tryptophan to lysine ratio on growth of young pigs fed wheat-barley or corn based diets. *Livestock Science*, 163: 102-109.

Nakano, T. and Tomlinson, N., 1967. Catecholamine and carbohydrate concentrations in rainbow trout (*Salmo gairdneri*) in relation to physical disturbance. *J. Fish. Res. Bd. Can*., 24: 1701-1715.

Navarre, O. and Halver, J. F., 1989. Disease resistance and humoral antibody production in rainbow trout fed high levels of vitamin C. *Aquaculture*, 79: 207-219.

Ndong, D., Chen, Y. Y., Lin, Y. H., Vaseeharan, B. and Chen, J. C., 2007. The immune response of tilapia, *Oreochromis mossambicus* and its susceptibility to *Streptococcus iniae* under stress in low and high temperatures. *Fish and Shellfish Immunology*, 22: 686-694.

Nelson, J. W. and Somogyi, M., 1975. Determination of glucose. *In*: Oser, B. L. (ed.), Hawk's physiological Chemistry, 14th edn. McGrow Publication, New York, USA, pp. 113.

Nelson, J. W., 1944. A photometric adaptation of the somogyi method of determination of glucose. *Journal of Biological Chemistry*, 153: 375.

Norouzitallab, P., Farhangi, M., Babapour, M., Rahimi, R., Sinha, A. K. and Baruah, K., 2008. Comparing the efficacy of dietary a-tocopherol with that of DL-atocopheryl acetate, both either alone or in combination with ascorbic acid, on growth and stress resistance of angelfish, *Pterophylum scalare*, juveniles. *Aquaculture International*. doi: 10.1007/s10499-008-9192-8.

Nose, T., 1979. Summary report on the requirements of essential amino acids for carp. *In*: Halver, J. E. and Tiews, K. (eds.), Finfish nutrition and fish feed *Technology*, Vol 1. Heenemann, Berlin, pp. 145-156.

Ochoa, S., 1955. Malic dehydrogenase and 'malic' enzyme. *In*: Coloric, S. P. and Kaplan, N.O. (eds.), Methods of enzymology, Vol. I, Academic Press, New York, pp. 735-745.

Orbach, A. and Laurencin, F. B., 1992. Effects of dietary oxidized fish oils and deficiency of anti-oxidants on the immune response of turbot *Scophthalmus maximus*. *Aquaculture*, 107: 221-228.

Ortuno, J., Esteban, M. A. and Meseguer, J., 2002. Lack of effect of combining different stressors on innate immune responses of seabream (*Sparus aurata* L.). *Veterinary Immunology and Immunopathology*, 84: 17-27.

Ortuno, J., Esteban, M. A. and Mesequer, J., 2003. The effect of dietary intake of vitamin C and E on the stress response of gilthead seabream (*Sparus aurata* L.). *Fish and Shellfish Immunol.*, 14(2): 145-156.

Overli, Ø., Harris, C. A. and Winberg, S., 1999. Short-term effects of fights for social dominance and the establishment of dominant-subordinate relationships relationships on brain monoamines and cortisol in rainbow trout. *Brain Behav. Evol.*, 54: 263-275.

Paladino, F. V., Spotila, J. R., Schubauer, J. P. and Kowalski, K. T., 1980. The critical thermal maximum: a technique used to elucidate physiological stress and adaptation in fish. *Rev. Can. Biol.*, 39:115-122.

Pankhurst, N. W. and Van der Kraak, G., 1997. Effects of stress on reproduction and growth of fish. *In*: Iwama, G. K., Pickering, A. D., Sumpter, J. P. and Schreck, C.B. (eds.), Fish Stress and Health in Aquaculture. Society for Experimental Biology, Cambridge, pp. 73-93.

Parent, A., Poitras, D. and Dube, L., 1984. Comparative neuroanatomy of central monoaminergic systems. *In*: Bjorklund, A. and Hokfelt, T. (eds.), Handbook of chemical neuroanatomy. Amsterdam, New York, Oxford: Elsevier, pp. 409-439.

Petr, T., 1999. Coldwater fish and fisheries in Afghanistan. *In*: Petr, T. (ed.), Fish and Fisheries at Higher Altitudes: Asia. FAO Fisheries Technical Paper No. 385. FAO, Rome, Italy, pp. 138-148.

Peuranen, S., Keinanen, M., Tigerstedt, C. and Vuorinen, P. J., 2003. Effects of temperature on the recovery of juvenile grayling (*Thymallus thymallus*) from the exposure to Al +Fe. *Aquatic toxicology*, 65:73-84.

Pickering, A. D., 1981. Introduction: the concept of biological stress. *In*: Pickering, A. D. (Ed.), Stress and fish. Academic press, London, pp.1-9.

Pickering, A. D., 1992. Rainbow trout husbandry: management of the stress response. *Aquaculture*, 100: 125-139.

Pickering, A.D., 1993. Growth and stress in fish production. *Aquaculture,* 111: 51-63.

Pickering, A. D., Pottinger, T. G. and Carragher, J. F., 1989. Differences in the sensitivity of brown trout *Salmo trutta* L. and rainbow trout, *Salmo gairdneri* Richardson to physiological doses of cortisol. *J. Fish Biol.*, 34: 757-768.

Post, R. L. and Sen, A. K., 1967. Colowick, S. P. and Kaplan, N. O. (eds.), Methods in Enzymology. Vol. 10, Academic Press Inc., New York. pp.762.

Pottinger, T. G., 1992. The influence of social interaction on the acclimation of rainbow trout, *Oncorhynchus mykiss* (Walbaum) to chronic stress. *J. Fish. Biol.*, 41: 435-447.

Prabhu, P. A. J., 2012. Nutritional strategies to counter effects of endosulfan and elevated temperature on productive and reproductive physiology in tilapia. Central Institute of Fisheries Education, Mumbai, M.F.Sc Thesis. p. 156.

Rahman, A. K. A., 1989. Freshwater fishes of Bangladesh. The Zoological Society of Bangladesh, Department of Zoology, University of Dhaka, Dhaka, pp.364.

Rahman, M. A., Zaher, M. and Azimuddin, K. M., 2008. Evaluation of growth, survival, and production of an endangered fish, *Labeo gonius* (Hamilton) fingerlings in earthen nursery ponds. *Journal of Applied Aquaculture*, 20 (1): 62-78.

Reddy, P. K. and Leatherland, J. F., 1998. Stress physiology. *In*: Leatherland, J.F. and Woo, P.T.K. (eds.), Fish diseases and disorders, Vol. 2: non-infections disorders. 1st edition, CABI Publishing, New York, pp. 279-301.

Reinhold, J.G., 1953. Manual determination of serum total protein, albumin and globulin fractions by Biuret method. *In*: Reiner, M. (ed.), Standard method of clinical chemistry. New York: Academic Press, p.88.

Renukardhyay, K. M. and Varghese, T. J., 1986. Protein requirement of the carps *Catla catla* and *Labeo rohita* (Ham). *Proceedings of the Indian Academy of Science (Animal Science)*, 95: 103-107.

Reubush, K. J. and Heath, A. G., 1996. Metabolic responses to acute handling by fingerling inland and striped bass. *J. Fish Biol.*, 49: 830-841.

Rick, W. and Stegbauer, H. P., 1974. Amylase measurement of reducing groups. *In*: Bergmeyer, H. V. (ed.), Methods of Enzymatic Analysis, 2nd edition, Vol. 2, Academic Press, New York, pp. 885-889.

Rios, F. S. and Fanta, E., 1998. Morphology of gill rackers and their ecological function in feeding of the Antarctic fish, *Notothenia neglecta* Nybelin. *Antarctic Record* (Nankyoku Shiryo), 42: 131-150.

Roberts, R. J., 1989. Nutritional pathology of teleosts. *In*: Roberts, R. J. (ed.), Fish pathology. London, Bailliere Tindall, pp. 337-362.

Roe, J. H. and Keuther, C. A., 1943. The determinations of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine (DNPH) derivative of dehydroascorbic acid. *Journal of Biological Chemistry*, 147: 399-407.

Rozas, G., Rey, P., Andre, M. D., Revolledo, E., Morfin, R., and Aldegunde, M., 1990. Distribution of 5-hydroxytryptamine and related compounds in various brain regions of rainbow trout (*Oncorhynchus mykiss*). *Fish Physiol. Biochem.*, 8: 501-506.

S‘eve, B., 1999: Physiological roles of tryptophan in pig nutrition. *Advances in Experimental Medical Biology*, 467: 729-741.

Sanders, B. M., 1993. Stress proteins in aquatic organisms: an environmental perspective. *CRC Critical Reviews in Toxicology*, 23: 49-75.

Santhakumar, M., Balaji, M. and Ramudu, K., 1999. Effect of sub-lethal concentration of monocrotophos on erythropoietic activity and certain haematological parameters of fish, *Anabus testudineus* (Bloch). *Bull. Environ. Contam. Toxicol.*, 63: 379-384.

Sarma, K., Pal, A. K., Ayyapan, S., Das, T., Manush, S. M., Debnath, D. and Baruah, K., 2010. Acclimation of *Anabas testuideneus* (Bloch) to three test temperatures influences thermal tolerance and oxygen consumption. *Fish Physiol. Biochem.*, 36, 85-90.

Sarma, K., Pal, A. K., Sahu, N. P., Ayyappan, S. and Baruah, K., 2009. Dietary high protein and vitamin C mitigates endosulfan toxicity in the spotted murrel, *Channa punctatus* (Bloch, 1793). *Sci. Total Environ.*, 407(12): 3668-3673.

Sayer, M. D. J. and Davenport, J., 1987. The relative importance of the gills to ammonia and urea excretion in five seawater and one freshwater teleost species. *J. Fish Biol.*, 31: 561-570.

Schindler, D. W., 2011. The cumulative effects of climate warming and other human stresses on Canadian freshwaters in the new millennium. *Canadian Journal of Fisheries and Aquatic Sciences*, 58(1): 18-29.

Schmidt-Nielsen, K., 1997. Animal physiology: adaptation and environment, 5th edn. Cambridge University Press, Cambridge, pp. 217-293.

Schneider, W. C., 1945. Determination of nucleic acids in tissue by pentose analysis. Methods in Enzymology, Vol. II, pp. 680-682.

Schreck, C. B., 1981. Stress and compensation in teleostean fishes: Response to social and physical factors. *In*: Pickering, A.D. (ed.), Stress and Fish. Academic Press, London, pp. 277-293.

Schreck, C. B., 1996. Immunomodulation: Endogenous factors. *In*: Iwama, G. and Nakanishi, T. (eds.), The Fish Immune System, Organism, Pathogen and Environment. San Diego: Academic press, pp. 311-337.

Schreck, C. B., Olla, B. L. and Davis M. W., 1997. Behavioural responses to stress. *In*: Iwama, O. K., Pickering, A. D., Sumpter, J. P. and Schreck, C. B. (eds.), Fish stress and health in aquaculture. Society for Experimental Biology, Cambridge, pp.145-170.

Segner, H., 2011. Moving beyond a descriptive aquatic toxicology: the value of biological process and trait information. *Aquatic Toxicology*, 105: 50-55.

Sen, P. R., Rao, N. G. S., Ghosh, S. R. and Rout, M., 1978. Observations on the protein and carbohydrate requirements of carps. *Aquaculture*, 13: 245-255.

Seyle, H., 1950. Stress and the general adaptation syndrome. *J. British Medicine*, 1: 1383-1392.

Sharp, G. J. E. and Secombes, C. J., 1993. The role of reactive oxygen species in the killing of the bacterial fish pathogen *Aeromonas salmonicida* by rainbow trout macrophages. *Fish and Shellfish Immunology*, 3: 119-129.

Sheridan, M. A. and Muir, N. A., 1988. Effect of epinephrine and nor-epinephrine on glucose release from Chinook salmon (*Oncorhunchus eshawytscha*) liver incubated *in vitro*. *J. Exp. Biol*., 248(2): 155-159.

Sidransky, H., Verney, E. and Murty, C. N., 1981. Effect of elevated dietary tryptophan on protein synthesis in rat liver. *J. Nutr.*, 111: 1942-1948.

Sies, H., 1986. Biochemistry of oxidative stress. *Angew. Chem. Int. Ed.*, 25: 1058-1071.

Singh, D. K. and Aggarwal, R. A., 1986. Synergistic effect of sulfoxide with carbaryl on the *in vivo* acetyl choline esterase activity and carbohydrate metabolism of snail *Lymnea acuminata*. *Acta. Hydrochem. Hydrobiol.*, 14(4): 421-427.

Smith, T. R. and Buckley, L. J., 2003: RNA-DNA ratio in scales from juvenile cod provides a nonlethal measure of feeding condition. *Transactions of American Fisheries Society*, 132: 9-17.

Soufy, H., Soliman, M., El-Manakhly, E. and Gaafa, A., 2007. Some biochemical and pathological investigations on monosex tilapia following chronic exposure to carbofuran pesticides. *Global Veterinaria*, 1: 45-52.

Spigarelli, S. A. and Smith, D. W., 1976. Growth of salmonid fishes from heated and unheated areas of Lake Michigan measured by RNA-DNA ratios. *In*: Esch, G.W. and Mc Farland, R.W. (eds.), ERDA Symposium Series (Conf. 750425) Augusta, Georgia, U.S.A., Thermal Ecology, vol. II. pp.100-105.

Stensløkken, K. O., Ellefsen, S., Larsen, H. K., Vaage, J. I., Nilsson, G. E., 2010. Expression of heat shock proteins in anoxic crucian carp (*Carassius carassius*): support for cold as a preparatory cue for anoxia. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, 298: 1499-1508.

Stowe, C. M., 1969. Cholinergic (parasympathomiatic) drugs: drugs acting on the autonomic nervour system. *In*: Jones, M. (ed.), Veterinary Pharmacology and Therapeutics, Oxford and IBH Publishing Co. pp. 265-350.

Suarez, R. K. and Mommsen, T. P., 1987. Gluconeogenesis in teleost fishes. *Can. J. Zoology*, 65: 1869-1882.

Summers, C. H., Larson, E. T., Summers, T. R., Renner, K. J. and Greenberg, N., 1998. Regional and temporal separation of serotonergic activity mediating social stress. *Neuroscience*, 87: 489-496.

Suzer, C., Aktulun, S., Çoban, D., Kamaci, O., Saka, S., Firat, K. and Alpbaz, A., 2007. Digestive enzyme activities in larvae of sharpsnout seabream (*Diplodus puntazzo*). *Comp. Biochem. and Physiol.*, 148: 470-477.

Takahara, S., Hamilton, B. H., Nell, J. V., Kobra, T. Y., Ogura, Y. and Nishimura, E. T., 1960. Hypocatalesemia, a new generis carrier state. *Journal of Clinical Investigation,* 29: 610-619.

Talwar, P. K. and Jhingran A. G., 1991. Inland Fishes of India and Adjacent Countries, Vol.1, Oxford and IBH Publishing, New Delhi, India, p. 541.

Tejpal, C. S., Pal, A. K., Sahu, N. P., Jha, A. K., Muthappa, N. A., Sagar, V. and Ranjan, M. G., 2009. Dietary supplementation of L-tryptophan mitigates crowding stress and augments the growth in *Cirrhinus mrigala* fingerlings. *Aquaculture*, 293: 272-277.

Tejpal, C. S., Sumitha, E. B., Pal, A. K., Murthy, S. H., Sahu, N. P. and Siddaiah, G. M., 2014. Effect of dietary supplementation of l-tryptophan on thermal tolerance and oxygen consumption rate in *Cirrhinus mrigala* fingerlings under varied stocking density. *J. Therm. Biol.*, 41:59-64.

Tomanek, L. and Somero, G. N., 1999. Evolutionary and acclimation-induced variation in the heat-shock responses of congeneric marine snails (genus *Tegula*) from different thermal habitats: implications for limits of thermo-tolerance and biogeography. *Journal of Experimental Biology*, 202: 2925-2936.

Tort, L., 2011. Stress and immune modulation in fish. *Dev. Comp. Immunol.*, 35: 1366-1375.

Tripathi, G. and Verma, P., 2003. Pathway-specific response to cortisol in the metabolism of catfish. *Comparative Biochemistry and Physiology, part B,* 136: 463-471.

Tsuchida, S., 1995. The relationship between upper temperature tolerance and final preferendum of Japanese marine fish. *J. Therm. Biol.*, 20: 35-41.

Ultsch, G. R., Ott, M. E. and Heisler, N., 1981. Acid base and electrolyte status in carp (*Cyprinus carpio*) exposed to low environmental pH. *J. Exp. Biol.*, 93: 65-80.

Vasudevan, D. M. and Sreekumari, S., 1998. Textbook of Biochemistry for Medical students, 3rd edn. Japee publishers, New Delhi, India.

Vaux, D. L., 1993. Toward an understanding of the molecular mechanisms of physiological cell death. *Proc. Natl. Acad. Sci.,* 90 (3): 786-789.

Verma, A. K., Pal, A. K., Manush, S. M., Das, T., Dalvi, R. S. and Chandrachoodan, P., 2007. Persistent sub-lethal chlorine exposure augments temperature induced immunosuppression in *Cyprinus carpio* advanced fingerlings. *Fish and Shellfish Immunology*, 22: 547-555.

Verma, A. K., Pal, A. K., Manush, S. M., Das, T., Dalvi, R. S., Chandrachoodan, P. P., Ravi, P.M. and Apte, S. K., 2006. Persistent sub-lethal chlorine exposure elicits the temperature induced stress responses in *Cyprinus carpio* early fingerlings. *Pesticide Biochemistry and Physiology*, 83(3): 229-237.

Vijayan, M. M. and Moon, T. W., 1994. The stress response and the plasma disappearance of corticosteroid and glucose in a marine teleost, the sea raven. *Can. J. Zool.*, 72: 379-286.

Vijayan, M. M., Pereira, C, Grau, E. G. and Iwama, G. K., 1997. Metabolic responses associated with confinement stress in tilapia: the role of cortisol. *Comp. Biochem. Physiol.*, 116C: 89-95.

Vijayaraghavan, S. and Rao, J. V. R., 1986. Starvational stress effects on tissue lactate and lactate dehydrogenase activity in *Anabas scandens* (Cuvier). *Comp. Physiol. Ecol.*, 2 (4): 233-236.

Walton, M. J, Coloso, R. M., Cowey, C. B., Adron, J. W. and Knox. D.,1984. The effects of dietary tryptophan levels on growth and metabolism of rainbow trout *(Salmo gairdneri)*. *British Journal of Nutrition,* 51: 279-287.

Wang, Q. J. S., Qiao, Z., Bai, D., Sun, J. and Qiao, X., 2012. Effect of lipopolysaccharide (LPS) and outer membrane protein (OMP) vaccines on protection of grass carp (*Ctenopharyngodon idella*) against *Aeromonas hydrophila*. *The Israeli Journal of Aquaculture – Bamidgeh*, 63: 655-662.

Wanger, E. J., Bosakowski, T. and Intelmann, 1997. Combined effect of temperature and high pH on mortality and stress responses of rainbow trout after stocking. *Transactions of the American Fisheries Society,* 126: 985-998.

Watson, R. R. and Petro, T. M., 1984. Resistance to bacterial and parasitic infections in the nutritionally compromised host. *CRC Critical Reviews in Microbiology*, 10: 297-315.

Webber, H. and Riordan, P. E., 1976. Criteria for candidate species for aquaculture. *Aquaculture*, 7: 107-123.

Wedemeyer, G. A. and Mcleay, D. J., 1981. Methods for determining the tolerance of fishes to environmental stressors. *In*: Pickering, A. D. (ed.), Stress and fish. Academic Press, pp. 247-268.

Wedemeyer, G. A., McLeay, D. J. and Goodyear, C. P., 1984. Assessing the tolerance of fish and fish population to environmental stress. The problems and methods of monitoring. *In*: Cairns, W. V., Hodson, P. V. and Nriagu, J. O. (eds.), Contaminant Effects on Fisheries. John Wiley and Sons Inc., New York, pp. 164-195.

Wedemeyer, G. A., Meyer F. P. and Smith, L., 1999. Environmental stress and fish diseases. Narendra Publ. House, Delhi, India. p.192.

Wendelaar Bonga, S. E., 1997. The stress response in fish. *Physiol. Rev.*, 77: 591-625.

Wiegertjes, G. F., Stet, R. J. M., Parmentier, H. K. and Van Muiswinkel, W. B., 1996. Immunogenetics of disease resistance in fish; a comparable approach. *Developmental and Comparative Immunology*, 20: 365-381.

Wilkie, M. P. and Wood, C. M., 1991. Nitrogenous waste excretion, acid–base regulation and ionoregulation in rainbow trout (*Onchorhynchus mykiss*) exposed to extremely alkaline water. *Physiol. Zool.*, 64: 1069-1086.

Wilkie, M. P. and Wood, C. M., 1996. The adaptation of fish to extremely alkaline environments. *Comp. Biochem. Physiol.*, 113B: 665-673.

Wilkie, M. P., Wright, P. A., Iwama, G. K. and Wood, C. M., 1993. The physiological responses of the Lahontan cutthroat trout (*Onchorhynchus clarki* henshawi), a resident of highly alkaline Pyramid lake (pH 9.4) to challenge at pH 10. *J. Exp. Biol.*, 175: 173-194.

Wilson, R. P., Allen, O. W., Robinson, E. H. and Poe, W. E., 1978. Tryptophan and threonine requirements of fingerling channel catfish. *Journal of Nutrition*, 108: 1595-1599.

Winberg, S. and Lepage, O., 1998. Elevation of brain 5-HT activity, POMC expression and plasma cortisol in socially subordinate rainbow trout. *Am. J. Physiol.*, 43: 645-654.

Winberg, S. and Nilsson, G. E., 1993. Roles of brain monoamine neurotransmitters in agonistic behaviour and stress reaction, with particular reference to fish. *Comp. Biochem. Physiol.*, 106C: 597-614.

Winberg, S., Nilsson, A., Hylland, P., Soderstrom, V. and Nilsson, G. E., 1997. Serotonin as a regulator of hypothalamic-pituitary-interrenal activity in teleost fish. *Neurosci. Lett.*, 230: 113-116.

Winberg, S., Nilsson, G. E. and Olsen, K.H., 1992. Changes in brain serotonergic activity during hierarchic bevaviour in Arctic charr (*Salvelinus alpinus* L.) are socially induced. *J. Comp. Physiol.*, 170: 93-99.

Winberg, S., Overli, Ø. and Lepage, O., 2001. Suppression of aggression in rainbow trout (*Oncorhyncus mykiss*) by dietary L-tryptophan. *J. Exp. Biol.*, 204:3867-3886.

Wittman, J. S., 1976. Alteration of glucose tolerance by dietary L-tryptophan in rats. *Journal of nutrition*, 106: 631-635.

Wolkers, C. P. B., Serra, M., Hoshiba, M. A. and Urbinati, E. C., 2011. Dietary L-tryptophan alters aggression in juvenile matrinxa˜ *Brycon amazonicus*. *Fish Physiol. Biochem.*, DOI 10.1007/s10695-011-9569-x.

Wood, C. M., 1989. The physiological problems of fish in acid waters. *In*: Morris, R., Taylor, E. W., Brown, D. J. A. (eds.), Acid Toxicity and Aquatic Animals. Cambridge University Press, Cambridge, pp. 125-152.

Wood, C. M., Perry, S. F., Wright, P. A., Bergman, H. L. and Randall, D. J., 1989. Ammonia and urea dynamics in the Lake Magadi tilapia, a teleost fish adapted to an extremely alkaline environment. *Respir. Physiol.*, 77: 1-20.

Wooten, I. D. P., 1964. Microanalysis. *In*: Churchill, J. and Churchill, A. (eds.), Medical Biochemistry, 4th edition, London, pp. 101-107.

Wroblewski, L. and Ladue, J. S., 1955. LDH activity in blood. *Proc. Soc. Exp. Biol. Med.*, 90: 210-213.

Yamashita, M., Yabu, T. and Ojima, N., 2010. Stress protein Hsp70 in fish. *Aqua-Bio Science Monographs*, 3(4): 111-141.

Yang, J. N. Y. and Carlson, J. R., 1972. Effects of high tryptophan doses and two experimental rations on excretion of urinary tryptophan metabolites in cattle. *J. Nutr.*, 102:1655 -1665.

Yu, C., 1995. Heat shock transcription factors: structure and regulation. *Annual Reviews of Cell Developmental Biology*, 11: 441-469.

Yu, Z., Magee, W. E. and Spotila, J. R., 1994. Monoclonal antibody ELISA test indicates that large amounts of constitutive Hsp-70 are present in salamanders, turtle and fish. *Journal of Thermal Biology*, 19: 41-53.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M. and Horvitz, H. R., 1993. The *C. elegans* cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell,* 75: 641-652.

Appendices

APPENDICES

Abbreviations		Description
ANOVA	:	Analysis of Variance
CRD	:	Completely Randomized Design
SE	:	Standard Error
%	:	Percentage
EE	:	Ether Extract
DM	:	Dry Matter
OM	:	Organic Matter
DE	:	Digestible Energy
TC	:	Total carbohydrate
CP	:	Crude Protein
FCR	:	Feed Conversion Ratio
FER	:	Feed Efficiency Ratio
PER	:	Protein Efficiency Ratio
SGR	:	Specific Growth Rate
DO	:	Dissolved Oxygen
CO_2	:	Carbon di-oxide
AST	:	Aspartate Amino Transferase
ALT	:	Alanine Amino transferase
GOT	:	Glutamate oxalo acetate transaminase
GPT	:	Glutamate pyruvate transaminase
LDH	:	Lactate dehydrogenase
MDH	:	Malate dehydrogenase
SOD	:	Superoxide dismutase
GST	:	Glutathione-s-transferace
AChE	:	Acetylcholine esterase
ALP	:	Alkaline phosphatase
ACP	:	Acid phosphatase
FDPase	:	Fructose-1, 6-Diphosphatase
G6PDH	:	Glucose-6-phosphate dehydrogenase
ATPase	:	Total adenosine triphosphatase

Hb : Hemoglobin
RBC : Red Blood Cells
WBC : White Blood Cells
NBT : Nitroblue tetrazolium
Hct : Haematocrit
Hsp : Heat shock protein
H & E : Haematoxylin and eosin
SEM : Scanning Electron Microscopy
CTM : Critical Thermal Methodology
LTM : Lethal Thermal Methodology
CT_{max} : Critical Thermal maximum
CT_{min} : Critical Thermal minimum
LT_{max} : Lethal Thermal maximum
LT_{min} : Lethal Thermal minimum
g : Gram
Kcal : Kilocalorie
mg : Milligram
n mole : Nano mole
ppm : Parts per million
U : Units
µg : Microgram
Cfu : Colony forming unit
ng : Nano gram
pg : Pico gram
dl : Deci liter
L : Liter
L-TRP : L-Tryptophan
MS_1 : Multiple stressors$_1$
MS_2 : Multiple stressors$_2$