EFFCT OF DIETARY SUPPLEMENTATION OF METHIONINE ON GROWTH, SURVIVAL AND IMMUNE RESPONSE OF INDIAN MAJOR CARP, *Labeo rohita*

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BY

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CERTIFICATE

This is to certify that the thesis entitled **"Effect of dietary supplementation of methionine on growth, survival and immune response of Indian major carp, *Labeo rohita*"** submitted by **Ms. Nilima Priyadarshini,** I.D. No. **MFK 1203** in partial fulfilment of the requirements for the award of **Master of Fisheries Science in Aquaculture** of the **Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar** is a record of bonafide research work carried out by her during the period of her study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma associateship, fellowship or other similar titles.

Mangalore
July, 2014

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Dedicated To

My

Beloved Parents

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*My acknowledgement will remain incomplete if don't acknowledge my sweet sister **Niharika** whose love is more precious than diamonds and pearls.*

*Finally, I would like to pay my sincere thanks to my adorable and affectionate father **Mr. Narendra Kumar Khuntia** and mother **Mrs. Nirupama Khuntia** for their financial, moral support and valuable assistance in my academic pursuits. Here I can say without reservation that whatever I am, it is because of prayers of my beloved parents. They are my worlds and also heaven and their hands are always raised for my well-being even at this moment of time. Their unfathomable love, prayers and help, both moral and financial, have rendered me sound environment during my studies and encouraged me at every step for my higher ideals of life. Words are lacking to express my obligation to my loving parents for their love and wish to see me glittering high on the skies of success.*

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LIST OF ABBREVIATIONS

AA	Amino acid
ANOVA	Analysis of variance
A.O.A.C.	Association of Official Analytical Chemists
CP	Crude protein
CL	Crude lipid
FAO	Food and Agriculture Organisation
FCR	Food Conversion Ratio
IMC	Indian Major Carp
NFE	Nitrogen Free Extract
PER	Protein Efficiency Ratio
PUFA	Poly unsaturated fatty acid
SGR	Specific Growth Rate
WG	Weight Gain

SYMBOLS

cm	Centimeter
g	Gram
hr	Hour
kg	Kilogram
l	Liter
m	Meter
mg	Milligram
ml	Milliliter
ppm	Parts per million
µg	Microgram

Introduction

I. INTRODUCTION

The global production of fish, crustaceans and molluscs has continued to increase and reached 144.6 million tonnes in 2008. While capture production has stayed around 92 million tonnes since 2001. India ranks second among top ten countries in aquaculture production with an average annual percent growth rate of 5.71 during 2006.

The world's population explosion is a major concern for all countries because the problem of feeding billions of people is becoming acute each day. Being surrounded by sea on three sides and possessing big rivers, bays, lakes and ponds, India has ideal conditions for improvement in fisheries. The role of aquaculture in helping to meet the world's food shortages has become more recently apparent. According to a survey of Indian Agriculture, India is the 3rd largest overall fish producer of the world with the total fish production of 7.58 million tons, 6th largest marine capture fishing nation with 2.99 million tons and occupies 2nd place in aquaculture production with 4.59 million tons after China (Anon, 2007). It also accounted for over 6.5 percent of global fish production.

Indian freshwater aquaculture constitutes mainly the culture of Indian major carps (IMC) namely, catla (*Catla catla*), rohu (*Labeo rohita*), mrigal (*Cirrhinus mrigala*). Carp culture is an age-old traditional practice in India. The appropriate technology of fish farming evolved through research investigations in carp culture has shown a major breakthrough by achieving high yields. Modernization of this technology has further uplifted the commercial production of carps. Carp culture in India constitutes 87% of total aquaculture production, consisting of 0.57 million tons of rohu (Ayyappan and Jena, 2003). Carps are the most cultured species in the world with 39% of production by volume. Among IMC, rohu (*Labeo rohita*) is the most preferred fish for freshwater aquaculture. It

is widely cultured in Indian subcontinent mainly in India and Bangladesh. Rohu has been contributing the highest production among the cultivable fishes in India during the last decade providing 35% of the total carp production in India (FAO, 2008).

Every farmer's goal in aquaculture is to maximize the production to get more profit. This could be achieved by the intensification of the farming systems. Diseases are one of the major constraints in intensive aquafarming which will come in the way of sustainable development of aquaculture industry. Disease is a result of the complex interactions between the host, the pathogen and the environment (Snieszko, 1974). In order for a disease to spread from either cultured fish to wild fish or vice-versa, with certain below criteria as described by Olivier (2002).

- Presence of pathogen both in fish and water source;
- Presence of susceptible host;
- Viability, in terms of number and longevity of pathogen in the environment;
- Viable infection route.

Many a time disease outbreak in fish is closely linked to environmental deterioration and stress associated with intensification of culture practises. Inadequate physico-chemical and microbial quality of pond water, poor nutritional status and very high stocking density can cause infection in fish by opportunistic pathogens. Unnoticed and unprecedented movement of brood stocks and seed between different regions has made the pathogen to activate and produce the disease in fish and other stressful conditions affecting the indigenous fish stocks.

In most of the Southeast Asian and Pacific countries, annual losses through diseases are largely unrecorded. But they are very substantial. The actual economic losses

in the aquaculture industry worldwide are estimated to be in excess of US$ 9 billion per year, which is roughly 15% of the value of world farmed fish and shellfish production. In Asia, disease has emerged as a major constraint to sustainable growth of aquaculture (FAO, 1996). In India, the occurrence of white spot and vibriosis since 1994 have caused losses to the tune of 10,000 to 12,000 tonnes which is estimated around US$ 30-50 million (Mohan and Basavarajappa, 2001).

The aquaculture industry in Asia is characterized by an enormous diversity of fish species and most Asian farms operate on a small scale where technical support, including disease diagnosis and training, is lacking. Consequently, treatment is generally decided without proper disease diagnosis and antibiotics are often improperly used. This has led to residual problems and the development of bacterial drug resistance. Poor husbandry practices are still in practice in many places, e.g., the use of trash fish as feed, or fry sourced from the wild or derieved from wild-caught broodstock. These practices open a door for pathogen infections. In addition, the increased trade of live aquatic animals and the introduction of new species for farming, without proper quarantine and risk analysis in place, have resulted in the spread of diseases within and between countries.

The use of disinfectants and antimicrobial drugs have shown limited success in the prevention and or control of aquatic animal diseases. It has been reported that antibiotics enhance growth and feed efficiency by destroying intestinal microflora and thus increasing the amino acid utilization by the host in some animal species (Rawlers *et al.*, 1997). Furthermore, there is a growing concern over the use or abuse of antibiotics and antimicrobials in aquaculture. Numerous studies have shown that most antibiotics including FDA-approved Oxytetracycline can suppress the natural immunity of fish, increasing the risk of infection from non-bacterial pathogens such as viruses, fungi and

parasites (Rijkers *et al.*, 1980, 1981; Siwicki *et al.*, 1989; Taffaella *et al.*, 1999; Luden *et al.*, 1999, 2002). The routine use of antibiotics as growth promoters is a subject for debate in animal farming and feed, and food industries. The use of low levels of antibiotics in animal feeds creates the possibility of transferring immunity to antibiotics used against bacterial pathogens in animals and humans. As a result of such concerns, the EU has banned the prescription-free use of all the antibiotic growth promoters (AGPs) from livestock production with effect from January 2006. Public opinion and regulatory authorities in most exporting countries now focus on the misuse of antibiotics in aquaculture and public attention has shifted towards production methods. Therefore, alternatives to AGP are sought worldwide in a variety of forms.

Prepared diets not only provide the essential nutrients that are required for normal physiological functioning but also may serve as a medium by which fish receive certain compounds that can alter endocrine activity, immunity and other physiological responses (Gatlin, 2002; Li *et al.*, 2005). Thus, in recent years there have been increased research efforts in developing dietary supplementation strategies, in which various growth promoting compounds like amino acids have been evaluated. In modern fish farming, artificial feeding accounts for more than 50% of the production cost. With increasing degree of intensification of fish farming there has been a progressive dependence on artificial feeding. Therefore, development of nutritionally balanced diets for each species farmed assumes great significance. Detailed information on nutritional requirements is known only for a few species cultured world wide. Thus, greater efforts need to be directed towards obtaining more information on nutritional requirements of cultured species (Dabrowski, 1979).

The nutrition of terrestrial animals has been well studied for many years, whereas research on fish nutrition began recently. Most of the early fish nutrition studies were directed towards cold water species particularly salmonids. Early fish researchers and culturists relied primarily on natural foods and fresh animals tissues to meet the nutritional requirements. Due to the paucity of information on fish nutrition, the composition of formulated fish feeds was based largely on the proximate composition of natural foods consumed by the fish or on the nutrient requirements of other animals. Feeds formulated on this basis were adequate at low stocking densities. However, as stocking densities of fish increased, the need for more precise nutritional information became apparent (Robinson and Wilson, 1985).

Protein is a major constituent of animal body. A liberal and continuous supply of protein is needed throughout life. The primary aim of fish culture is to efficiently transform dietary protein into tissue protein. Natural diets of fish are rich in protein. Generally, fish require a high percentage of protein in the diet than birds and mammals. This may be because, fishes utilize carbohydrates less efficiently. The amount of protein needed to produce maximum growth has been investigated with purified test diets (NRC, 1983).

In formulated diets, protein is the most expensive component. Protein is a complex organic molecule consisting of more than twenty amino acids. When ingested protein is digested, amino acids are released into the body pool. They are absorbed for tissue protein synthesis. Methionine is one of the essential amino acids needed for good health, but cannot be produced in the body, and so must be provided through the diet. One of the important functions of methionine is its ability to be a supplier of sulfur and other compounds required by the body for normal metabolism and growth. Sulfur is a key element and vital to fish life. Without an adequate intake of sulfur, fish body will not be

able to make and utilize a number of antioxidant nutrients. Methionine is also a methyl donor, capable of giving off a molecule with a single carbon atom with 3 tightly connected hydrogen atoms, called a methyl group which fish need for a wide variety of chemical and metabolic reactions inside their body.

Some information is available on the qualitative and quantitative gross protein requirements of Indian major carps. Adults of Indian major carps require 30% dietary protein for proper growth and survival. Fingerlings and fry of these carps require 35% and 40% dietary protein, respectively, for good growth (Sen *et al.*, 1978; Renukaradhya and Varghese, 1986). Mondal *et al.* (2000) reported that catla fry require 38.5% dietary protein for optimal growth and survival. Indian major carps, like other animals, do not have an absolute requirement for protein but require a balanced mixture of indispensable and dispensable amino acids (Murthy and Varghese, 1998). *Labeo rohita* is commercially important Indian major carp cultured widely all over India, often in polyculture with other major carps. In order to formulate a balanced fish diet, it is necessary to have complete information on its amino acid requirements.

The present investigation was undertaken to study the effect of dietary supplementation of amino acid, methionine on growth, survival and immune response of Indian major carp, *Labeo rohita* with the following objectives:

1. To evaluate the effect of dietary supplementation of methionine on growth and survival of rohu, *Labeo rohita.*
2. To investigate the effect of dietary supplementation of methionine on immune response of rohu, *Labeo rohita.*

3. To evaluate the effect of dietary supplementation of methionine on biochemical composition and disease resistance of rohu, *Labeo* rohita, challenged with *Aeromonas hydrophila*.

Review of Literature

II. REVIEW OF LITERATURE

The term health management is very broad and encompasses wider areas like water quality maintenance, proper nutrition, selection of good quality brood stock and seed. Health management includes preventive and therapeutic strategies. Preventive strategies include all measures to prevent the occurrence of disease. Fish must not only survive but also should grow rapidly. A wide variety of parasites and pathogens do infect fish. Most disease causing agents are naturally present in low numbers and normally do not cause problems and sometimes make the situation worst and force the farmers to go for dreadful antibiotics, which is not healthy for aquaculture development. In this context, fighting the disease through enhancing the immunity gains importance (Stephen and Anantharaja, 2007).

2.1 Nutrition and disease resistance

Large quantities of fish were being caught from nature and it was possible to satisfy the consumer demand for fish until some years ago. However, demand for domestic fish for food has increased to such a level that fish farming has emerged as a significant animal husbandry industry. Fish cultural operations provide greater possibilities for management and manipulation. The farming of large number of animals under captive conditions requires an elaborate knowledge of their nutrition. Nutrition plays an important role in health management. In aquaculture systems, where stocking density exceeds the food availability, supplementation of food becomes essential. Food, both natural and supplemental, should provide all essential nutrients in right quantities to the farmed organisms. Lack of essential nutrients leads to nutritional disorders which produce gross

non-specific clinical signs and pathological changes (Schaperclaus, 1991; Rao *et al.*, 1992).

Diseases cause heavy economic losses in fish culture due to mortality, morbidity, poor product quality and costs associated with chemotherapy. Aquaculturists are therefore, interested in developing cost-effective management strategies that can either prevent the outbreak or reduce the severity of epizootics. One management strategy currently under exploration is nutritional modification. The influence that dietary factors may have on disease outbreaks in cultured fishes has been recognized since many years. Earlier, investigators concentrated on the effects of various diets on the incidence and severity of common infectious disease. Later, they attempted to determine the mechanisms for some of the observed nutritional effects (Ellis, 1999; Muiswinkel *et al.*, 1985).

Profound changes in the immune response are some of the earliest manifestation of malnutrition. Studies in higher vertebrates have shown that these changes arise through effects upon the thymolympatic system which can be seen automatically in the form of thymic involution, splenic atrophy and thinning of intestinal lymphoid tissue. Some of these changes occur directly as a consequence of general weight loss and amino acid deficiency, others arise indirectly in response to stress-mediated alterations in steroid catabolism (Mc Farlane and Path, 1977). The visible changes in thymolympatic morphology are reflected immunologically by decreased phagocytic index, impaired hypersensitivity responses, subnormal lymphocyte transformations, altered immunoglobulin synthesis and other phenomena which render the animal susceptible to diseases (Raa *et al.*, 1992; Lillehaug, 1989).

In homeotherms, specific dietary deficiency is known to modulate the immune system. Such specific nutritional factors include vitamins, proteins, lipids and minerals (Johnston, 1985; Chandra, 1988). Most of the research which has been conducted on nutrition and immunity has focused on mammals and birds; however, in the recent years many investigators have conducted similar studies employing fish. Our knowledge of the fish immune system and nonspecific disease resistance factors has increased as also the methodology for examining mechanisms of diet-induced effects. In early fish nutrient requirement studies, requirement was determined based strictly on growth, feed conversion and lack of deficiency syndrome. Of late attention has been focused on the complex interactions of nutrients, physiological effects, disease susceptibility and overall health (Blazer, 1992).

Production from aquaculture to a great extent depends upon the type and quality of feeds used. Diets which are capable of maximizing fish or shellfish production are manufactured and employed to derive higher profits from aquaculture. In this context use of growth promoters and feed additives as a means to enhance fish and shellfish production have gained considerable importance. Growth promoters are non-nutritive substances which are incorporated at very low levels in the feed; they readily enhance growth of an animal. Growth promoters have been found to increase efficiency of feed utilization (Viola and Arieli, 1987). Presently, a variety of substance which is termed as growth promoters are being used in aqua feeds. Important among them are hormones, antibiotics, enzyme extracts and many other non-hormonal substances. In this chapter, literature on the use of growth promoters and feed additives in aquaculture is reviewed (Blazer and Wolke, 1982; Blazer *et al.*, 1989).

2.2 Growth:

Growth depends on a number of factors. Among which food ration and the weight of the fish are of special important. When food is insufficient for both maintenance and growth, growth will be inhibited or will cease entirely. In order to determine the amount of food required for both maintenance and growth, it is necessary to know the maximum rate of growth possible when food is not limiting. This does not mean that at this growth rate food is utilized efficiently. It is often possible to achieve a high rate of growth at the expense of excessive food and low utilization so as to make this gain uneconomical (Hepher, 1988).

Good nutrition in animal production systems is essential to economically produce a healthy, high quality product. In fish farming, nutrition is critical because feed represents 40-50% of the production costs. Fish nutrition has advanced dramatically in recent years with the development of new, balanced commercial diets that promote optimal fish growth and health. The development of new species-specific diet formulations supports the aquaculture industry as it expands to satisfy increasing demand for affordable, safe, and high quality fish and seafood products (Halver, 1957; Dupree, 1966; Kitamura *et al.*, 1967; Raa *et al.*, 1992).

Prepared or artificial diets may be either complete or supplemental. Complete diets supply all the ingredients (proteins, carbohydrates, fats, vitamins, and minerals) necessary for the optimal growth and health of the fish. Most fish farmers use complete diets, those containing all the required protein (18-50%), lipid (10-25%), carbohydrate (15-20%), ash (<8.5%), phosphorus (<1.5%), and trace amounts of vitamins, and minerals. When fish are reared in high density indoor systems or confined in cages and cannot forage freely on

natural feeds, they must be provided a complete diet (Yamamoto *et al.*, 1978; Soliman *et al.*, 1986; Chatterjee, 1973; Mahajan, 1980).

Growth is also a function of body size. Body weight or length is the main parameters by which fish farmers determine the feeding level. If feed ration is to produce optimum growth, it is essential to learn the relationships between body weight and growth rate. Except food and weight, growth also depends on a number of other factors, which often interact with food ration and body weight (Brett and Grovers, 1979).

It can thus be concluded that when the diet composition is compatible with the requirements for growth, the net efficiency of utilization of metabolizable energy for growth is about 40-50%, which is somewhat lower than the efficiency of utilization of metabolizable energy for maintenance. However, when the food is not suitable, such as with diets low in protein and high in carbohydrate, when fish are fed excess, or when environmental conditions are unfavorable, efficiency decreases rapidly. Carbohydrate rich diets may still have relatively high efficiency with species that tend to accumulate fat, such as common carp (Hepher, 1988).

2.3 Growth promoters:

Many growth promoters have been employed for the purpose of enhancing growth of cultured animals. They can be broadly categorized into hormonal and non-hormonal types;

2.3.1 Hormonal growth promoters

Dietary hormonal manipulation is one of the means by which growth rate of an animal can be accelerated. There are mainly three groups of hormones, namely, pituitary

growth hormones, steroid hormones and thyroid hormones. They have been shown to enhance growth in fish and shellfish when used singly or in combinations. The usual routes of hormone administration are injection, immersion treatment and through feeds. Donaldson *et al.*, (1979) reviewed the work on hormonal enhancement of growth in fishes.

2.3.2 Non-hormonal growth promoters

Even though hormones have been proved to have an anabolic effect, they are not usually recommended for use in commercial aquaculture operations due to the feare of possible residual hormones in the flesh. Non-hormonal feed additives include vitamins, antibiotics, minerals, lipids and fatty acids, feed attractants, probiotics, prebiotics, protein and amino acids.

As fish culture technology evolved, intensive efforts were made to achieve faster growth of fish leading to higher production. This resulted in the production and use of supplementary fish feeds in addition to enhancement of natural food production through pond fertilization. Nutritionally balanced diets and proper feeding strategies are the important management tools in modern aquaculture. To formulate low cost nutritionally balanced diets for fish, information on nutritional requirements of the cultivated species is a prerequisite. A well balanced feed not only results in higher fish production, but also provides the nutrients necessary to prevent occurrence of disease, besides helping the fish in withstanding environmental stress. Further, nutritionally balanced feed given at the required feeding rate helps in avoiding excess use of one or more nutrients in the diet, which is wasteful.

The nutrients required by fish for growth, reproduction and other normal physiological functions are similar to those of higher animals. They need protein, amino

acids, lipids, carbohydrates, minerals, vitamins and growth factors. These nutrients may come from feeds, picked up from nature or supplied from outside. If large number of fish are held in artificial confinement (as in intensive culture systems), where natural food supply is insignificant, the feed must be nutritionally complete. Protein constituted by amino acids is the important component for growth promotion. These are the expensive nutrients in formulated feeds. Literature available on the gross protein requirements of fishes in general and the amino acid requirements in particular have been reviewed in this chapter.

2.3.2.1 Vitamins

Vitamins are organic compounds necessary in the diet for normal fish growth and health of fish. They often are not synthesized by fish in sufficient quantities and must be supplied in the diet. The two groups of vitamins are water-soluble and fat-soluble. Water-soluble vitamins include: the B vitamins, choline, inositol, folic acid, pantothenic acid, biotin and ascorbic acid (vitamin C). Of these, vitamin C probably is the most important because it is a powerful antioxidant and helps the immune system in fish (Srinivasa, 2000; Sobhana, 1997; Rosenlund *et al.*, 1990; Chavez de Martinez, 1990; He *et al.*, 1992; Deshimaru and Kuroki, 1979; Gunther, 1990).

The fat soluble vitamins include A vitamins, retinols (responsible for vision); the D vitamins, cholecalciferols (bone integrity); E vitamins, the tocopherols (antioxidants); and K vitamins such as menadione (blood clotting, skin integrity). Of these, vitamin E receives the most attention for its important role as an antioxidants. Deficiency of each vitamin has certain specific symptoms, but reduced growth is the most common symptom of any vitamin deficiency. Scoliosis (bent backbone symptom) and dark coloration may result

from deficiencies of ascorbic acid and folic acid vitamin, respectively (Tacon, 1990; Sinha and Sinha, 1994).

Table 1. Vitamin requirements of carps, tilapias, Asian catfish and IMC (mg or IU/kg)

Vitamin	*Cyprinus carpio*	*Oreochromis niloticus*	*Clarias batrachus*	Indian Major Carp (IMC)
Vitamin A (IU)	4,000-20,000			1500
Vitamin D (IU)	Not required			400-500
Vitamin E (mg)	100-300	50-100		40-50
Vitamin K	Not required			5-10
Thiamin (mg)	Required		Not required	
Riboflavin (mg)	4-10		Required	6-8
Pyridoxine (mg)	5.4		Required	6-8
Pantothenate (mg)	30-50		Required	
Nicotinic acid (mg)	28		Required	
Biotin (mg)	1			5-8
Folic acid (mg)	Not required		Required	0.5-1
Cyanocobalamin (mg)	Not required	Not required		
Inositol (mg)	440			300-350
Choline (mg)	4000			500-600
Ascorbic acid (mg)	Not required	1250	Required	300-1000
Ref. from information summarized by Tacon (1990)				Ref. H. S. Murthy (2002)

Vitamins are low molecular weight organic compounds and they are essential for growth and disease resistance in fish.

2.3.2.1.1 Vitamin- A

It is an acyclic polyenoic alcohol and found only in animal kingdom. There was no effect of vitamin A on the growth of *salmo salar*. (Gisdale *et al*., 1991).

2.3.2.1.2 Vitamin-B complex

There are not many studies on these vitamins which suggest growth promoting effect on fish and shellfishes. Tanks holding Indian major carp fry were added with vitamin B-complex or yeast (56 mg/ liter). It was found that a higher survival rate was obtained, which was double that of the control (Das and Krishnamurthy, 1965b; Das, 1959; 1960a; 1960b; 1967).

2.3.2.1.3 Vitamin-C

It is known that fishes only to some extent can synthesise vitamin-C or ascorbic acid, so it must be supplied from outside. No hypervitaminosis with vitamin C up to 500 mg/kg diet in channel catfish was reported by Lovell (1983). Koenig (1984) observed growth promoting effect in fishes by the addition of vitamin C, due to better protein synthesis. Anadu *et al*. (1990) reported the best growth, FCR and PER in the case of *Tilapia zilli* when vitamin C was supplemented in the diet at a level of 300 mg/kg diet. Shiau and Jan (1992) opined that the highest weight gain in shrimp fed the diet containing 250 mg/kg of vitamin C. It was found that ascorbic acid induced better growth in *Clarias batrachus* fry at 69 mg/kg diet (Mishra and Mukhopadhyay, 1996; Hilton, *et al*., 1977). Gouillou *et al*. (1988) reported that ascorbic acid at 45 mg/kg diet induced best growth

performance in *Cyprinus carpio* larvae. Dietary ascorbic acid at 106 mg/kg diet in the form of L-ascorbyl-2-monophosphate-Na/kg diet and 48.4 mg of L-ascorbyl-2-monophosphate-mg/kg diet gave better growth in *Penaeus monodon* (Tsai-Shen and Shi-Yen, 1998).

2.3.2.1.4 Vitamin-D

The compounds which are of greatest physiological interest are vitamin D_2 (ergocalciferol) and vitamin D_3 (Cholecalciferol). Andrews *et al.* (1980) reported that channel catfish exhibited maximum growth rate when fed 1000-4000 IU cholecalciferol/kg.

2.3.2.1.5 Vitamin-E

Vitamin-E is also referred to as tocopherol. No additional improvement in growth was observed by supplementing vitamin E beyond the optimum level in the diet in case of Atlantic salmon (Lall *et al.*, 1988). Roem *et al.* (1990) reported a significant lower weight gain and food conversion in *Oreochromis aureus* fed a diet deficient in vitamin E.

2.3.2.2 Minerals

Minerals are essential chemical elements which are involved in the building of animal organs and are necessary in various physiological functions. Their main functions include formation of skeletal structure, electron transfer, regulation of acid-base equilibrium, and hormones and enzymes. Watanabe *et al.*, (1997) reported that elements are essential ingredients in diets of fish, although in small quantities.

Table 2. Mineral requirements of carps and tilapias

Mineral	Carps	Tilapias
Calcium	0.028%	0.65%
Phosphorus	0.6-0.7%	0.5-0.9%
Magnesium	0.04-0.05%	0.06-0.08%
Zinc	15-30 mg/kg	10 mg/kg
Copper	3 mg/kg	3-4 mg/kg
Manganese	12-13 mg/kg	12 mg/kg
Ref. from information summarized by Tacon (1990) and Jantrarotai (1996)		

2.3.2.2.1 Calcium

Higher content of calcium in the diet produced highest survival and growth in freshwater prawn, *M. rosenbergii* (Zimmermann *et al.*, 1993).

2.3.2.2.2 Phosphorous

Phosphorous level in water is low in general and hence it must be supplemented in the diet. When Hepher and Sandbank (1984) included 6.5% dicalcium or monocalcium phosphate in the diet for common carp and recorded higher growth rate.

2.3.2.2.3 Zinc

Zinc is one of the structural and functional components in enzymes. Dietary inclusion of zinc leads to an increase in food consumption, growth rate and survival of carp when it was incorporated at 15 to 30 ppm (Ogino and Takeda, 1976). Rath and Dube

(1994) reported higher survival, FCR, SGR, condition factor and growth in *M. rosenbergii* when fed a diet containing 9 mg zinc/kg.

2.3.2.2.4 Cobalt chloride ($CoCl_2$)

It is a part of vitamin B12 and is essential for normal activity of animals. Cobalt chloride at 5 ppm concentration yielded better growth in carps (Rajagopalswamy *et al.*, 1995).

2.3.2.2.5 Selenium

Selenium is also an integral part of enzymes. The quality of feed can be improved by the addition of selenium (Froeslie *et al.*, 1985).

2.3.2.2.6 Mineral mixture

The addition of phosphorous, calcium, magnesium, iron, zinc, manganese, iodine, cobalt, sodium and potassium in feed resulted in 5 to 10 % increase in growth in *Salmo gairdneri* (Kanidev *et al.*, 1986). 4% mineral mixture in the diet for *Cyprinus carpio* enhanced growth significantly (Stiffens *et al.*, 1988).

2.3.2.3 Feed attractants and stimulants

Feed attractants have been found to stimulate feeding, thereby enhancing growth. According to Mackie and Mitchell (1985), stimulants consist of chemicals like L-amino acids such as glycine, betaine and inosine. Supplementation of diets with DMPT (Dimethyl-β-propiothetin) lead to increased feeding activity and growth of different fishes. Jasmine *et al.* (1993) reported that DMPT and betaine can effectively increase body weight and moulting rate in *Penaeus indicus*. Hartati and Briggs (1993) reported higher growth

rate, feed intake, feed assimilation, FCR and survival in *P. monodon* when fed a semi-purified diet containing 1.55% of taurine and amino acid mixture. Sheenan, (1997) reported higher feeding rate and a 17% higher growth in *M. rosenbergii* when betaine was added to rearing water.

2.3.2.4 Probiotics

Microorganisms are naturally present in the digestive system of the animals. Some microbes aid digestion, others can potentially cause pathogenesis. The implication of gut ecology for nutrition, feed conversion and disease control, the microbial ecology of the gut merits greater attention. Use of antibiotics disturbs the microbiological balance of gut flora eliminating most of the beneficial flora. On stopping the antibiotic treatment, pathogens begin to re-establish themselves in the intestine. Overgrowth of these organisms and subsequent invasion of the system by pathogenic organisms cause inflammatory, immunological, neurological and endocrinological problems. Use of probiotics will help to build up the beneficial bacteria in the intestine and competitively exclude the pathogenic bacteria. These bacteria also release enzymes, which help in the digestion of feed. The concept of using probiotics or direct fed microbials in animal feed particularly poultry and aquaculture is slowly becoming popular. The common organisms in probiotic products are *Aspergillus oryzae, Lactobacillus acidophilus, L. bulgaricus, L. plantarium, Bifidobacterium bifidium, Streptococcus lactis* and *Saccharomyces cerevisiae.* These products can be administered through water or incorporated in the feed. Probiotics have been particularly useful in the early stages of chick growth since the gut of the newly hatched chick is sterile and administering probiotics through water at this stage helps to build up beneficial bacteria much faster than the normal course (Conway, 1989; Dopazo *et*

al., 1988; Fukami *et al.*, 1997; Fuller, 1989; Gilliland, 1979; Gomez-Gil *et al.*, 2000; Gram *et al.*, 1999; Holzapfel *et al.*,1998; Huis In Tveld *et al.*, 1994; Jong, 1993; Kozasa, 1986).

The most important feature of probiotics is that the vegetative or the spore forms have to be viable to be able to multiply in the gut. Secondly they should be resistant to antibiotics, which are administered so that the gut ecology could be maintained. Genetic engineering would help to develop probiotics with special properties like secreting enzymes and vitamins in large quantities. Such products would be the future generation feed additives (Krovacek *et al.*, 1987; Lin, 1995; Rana, 1997; Ringo and Gatesoupe, 1998).

Probiotics have also been used in a big way as pond cleaners in aquaculture. Probiotic bacteria directly uptake or decompose the organic matter or toxic material and improve the quality of water. The microbial cultures produce a variety of enzymes like amylase, protease, lipase and cellulose in high concentrations than the native bacteria, which help in degrading waste. These bacteria have a wide range of tolerance for salinity, temperature and pH which usually exists in aquaculture operations. The use of several antibiotics in aquaculture is banned due to rejection of export consignments of marine products. Hence usage of probiotics is propagated to counter the effect of viral and bacterial infections in commercial aquaculture. The pond probiotics also have a special blend of denitrifying bacteria that remove the algae's primary source of food, nitrogen from the water. This drastic reduction in nitrogen concentration makes it difficult for the algae to bloom. The balance between phytoplankton, zooplankton and beneficial bacteria during culture period play a crucial role in the maintenance of pond health. There is yet no definitive parameter to judge the efficacy of probiotics. A quick and easy microbiological testing kit would be very useful in evaluating pond health on a daily basis (De la Bonda *et al.*, 1992; Vanbelle *et al.*, 1990; Tannock, 1999).

In cattle there is no supplemental probiotic given since the ruminant animals employ microbial fermentation to digest the food. By the use of genetic engineering technique one could alter rumen microorganisms and populations to provide more efficient feed conversion, improved milk composition and removal of toxins. The usage of probiotics has been a subject of intense research all over the world and has been accepted as an alternative for antibiotics (Burgents *et al*., 2004; Subasinghe, 1997; Verschuere *et al*., 2000).

2.3.2.4.1 G-probiotics

G-probiotic is a live microbial feed additive manufactured by Vetcare Pvt. Ltd., (Bangalore, India). It contains useful microbes such as *Lactobacillus acidophilus* and *Streptococcus faecium*, betaglucanase and liver extract. Besides it is a very good source of vitamin B-complex. G-probiotic resulted in the better growth, FCR and survival in *Cyprinus carpio* when compared to control (Manjo Kumar, 1994). It was found that, 2 g/kg diet showed better performance in common carp. Hanumanthappa, (1998) tested G-probiotic at 1, 2 and 3% diet for *Catla catla*. He reported best growth in the group fed 3 g G-probiotic/kg diet. Better specific growth rate, food conversion rate and protein efficiency ratio in the group fed 7.5g G-probiotic per kg diet was reported in Tilapia, *O. mossambicus* (Ramachandra Naik *et al*., 1999).

2.3.2.5 Prebiotics

The concept of prebiotics in feed is fairly recent. Prebiotics are non-digestible components and used as basically feed for probiotics where they are resistant to attack by endogenous enzymes and hence reach the site for proliferation of gut microflora. Some of the prebiotics, which are currently used in animal feed, are Mannan-oligosaccharides

(MOS). Mannan-oligosaccharides are mainly obtained from cell walls of yeasts. Other sources of MOS are copra or palm kernel meal. MOS interferes with the colonization of the pathogens. Cell surface carbohydrates are primarily responsible for cell recognition. Bacteria have lectins (glycoprotein) on the cell surface that recognize specific sugars and allow the cell to attach to that sugar. Binding of *Salmonella, E. coli* and *Vibrio* sp. is shown to be mediated by a mannose specific lectin like substance present on the bacterial cell surface. Similarly fructo-oligosaccharides from Chicory have been used as prebiotics to competitively exclude pathogenic bacteria (Xu *et al.*, 2003). The pH of the lumen gets reduced thus preventing the entry of pathogenic bacteria. The concept of using prebiotics has not yet been accepted but the advantages of prebiotic is that it can stand high pelletizing temperatures in the feed and also have a long shelf life.

2.3.2.6 Lipids

Fats form the major group of dietary components after proteins. In general, fats form a highly effective source of energy for fish. They also play a role as metabolic regulators. Lipids are indispensable nutrients for growth and survival of fishes and crustaceans (Kanazawa, 1985). There are reports highlighting the importance of lipid supplementation for growth enhancement. Using a 2:1 cod liver oil/corn oil mixture Sheen and D' Abramo (1989) found that 6% inclusion was optimal, while zero, 10 and 12% levels depressed growth in *M. rosenbergii*. The growth, survival and PER increased with increase in dietary lipid level from zero to 12% in juveniles of Indian white prawn, *Penaeus indicus*, while no significant improvement in growth occurred by inclusion of lipid at levels above 12% (Chandge and paul, 1997). Cowey and Sargent (1972); Cowey (1979) obtained 10-20% lipid in fish diets gives optimum growth without producing an excessively fatty carcass. When rainbow trouts were fed diets having lipid (5-20%) and

protein levels (16-48%), the optimum ratio of protein to lipid was found to be 35% protein, 18% lipid (Takeuchi *et al*, 1978a,b). However, carp fed diets with a fixed protein level of 32%, with lipid varying from 5% to 15% and with corresponding decrease in carbohydrate, did not show increased growth or feed conversion rates (Takeuchi *et al.*, 1979).

2.3.2.6.1 Essential fatty acids

Fatty acids are required for a number of essential compounds in the animal body (e.g. membrane phospholipids). Most of the fatty acids can be synthesized by the animals de novo from acetate as a precursor. Fatty acids produced in this way are saturated, but they usually undergo a partial desaturation to form monoenoic acids of varying chain length. Lipids are high-energy nutrients that can be utilized to partially spare (substitute for) protein in aquaculture feeds. Lipids supply about twice the energy as proteins and carbohydrates. Lipids typically comprise 15% of fish diets, supply essential fatty acids (EFA) and serve as carriers for fat-soluble vitamins (Borlongan and Pazaro, 1991; Takeuchi *et al.*, 1979; Paul *et al.*, 1998).

A recent trend in fish feeds is to use higher levels of lipids in diets. Although increasing dietary lipids can help reduce the high costs of diets by partially sparing protein in the feed, problems such as excessive fat deposition in the liver can decrease the health and market quality of fish. Lipids or fats are required nutrients for fish and supply energy and essential fatty acids. They can also be an important consideration in the manufacture of pellets, especially where extrusion technology is used. Excess dietary lipid can lead to unwanted accumulation of visceral and muscle fat in harvested fish (NRC, 1993 and Tacon, 1990).

Practical diets for channel catfish typically contain 5-6% lipid, with about 3-5% coming from dietary ingredients and the rest sprayed on to pellets after manufacture, to control dust (Robinson and Li 2002). Channel catfish seem to require n-3 fatty acids (1-2% of diet) but not n-6 fatty acids (Robinson and Li 2002). For hybrid *Clarias* catfish, Jantrarotai and Somsueb (1995) and Jantrarotai *et al* (1995) reported an optimal level of 4-10% for dietary lipid, 0.8-0.9 for n-3 fatty acids and 1.0-1.5% for n-6 fatty acids.

In general, carps seem to have gross requirements for lipid of less than 10% (7-8% for Indian major carps, according to Murthy, 2002) and require both n-3 and n-6 fatty acids at 1% of each in the diet (Murthy 2002; Takeuchi *et al.* 2002). Although lipid has a protein-sparing effect for tilapia, level above 12% depressed growth (Shiau, 2002). Although requirements for both n-3 and n-6 fatty acids have been reported. However, there have been some contradictory results, this may be a future research area in tilapia nutrition (Shiau, 2002).

Simple lipids include fatty acids and triacylglycerols. Fish typically require fatty acids of the omega 3 and 6 (n-3 and n-6) families. Fatty acids can be: a) saturated fatty acids (SFA, no double bonds), b) polyunsaturated fatty acids (PUFA, >2 double bonds), or c) highly unsaturated fatty acids (HUFA, >4 double bonds). Marine fish oils are naturally high (>30%) in omega 3 HUFA, and are excellent sources of lipids for the manufacture of fish diets. Lipids from these marine oils also have beneficial effects on human cardiovascular health. (Tom, 1989; Yone, 1982; Sargent *et al.*, 1989).

Marine fish typically require n-3 HUFA for optimal growth and health, usually in quantities ranging from 0.5-2.0% of dry diet. The two major essential fatty acids (EFA) of this group are eicosapentaenoic acid (EPA: 20:5 n-3) and docosahexaenoic acid (DHA:

22:6 n-3). Freshwater fish do not require the long chain HUFA, but often require an 18 carbon n-3 fatty acid, linolenic acid (18:3 n-3), in quantities ranging from 0.5 to 1.5% of dry diet. This fatty acid cannot be produced by freshwater fish and must be supplied in the diet. Many freshwater fish can take this fatty acid, and through enzyme systems elongate (add carbon atoms) to the hydrocarbon chain, and then further desaturate (add double bonds) to this longer hydrocarbon chain. Through these enzyme systems, freshwater fish can manufacture the longer chain n-3 HUFA, EPA and DHA, which are necessary for other metabolic functions and as cellular membrane components. Marine fish typically do not possess this elongation and desaturation enzyme systems, and require long chain n-3 HUFA in their diets. Other fish species, such as tilapia, require fatty acids of the n-6 family, while still others, such as carp or eels, require a combination of n-3 and n-6 fatty acids (Borlongan and Pazaro, 1991; Huisman, 1976; Kiessling *et al*, 1989; Storebakken and Austreng, 1987a,b; Reigh and Stickney, 1989; Weatherly and Gill, 1983).

2.3.2.6.2 Phospholipids

Hung *et al*., (1987) reported that feeding supplemental soy phospholipids in a purified diet resulted in increased growth of juvenile white sturgeons. *M. rosenbergii* larvae when reared for 12 weeks on a semi-purified diet supplemented with lecithin between 0 and 10% did not lead to significant difference in either weight gain or survival (Hilton *et al*., 1984). Kanazawa *et al*., (1985) reported that growth and survival rate of *Penaeus japonicas* larvae improved by the addition of soyabean phosphotidyl choline to diets containing 18:1 ω-9 essential fatty acids as lipid source. The level of lecithin significantly affected mean weight gain and survival rate in juveniles of *Penaeus indicus* (Taki *et al*., 1985). Maclean *et al*. (1994) observed that percentage weight gain and survival rate significantly increased as the level of lecithin increased from 0.2%, regardless

of source of lipid in juveniles of *Penaeus monodon*. Briggs *et al*., (1988) reported that there was no advantage of supplementing cholesterol and lecithin to the diet in case of *M. rosenbergii* juveniles. Chen *et al*. (1992) opined that phospholipids supplementation improved water stability of pellet and growth of shrimp significantly (Chen, 1993). The optimum supplemental levels of cholesterol and phosphotidyl choline for *Penaeus monodon* better growth, feed efficiency and survival were observed by the incorporation of lecithin into the diet (Briggs *et al*., 1994). Coutteau *et al*., (1996) reported that the growth of *Penaeus vannamei* fed 1.5% soyabean phosphotidyl choline or 6.5% of deoiled soy lecithin was significantly higher than that fed a deficient diet. Higher growth rate, metabolic rate and assimilation efficiency and increased feed efficiency were observed in prawns fed 2.5% cholesterol diet (Samuel *et al*., 1997). Paul *et al*., 1998, opined that the inclusion of dietary phospholipids at 4% in the larval diet is necessary for rapid growth and high survival of Indian major carps.

2.3.2.7 Carbohydrates

Carbohydrates (starches and sugars) are the most economical and inexpensive sources of energy for fish diets. Although not essential, carbohydrates are included in aquaculture diets to reduce feed costs and their binding activity during feed manufacturing. Dietary starches are useful in the extrusion process makes it more biologically available to fish.

In fish, carbohydrates are stored as glycogen that can be mobilized to satisfy energy demands. They are a major energy source for mammals, but are not used efficiently by fish. For example, mammals can extract about 4 kcal of energy from 1 gram of carbohydrate, whereas fish can only extract about 1.6 kcal from the same amount of

carbohydrate. Up to about 20% of dietary carbohydrates can be used by fish. Carbohydrates are included in feeds to serve as bulking agents, for binding purpose, and also as protein sparing energy sources (Jauncey and Ross, 1982; Rodriguez *et al.*, 1996). It was found that prawns contain cellulose and chitinase enzymes and carbohydrases, can thus spare protein (Nobarikawa, 1978). The highest weight gain was recorded after 60 days of rearing in *M. rosenbergii* fed potato starch or soluble starch as source of carbohydrates (Gomez Diaz *et al.*, 1990). The group receiving glucose as the carbohydrate source showed least weight gain. Growth, FCR and survival of *Penaeus indicus* improved with the increase in dietary carbohydrate levels from 10 to 40% (Ali, 1982). Level of 20% trehalose resulted in higher weight gain and survival of *Penaeus monodon* (Alva and Pascual, 1987). Maximum growth, higher conversion efficiency and increased nutrient retention were recorded in young catfish, *Heteropneustes fossilis* at 20% dietary carbohydrate (Erfanullah and Jafri, 1995; 1998). Inclusion with relatively high levels of carbohydrate in formulated fish feed is preferred in view of its protein sparing action that may make the diet more cost effective (Hidalgo *et al.*, 1993).

Erfanullah and Jafri, 1995, reported more pronounced protein sparing by carbohydrate at suboptimal levels of protein than at optimal levels in fingerling *Labeo rohita*. Similar result was achieved in common carp where four diets were formulated reducing the fish meal component by 100 g/kg from 300 to 0 g/kg including proportionately increasing quantities of maize with diets containing 190-320 g/kg protein level (Keshavanth and Renuka, 1998).

Ufodike and Matty (1983) optined that carp per formed well when cassara or rice was included at 450 g/kg diet in a 300 g protein/kg diet, rice inducing higher growth than

cassara. Increased amylase activity was recorded in carp fed with increase level of starch (Kawai and Ikdea, 1972; Anderson, 1998; Wilson, 1994).

2.3.2.7.1 Fibre

It is known that fibre addition in feed enhances growth by increasing the retention time of feed in the gut for the enzymes to act. (Kono *et al.*, 1987) a slight growth enhancement in sea bream and yellow tail by supplementing the diet with 10% cellulose. Addition of 2.5-5% α-cellulose addition in diet resulted in higher growth rate, survival and PER in *Oreochromis mossambicus* (Diounddick and Stom, 1990).

2.3.2.7.2 Chitin

Efficiency of chitin supplementation on the growth of red sea bream, Japanese eel and yellow tail was examined among different levels, 10% chitin produced the best result (Kono *et al.*, 1987). Fox (1993) reported that feeding a diet containing chitin varying from 0 to 16% to *P. monodon* for 50 days resulted in poor weight gain, SGR, FCR, survival and chitinase enzyme activity.

2.3.2.8 Proteins

Protein is very important constituent of the diet, both quantitatively and qualitatively. It is the building material for growing animals. The three basic functions of a protein are that of maintenance of tissues, repletion of depleted tissues and growth (Cowey and Sargent, 1972). Wilson (1985) reported that optimum protein requirement for maximum growth in fishes ranges between 30 and 35% and in prawns from 30 to 40% (Bhenana and Mathews, 1995). "Fermosin" (biosynthetic protein product from yeast) was added up to 18% of diet and fed to young carps for 6.5 months resulted in better growth

(Rickter *et al.*, 1983). Blood meal (80% protein content) at 10% level enhanced total fish production, weight gain and final weight in *Oreochromis niloticus* reared in cages (Otubusin, 1987). Squid meal at 10% level induced higher weight gain and better food conversion ratio in *P. monodon* (Cruz *et al.*, 1992).

Best growth rate and feed to gain ratio were obtained at 550 g protein/kg in diet in of *Sparus aurata* (Sanintha *et al.*, 1996). The growth of shrimps (*Penaeus merguensis*) increased with increase in dietary protein level from 34.5 to 42% and declined with further increase (Sadhana and Neelakantan, 1996). A level of 45% protein was found to be better for achieving maximum growth in juvenile Florida pompano, *Trachinotus corolinus* (Lazo *et al.*, 1998). Relative growth, specific growth and survival rate of grey mullet, *Liza macrolepis* were the highest in fish which received 40% dietary protein (Gopal *et al.*, 1999). Jayaprakas and sunil (1999) reported superior growth with 35% protein in case of Asian cichlid, *Etroplus suratensis*.

Protein is the basic component of animal tissues and is, therefore, an essential nutrient for both maintenance and growth. At maintenance level the fish requires protein for replacement of worn-out tissues and proteinaceous products such as intestinal epithelial cells, enzymes and hormones, which are vital for the proper function of the body, and are recycled quite rapidly. The requirement of protein for the synthesis of new tissues is obvious, since protein constitutes 45-75% of the tissue dry matter. The capacity of the fish to synthesize protein *de novo* from carbon skeletons is limited, and most of the protein must, therefore, be supplied through the diet. Thus, the content of protein in the diet and its ratio to the metabolizable energy becomes prime importance (Kaushik, 1980; Ogino and Yang, 1980).

Because protein is the most expensive part of fish feed, it is important to accurately determine the protein requirements for each species. Proteins are formed by linkages of individual amino acids. Although over 200 amino acids occur in nature, only about 20 amino acids are common. Of these, 10 are essential (indispensable) amino acids that cannot be synthesized by fish. The 10 essential amino acids that must be supplied by the diet are: arginine, histidine, isoleucine, leuucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. Of these, lysine and methionine are often the first limiting amino acids. Fish feeds prepared with plant (soyabean meal) protein typically are low in methionine; therefore, extra methionine must be added to soyabean- meal based diets in order to promote optimal growth and health. It is important to know and match the protein requirements and the amino acid requirements of each fish species reared (Kaushik, 1980; Ogino and Yang, 1980; Houlihan *et al.*, 2001; NRC, 1993).

Protein levels in aquaculture feeds generally fall in the range of 40-50% for marine shrimp, 28-32% for catfish, 32-38% for tilapia, 38-42% for hybrid striped bass. Protein requirements usually are lower for herbivorous fish and omnivorous fish (plant-animal eaters) than that of carnivorous fish, and are higher for fish reared in high density (recirculating aquaculture) than low density (pond aquaculture) systems. (Houlihan *et al.*, 2001).

Protein requirements generally are higher for smaller and younger fish. As fish grow larger, their protein requirements usually decrease. Protein requirements also vary with rearing environment, water temperature and water quality, as well as the genetic composition and feeding rates of the fish. Protein is used for fish growth if adequate levels of fats and carbohydrates are present in the diet. If not, protein may be used for energy and life support rather than growth (Murthy, 2002).

Proteins are composed of carbon (50%), nitrogen (16%), oxygen (21.5%), and hydrogen (6.5%). Fish are capable of using a high protein diet, but as much as standard values of the protein may be lost to the environment. Most nitrogen is excreted as ammonia (NH_3) by the gills of fish, and only 10% is lost as solid wastes. Accelerated eutrophication (nutrient enrichment) of surface waters due to excess nitrogen from fish farm effluents is a major water quality concern of fish farmers. Effective feeding and waste management practices are essential to protect downstream water quality (Mondal *et al.*, 2000). Mohanty *et al.* (1990), obtained the practical diet containing 40% protein prepared from fish meal and groundnut cake and gave excellent growth when fed to *Labeo rohita* fry.

The amount of protein required for maintenance of fish can be measured by feeding fish a diet containing just enough protein to balance the loss due to the recycling of tissues, enzymes, etc., so that the protein content of the body will remain unchanged (Ogino and Yang, 1980 and Kaushik, 1980).

Many experiments have been conducted to determine the optimal level of protein in the diet for various fish species. However, the interpretation of the results of these experiments is not always easy. Since protein requirement is affected by, and interacts with, many factors such an environmental conditions, specific physiology and feeding habits, as well as age and developmental stage of the fish, the results of one experiment, under a certain set of experimental conditions, are not necessarily true for different set of conditions (Smith, 1975; Ogino and Yang 1980; Kanazawa, 1985).

Table3. Endogenus nitrogen excretion (mg N/100 g body wt/day) by various fish species.

Fish species	Fish weight	Temperature °C	N_E	Source
Carassius auratus (goldfish)	1.8-1.95	17	10.8	Nose (1961)
Cyprinus carpio (common carp)	78-370	20-27	10.5-13.0	Ogino and Yang (1980)
Salmo gairdneri (rainbow trout)	5.3	17	13.9	Nose (1961)
Oreochromis mossambicus (tilapia)	1.6-1.8	27	10.5	Jauncey (1982)

The most expensive nutrient to supply is usually protein. Carnivorous species tend to have a higher protein requirement than omnivores or herbivores, and are more expensive to feed. Earlier life stages such as fry and fingerlings also require relatively more protein than juveniles and immature adults. Published requirements for protein for several species are summarized in Table 3. Fish do not require protein as such, but rather a well balanced mix of essential and non-essential amino acids. The published requirements for essential amino acids are presented in Table 4 (Shiau and Jan, 1992; Jantrarotai, 1996; Takeuchi *et al.*, 2002; Murthy, 2002; Paripatananont, 2002; NRC, 1993).

Table 4. Dietary protein requirement of carps, Tilapias and Asian catfish.

Species	Requirements (%)	Size
Cyprinus carpio	30-38	Fingerling/juveniles
Ctenopharyngodon idella	28-35	Fingerling
Hypophthamichthys molitrix	37-42	Fry/fingerling
Aristichthys nobilis	30	Fry
Catla catla	35-47	Fry
	40	Fingerling
	30	Adults
Labeo rohita	40	Fry
	35	Fingerling
	30	Adults
Cirrhinus mrigala	40	Fry
	35	Fingerling
	30	Adults
Barbodes gonionotus	35	Fingerling
Oreochromis niloticus	45	Fry
	30-36	Fingerling
	28-35	Juveniles
Oreochromis mossambicus	50	Fry
	30-40	Fingerling
	29-35	Juveniles
Clarias batrachus	40	

Clarias macrocephalus/gariepinus	35	
Pangasius hypophthalmus	27-29	Fingerling
	>18	Juveniles
	35	Fingerling
	20	Juveniles
Channa sp.	43	Fry
	36	Fingerling

From information summarized by (Takeuchi *et al.*, 1978a; 1978b; 1992; Shiau and Jan, 1992; Jantrarotai, 1996; Takeuchi *et al.*, 2002; Murthy, 2002; Paripatananont, 2002).

2.3.2.9 Amino acids

Information on gross protein requirements of fishes without knowledge on the amino acid profile of the diet and their requirement is of limited value, because fish require a well-balanced mixture of essential amino acids (EAA) and non-essential amino acids. The amount of dietary protein required by fishes is directly influenced by the essential amino acid pattern of the diet. Further, the amount of protein that should be provided in practical diets depends largely on digestibility and amino acid composition (NRC, 1977).

In general, there are 18 amino acids that are found in most plant stuffs and animal proteins contain 22 to 26 amino acids. The amino acids can be divided into essential and non-essential groups. The former are those that an animal cannot synthesize in sufficient quantity to support maximum growth. The latter are those that can be synthesized by the animal in sufficient quantity to support maximum growth. Most monogastric animals,

including fish, require the same 10 essential amino acids, namely, arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. The non-essential amino acids are glycine, alanine, aspartic acid, cystine, glutamic acid, proline, serine and tyrosine (Lovell, 1989).

The quantitative EAA requirements of fishes have traditionally been determined starting from Halver and co-workers by feeding graded levels of one amino acid at a time keeping other amino acids at the required levels so as to elicit growth response or dose response curve (Mertz, 1972; Ketola, 1982; Cowey and Luquet, 1983). Several workers have used free amino acid levels in body pools i.e., blood or plasma (Kaushik, 1979) or oxidation of radioactively labelled amino acids administered orally or by injection (Walton *et al.*, 1982) as the criteria for estimating dietary requirement.

Crystalline amino acids either alone or in combination with selected intact protein sources such as casein, gelatin, zein, gluten or fish meal were used in experimental test diets. Recent studies have favoured the use of higher proportion of intact protein sources in amino acid test diets (Kaushik, 1979; Jackson and Capper, 1982; Borlongan and Coloso, 1993). The overall amino acid profile of the dietary protein component is usually carefully controlled so as to stimulate the amino acid profile of a specific reference protein with the exception of the test amino acid (Tacon and Cowey, 1985). Halver (1957) formulated amino acid test diet for the first time and fed successfully to chinook salmon. He compared test diets containing 70% crystalline L-amino acids so as to have amino acid profile similar to whole chicken egg protein, salmon egg protein and salmon yolk sac fry protein. Few other workers employed amino acid test diets similar to those developed by Halver (1957) and studied the amino acid requirements of other species. Aoe *et al.* (1970) reported a marked reduction in growth rate of common carp corresponding to the relative increase in

free amino acids in the test diets. However amino acid diets have to be neutralized for better utilization by common carp and channel cat fish (Nose *et al.* 1974 and Wilson *et al.*, 1977).

The essentiality of various amino acids for fish has been determined either by growth studies or by ^{14}C labelling studies. Most of these studies have involved feeding purified diets containing crystalline amino acids. Essentiality of each amino acid is determined based on growth response associated with the deletion of that amino acid from the diet.

Cowey *et al.* (1970) studied the qualitative amino acid requirements of plaice, *Pleuronectes platessa* and sole, *Solea solea* using intraperitoneal injections of uniformly labelled ^{14}C-glucose. Formation of radio active labelled aspartic acid, glutamic acid, cystine, serine, glycine, alanine and proline over a six day period indicated that sufficient amounts of this amino acid can be produced by yearling plaice and sole through intermediary metabolism, thus suggesting non-essentiality of these amino acids. On the other hand, arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine were not incorporated from labelled ^{14}C glucose, thus implying their dietary essentiality. Similar results have been reported by Metailler *et al.* (1973) with sea bass, *Dicentrachus labrax*. All the finfishes that have been studied to date have shown requirement for the same ten amino acids namely arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine considered indispensable for normal growth (Wilson, 1985).

Most of the quantitative amino acid requirement values reported have been estimated based on the conventional growth response curve. A few investigators have also

shown a high degree of correlation of serum free amino acid levels to dietary amino acid intake. For example, serum content of certain amino acids remained very low until the requirement for that amino acid was met and then increased to very high levels when excessive amounts of the amino acid were fed (Harding *et al.*, 1977; Cowey and Sargent, 1979; Robinson *et al.*, 1980a, 1980b; Wilson, 1985).

Most investigators employed the basic method developed by Halver and co-workers (Mertz, 1972) to determine the quantitative amino acid requirements of fish. Diets were designed to contain protein levels at or slightly below the optimum requirement level for that species to assure maximum utilization of the limiting amino acid. This procedure has been used successfully in determining the quantitative amino acid requirements of chinook salmon and coho salmon (Halver *et al.*, 1957), sockeye salmon (Halver and Shanks, 1960), rainbow trout (Shanks *et al.*, 1962), and Japanese eel (Arai *et al.*, 1972). Nose *et al.* (1974) and Wilson *et al.* (1980) have used the basic method of Halver but modified the methodology by neutralizing the diets with sodium hydroxide for maximum utilization by warm water species, namely common carp and channel cat fish respectively.

Several other investigators have had varying degrees of success by using semi-purified and practical test diets to determine the amino acid requirements of fish. The semi-purified diet includes an imbalanced protein as the major source of intact amino acids, such as corn gluten (Halver *et al.*, 1957; Ketola, 1983; Walton *et al.*, 1984a) or zein (Kaushik, 1979; Dabrowski, 1981) which is deficient in certain amino acids. The practical diet involves normal feed stuffs to supplement the bulk of the amino acids in the test diets. These may be formulated with a fixed amount of desired protein level and the remaining amount of the protein equivalent is made up of crystalline amino acids (Luquet and Sabaut, 1974; Jackson and Capper, 1982; Thebault, 1983; Hughes *et al.*, 1983; Walton *et al.*,

1984a, 1984b). Halver (1957) reported the similarity of amino acid composition of eggs and larval stages of salmon to the requirements of the same species. Working on the amino acid supplementation of casein in diets of Atlantic salmon (*Salmo salar*) fry and soyabean meal for rainbow trout (*Salmo gairdneri*) fingerlings, Rumsey and Ketola (1975) observed improved weight gain in rainbow trout fingerlings fed amino acid supplemented diet, thereby suggesting the similarity between amino acid profiles of rainbow trout eggs and dietary amino acid requirements of rainbow trout juveniles. The recent trend is to use the amino acid composition of body or carcass as the reference to formulate amino acid diets for fish. There exists a close similarity between the amino acid composition of carcass and the requirement of the species (Cowey and Tacon, 1983; Wilson and Poe, 1985; Coloso *et al*., 1986; Borlongan and Benitez, 1990; Borlongan, 1991).

Cowey and Luquet (1983) and Cowey and Tacon (1983) reviewed the various problems involved in accurate determination of the amino acid requirement of fish based on growth studies: (a) imprecise interpretation of growth response curves; (b) the growth rates commonly observed with amino acid test diets being generally lower than those observed with intact protein diets and (c) some of the crystalline amino acids in the test diets being leached during the feeding studies. Wilson (1986) has opined that the requirement values obtained by the use of these types of diets should only be used as estimated values because of the many inherent problems in the procedure.

Some of these are variation in protein digestibility coefficients, rate of passage and digestion of various proteins varies and slower than the rate when supplemented with amino acids and the imbalanced proteins commonly used namely zein and corn gluten contain high levels of leucine which may depress the rate of absorption of other amino

acids. An accurate analysis of total protein content of the test diets is necessary due to the relationship between the amino acid requirement and level of protein intake.

Essential amino acid requirements have also been determined based on carcass deposition and concentration of serum-free amino acids in the body. Ogino (1980) determined the essential amino acid requirement of rainbow trout and common carp on the basis of the observed daily carcass deposition of individual amino acids. Essential amino acid deficient diets were significant in causing deficiency syndromes in some species. Scoliosis appeared in salmon when low levels of tryptophan were present in the ration (Halver *et al.*, 1957; Halver and Shanks, 1960). Majority of the species studied so far showed poor growth when an essential amino acid deficient diet was given. Fin erosion was observed in rainbow trout fed lysine deficient diets (Walton *et al.*, 1984a). Walton *et al.* (1984b) reported scoliosis and eye cataracts in rainbow trout fed tryptophan deficient diets.

Toxic effects of gross imbalance of some of the indispensable amino acids were also observed when isoleucine was three times in excess of requirement of three fold excess leucine was fed. High level of valine also inhibited growth in salmonids (Chance *et al.*, 1964). Wilson *et al.* (1980) observed a marked increase in valine and isoleucine in plasma when leucine deficient diet was fed to catfish. Cho *et al.* (1991) reported that rainbow trout developed scale and vertebral abnormalities when fed with excess leucine in the diet (13.4%). They opined that gross lesions could be due to the toxic effect of leucine and not due to branched chain amino acid antagonism.

Amino acid test diets did not work well when the protein component was exclusively made of crystalline acids in young carp (Aoe *et al.*, 1970), cat fish (Dupree and

Halver, 1970) and red sea bream (Yone *et al.*, 1974). Little or no growth resulted when these diets namely, amino acid mixtures or hydrolysates of casein, gelatin or other proteins were incorporated in the ration as the major protein component. An appetite stimulating effect has been reported in sea bream upon addition of alanine, tyrosine or serine to the test diet, (Yone *et al*, 1974).

By using purified, semipurified or practical diets, quantitative dietary requirements of essential amino acid for a number of species have been determined by various workers.

2.3.2.9.1 Arginine

Quantitative dietary arginine requirement for channel catfish (*Ictalurus punctatus*) has been determined as more than 3.9% of dietary protein by Andrews *et al.* (1977). However, Robinson *et al.* (1981) recorded 4.3% of dietary protein as the requirement for the same species. Chinook salmon and Coho salmon require 6% and 5.8% arginine respectively (Klein and Halver, 1970) and eel requires 3.9% arginine (NRC, 1977). For common carp, the arginine requirement was found to be 1.6% of the diet or 4.3% of the dietary protein. This level is considerably lower than that of chinook salmon and almost same as that of eel, which is about 4.5% of the dietary protein or 1.7% of diet (Nose, 1979).

Salinity was found to influence the arginine requirement of rainbow trout (Kaushik, 1979). He found the requirement to be 3.3% of protein in freshwater and 2.8% of protein in 20 ppt salinity, which decreased to 2.2% of protein as salinity increased. Kim *et al*, (1983) reported the arginine requirement of rainbow trout as 4% of dietary protein, whereas higher values of 5.9% by Ketola (1983) and 3.6% Walton *et al*, (1986) were recorded. Jackson and Capper (1982) determined the dietary requirement of arginine for tilapia, *O.*

mossambicus as 1.59% which is lower than the reported values of 1.8% by Jackson *et al.* (1982) and 2.82% by Jackson *et al.* (1983) for the same species. Arginine was found to be an indispensable amino acid for salmonids (Halver *et al.*, 1962). Arginine is a limiting amino acid in many of the feed ingredients and its requirement was found to be 6% of dietary protein for maximum growth in many of the species (Halver, 1975). Further, arginine can be used as a nitrogen supplement to spare other protein ingredients of the diet and in protein components of larval diets. Luquet and Sabaut (1974) elucidated the dietary requirement of gilt head bream to be 5% of dietary protein.

Santiago and Lovell (1988) reported the dietary arginine requirement of Nile tilapia, *O. niloticus* as 1.18% of the diet corresponding to 4.2% of the dietary protein, using casein and gelatin as a source of intact protein supplemented with crystalline amino acids. Borlongan (1991) using the same type of diet estimated the dietary requirement of arginine for the growth of milkfish, *Chanos chanos* as 2.10% of the diet or 5.25% of the dietary protein. Fry of the Indian major carp, *C. catla* was found to require 1.92% of arginine in the diet corresponding to 4.8% of dietary protein when fed with diets containing only crystalline amino acids as the source of protein (Ravi and Devaraj, 1991). Dietary requirement of arginine for rohu, *L. rohita* was 23 grams of the diet (Murthy and Varghese, 1995).

2.3.2.9.2 Histidine

Histidine is found to be an indispensable amino acid for the normal growth of all the species studied so far. Working on the nutrition of salmonid fishes, Klein and Halver (1970) found that the histidine protein. Both carp and eel were found to require 2.1% of arginine when expressed as percentage of dietary protein (Nose, 1979; NRC, 1981). Nose

(1979) was of the view that a large amount of free histidine found in tissues might serve as a reservoir for histidine need.

Wilson *et al.* (1980) investigated the histidine requirement of channel catfish. They reported it to be as low as 0.37% of the diet or 1.54% of protein. Rainbow trout was found to require 2.4% of dietary protein for normal growth (Ogino, 1980). Muscle carnosine concentration of chinook salmon was found to be altered by dietary histidine. It depleted when a histidine deficient diet was fed (Lukton, 1958), however, Wilson *et al.* (1980) could not detect carnosine in muscle tissue of channel catfish regardless of the dietary level of histidine. Jauncey *et al.* (1983) reported the histidine requirement of *Oreochromis mossambicus* to be 1.05% of dietary protein, whereas another species of tilapia, *Oreochromis niloticus* found to require 0.48% of the diet or 1.72% of dietary protein (Santiago and Lovell, 1988). Ravi and Devaraj (1991) estimated the dietary histidine requirement for fry of catla as 0.98% of diet, which corresponds to 2.45% of dietary protein. Milkfish found to require 2% histidine in the dietary protein for normal growth (Borlongan and Coloso, 1993). Dietary requirement of histidine for rohu, *Labeo rohita* was 9 grams of the diet (Murthy and Varghese, 1995).

2.3.2.9.3 Isoleucine

Isoleucine is one of the branched chain amino acids which is indispensable for many of the species studied. General requirement range is 2.0 to 2.6% of isoleucine as a percentage of dietary protein for most of the species. Chinook salmon was found to require 2.2% of isoleucine in the dietary protein (Chance *et al.*, 1964). Nose (1979) reported that common carp required 2.5% isoleucine in the dietary protein. Lake trout, *Savelinus namaycush* required a lower level of 1.54-2.06% of isoleucine in the dietary protein

(Hughes *et al.*, 1983). Wilson *et al.* (1980) elucidated the isoleucine requirement of channel catfish as 2.6% of the dietary protein for optimum growth. Almost the same value has been reported by NRC (1983) i.e., 2.58% for the same species.

Rainbow trout has a dietary requirement of 2.4% for isoleucine in the protein for normal growth (Ogino, 1980). Jauncey *et al.* (1983) quantified the isoleucine requirement to be 2.01% for the growth of Mossambique tilapia. Studying on the amino acid requirement of Nile tilapia, Santiago and Lovell (1988) determined the isoleucine requirement as 3.11% of protein. Leucine and isoleucine interaction was not apparent in Nile tilapia, whereas isoleucine requirement of chinook salmon increased when dietary leucine was in excess of the requirement (Chance *et al.*, 1964). Requirement of catla fry was found to be around 2.35% isoleucine of the dietary protein (Ravi and Devaraj, 1991). Borlongan and Coloso (1993) reported that milk fish requires 4.0% isoleucine in the protein for optimum growth.

2.3.2.9.4 Leucine

With the exception of eel, the leucine requirement of most of the fishes is in the order of 3.3 – 4% of dietary protein. Chance *et al.* (1964) opined that chinook salmon requires 3.9% leucine in the protein for normal growth. Common carp has a dietary requirement of 3.3% of protein (Nose, 1979); this value is lower than 5.3% reported for eel (NRC, 1981). Wilson *et al.* (1980) reported 3.5% leucine as the dietary requirement of channel catfish, which is nearer to the requirement value of Mossambique tilapia i.e., 3.4% (Jauncey *et al.*, 1983). Lake trout found to require 3.66% leucine in the dietary protein for optimum growth (Hughes *et al.*, 1983). Santiago and Lovell (1988) investigated the leucine requirement of Nile tilapia as 3.39% of protein, which is same as that of the requirement

reported for Mossambique tilapia. Ravi and Devaraj (1991) estimated the dietary requirement of fry of catla as 3.7% leucine in the dietary protein. Milkfish has a dietary requirement of 5.11% leucine (Borlongan and Coloso, 1993).

Excess dietary isoleucine reduced the growth of Chinook salmon fingerlings when fed with sub – optimal levels of leucine (Chance *et al.*, 1964). It was reported by Wilson *et al.*, (1980) that the serum free leucine level in channel catfish remained constant regardless of dietary leucine intake. There was however, a marked effect of dietary leucine on the serum free – isoleucine and valine levels; nearly a six fold increase in the concentration of isoleucine and valine levels was observed. This observation indicates that leucine may facilitate the tissue uptake of the other two branched – chain amino acids.

2.3.2.9.5 Lysine

Lysine is an indispensable amino acid for growth of all fish species studied so far. Chinook salmon was found to have dietary requirement of 20 g lysine/kg diet containing 40 percent crude protein (Halver *et al.*, 1958). Robinson *et al.* (1980) reported that channel catfish requires 15g lysine/ kg diet containing 300 g crude proteins. Rainbow trout found to have a dietary requirement of 2.1% lysine in the diet which corresponds to 5.25g protein (Ogino, 1980). Jackson and Capper (1982) inferred that *Sarotherodon mossambicus* has a requirement of 1.62% dietary lysine. A few workers have studied the dietary lysine requirement of rainbow trout and varying results have been obtained. Kim and Kayes (1982) opined that lysine requirement of Mossambique tilapia was 1.3% of diet or 3.7% of the dietary protein, whereas, another study suggests that a level of 6.1% of protein is the quantitative requirement for the same species (Ketola, 1983). Walton *et al.* (1984a) found

excellent agreement between requirement values determined by growth studies and amino acid oxidation values in the above species.

Deficiency syndroms in fish fed lysine deficient diets have been reported. Ketola (1979) observed fin rot in rainbow trout fed a diet containing corn gluten meal, which is deficient in lysine. Depressed rates of collagen formation were recorded by Millikin (1982), when fish were fed lysine deficient diet. Very high mortality and incidence of caudal fin erosion in fish fed lysine deficient diets were observed by Ketola, (1983), but this syndrome was not noticed in fish fed arginine deficient diets. Borlongan and Benitez (1990) reported that milkfish require 2.0% dietary lysine in the diet for better growth. Growth and survival of hybrid stripped bass was found to be better when fed with a diet containing 1.4% lysine corresponding to 4.03% of dietary protein (Keembiyehetty and Gatlin, 1992). Tibaldi and Lanari (1991) reported that fingerling sea bass (*Dicentrarchus labrax*) grow well with a diet having 2.17% lysine *i.e.*, 4.82% of dietary protein in the diet. According to Borlongan and Coloso (1993), milkfish has a dietary requirement of 4% lysine as percentage of dietary protein. Dietary requirement of lysine for rohu, *L. rohita* was 2.24% of the diet (Murthy and Varghese, 1997). The best growth and specific growth rate were recorded in mrigal, *Cirrhinus mrigala* fed the diet containing lysine (Benakappa and Varghese, 2002).

2.3.2.9.5.1 Lysine – Arginine interaction

Lysine – Arginine antagonism has been observed in a few species. Kaushik and Fauconneau (1984) found some biochemical evidence for metabolic antagonism between lysine and arginine in rainbow trout. Increase in dietary lysine intake affected plasma arginine, urea levels and ammonia excretion. They opined that these changes were due to a

decrease in the relative rate of arginine degradation as the level of lysine increased. However, such arginine and lysine interactions were not observed in channel catfish (Robinson *et al.*, 1981) and rainbow trout (Kim *et al.*, 1983).

2.3.2.9.6 Phenylalanine

Phenylalanine is an indispensable amino acid for fish. Phenylalanine and tyrosine are together called aromatic amino acids. Some scientists considered tyrosine as a semi-essential amino acid and most often the sum of the two amino acids is reported to be the requirement for total aromatic amino acids. Fish can readily convert phenylalanine into tyrosine or utilize, dietary tyrosine to meet their metabolic needs of phenylalanine. Dietary requirement for phenylalanine is determined either in the absence of tyrosine or with test diets containing very low levels of tyrosine (Halver, 1970). Chance *et al.* (1964) reported the total aromatic amino acid (Phenylalanine+Tyrosine) requirement for chinook salmon as 5.1% and opined that tyrosine spared a part of the phenylalanine requirement. When the tyrosine was excluded from the diet, common carp showed poor growth; however, a rapid growth response was observed on the addition of phenylalanine upto 2.5%. Further, addition of phenylalanine beyond 2.5% did not stimulate growth (Nose, 1979). Eel was found to require 2.2% phenylalanine in the diet when tyrosine is present and 5.8% of the dietary protein in the absence of tyrosine (Nose, 1979).

Eel was found to have a dietary requirement of 5.8% total aromatic amino acids (NRC, 1981). Ogino (1980) recorded the requirement of rainbow trout as 5.2% for aromatic amino acids in the dietary protein. Mossambique tilapia was found to have a dietary requirement of 2.5% total aromatic amino acid as percent of dietary protein (Jauncey *et al.*, 1983). Robinson *et al.* (1980a) determined the phenylalanine requirement

for channel catfish as 1.2% of diet when 0.3% tyrosine was in the diet. Borlongan (1992) working on the aromatic amino acid requirements of milkfish reported that it requires 1.9% dietary phenylalanine with the tyrosine level being 0.45% in the diet. With 1.2% dietary tyrosine the phenylalanine requirement was found to be 1.26-1.9 of the diet. Total aromatic amino acid requirement of milkfish worked out to 5.22% of dietary protein and replacement value of tyrosine for phenylalanine was computed to be 46%.

2.3.2.9.7 Valine

Valine is also an indispensable amino acid in all the fishes that have been studied so far. Wilson *et al.* (1980) reported that channel cat fish has a dietary requirement of 2.96% valine when expressed as percentage of protein. Rainbow trout was found to require 3.1% valine in the protein (Ogino, 1980). Almost the same level (3.2%) of valine was reported to be necessary for proper growth and survival of chinook salmon (Chance *et al.*, 1964). Nose (1979) observed that common carp grows better with the diet having 3.6% valine in the dietary protein, whereas eel is known to have a dietary requirement of 4% valine in the protein (NRC, 1981). Jauncey *et al.* (1983) opined that Mossambique tilapia grows well with 2.2% valine in the dietary protein. Santiago and Lovell (1988) studying the amino acid requirements of Nile tilapia observed that the fish requires 2.8% valine in the dietary protein (0.78% of diet). Catla fry is known to have a dietary requirement of 3.35% valine in the dietary protein or 1.5% valine in the whole diet (Ravi and Devaraj, 1991). Borlogan and Coloso (1993) reported that milkfish has a dietary requirement of 3.55% valine as a percent of protein for normal growth.

2.3.2.9.8 Threonine

Threonine is an indispensable amino acid for all the fishes studied so far. Delong *et al.* (1962) determined the threonine requirement of chinook salmon as 0.9% of dry diet when fed a 40% crude protein diet (2.25% of protein). Channel catfish was found to have a dietary requirement 0.53% of threonine in the diet or 2.2% of protein (Wilson *et al.*, 1978). Common carp and eel were found to require same levels of threonine for normal growth i.e., 1.5% of the diet or 3.9% the dietary protein, which is higher than the levels reported for chinook salmon and channel cat fish (Nose, 1979). Threonine requirement of chum salmon fry was reported as 1.2% of the diet (3.0% of protein) by Akiyama *et al.* (1985b). Santiago and Lovell (1988) determined the dietary requirement of threonine for Nile tilapia, *O. niloticus* to be 1.05% of the diet which corresponds to 3.75% of dietary protein. Milk fish, *Chanos chanos* was found to have a dietary requirement of 1.80% threonine in the diet or 4.50% dietary protein as reported by Borlongan (1991). Ravi and Devaraj (1991) estimated that fry of catla require 1.98% of threonine in the total diet which is equal to 4.95% of the dietary protein. Similarly dietary requirement of threonine for rohu, *Labeo rohita* was 1.7% of the diet (Murthy and Varghese, 1996). The best growth and specific growth rate were recorded in mrigal, *Cirrhinus mrigala* fed the diet containing 1.7% threonine (Benakappa and Varghese, 2002).

Quantitative amino acid requirement of the fish species that have been studied so far was found to vary significantly when expressed as percent of total diet, but remarkable closeness appears when expressed as a percentage of dietary protein. In addition to species difference, the sources of protein (purified or practical ingredient), protein level, size and age of fish, temperature and experimental conditions also affect the quantitative amino acid requirements.

2.3.2.9.9 Tryptophan

Tryptophan has been shown to be an essential dietary amino acid for those fish species studied so far (Ketola, 1982). Quantitative requirement appears to vary with species, it being 2g/kg diet for chinook salmon and 0.2 to 0.25% for sockeye salmon and coho salmon (Halver, 1965). Wilson *et al.* (1978) observed that channel catfish fingerlings showed poor weight gain, when fed with a diet having only 0.05% of tryptophan, but weight significantly improved when fed on a diet containing 0.12% of tryptophan. Japanese eel was found to have a dietary requirement of 4g tryptophan/kg diet and common carp 0.8% for normal growth (Nose, 1979). Akiyama *et al.* (1985) opined that young ones of chum salmon (*Onchorhynchus keta*) require 0.29% tryptophan in the diet (0.73% of dietary protein) for proper growth. Rainbow trout attained maximum weight when fed a diet having 0.58% of the amino acid (Poston and Rumsey, 1983). Walton *et al.* (1984) reported the tryptophan requirement of rainbow trout to be 0.25% of the diet. Santiago and Lovell (1988) reported that a minimum quantity of 0.28% tryptophan in the diet or 1.0% of dietary protein was required for the normal growth of tilapia. Fry of catla was found to have a dietary requirement of 0.38% tryptophan in the diet, corresponding to 0.95% of dietary protein (Ravi and Devaraj, 1991). Borlongan and Coloso (1993) investigated the tryptophan requirement of milkfish as 0.6% of dietary protein.

Anatomical syndromes of tryptophan deficiency have been well documented in trouts and salmons. Halver and Shanks (1960) and Shanks *et al.* (1962) reported scoliosis and lordosis in sockeye salmon, but not in chinook salmon fed tryptophan deficient diets. Kloppel and Post, (1975) observed scoliosis, abnormal deposition of calcium in kidney and hyperemia in rainbow trout fed tryptophan deficient diets. They opined that hyperemia might be attributed to lack of serotonin resulting from a deficiency of its precursor

tryptophan. They reported that tryptophan is a major constituent of protocollagen which is supposed to be the precursor of collagen. These deformities were found to be reversible when the fish were fed adequate tryptophan. Wilson *et al.* (1978), while studying the deficiency syndromes of tryptophan in channel catfish, did not observe any anatomical or pathological deformities in fish fed tryptophan deficient diets. Anorexia and scoliosis were not observed in rainbow trout fed tryptophan deficient diet (Poston and Rumsey, 1983). Other tryptophan deficiency signs observed in rainbow trout include renal calcinosis (Kloppal and Post, 1975), caudal fin erosion, cataract and short-gill opercula (Poston and Rumsey, 1983) and increased levels of calcium, magnesium, sodium and potassium in the liver and kidney (Walton *et al.*, 1984b).

2.3.2.9.10 Methionine

Methionine is a sulphur containing amino acid is cystine which is considered as non-essential. Sometimes it is referred to as semi-essential amino acid. Quantitative dietary methionine requirements for several fish species have been shown to depend on dietary cystine concentrations. Cystine can substitute for a part of methionine requirement, since conversion of methionine to cystine is a common path way of intermediary metabolism in many terrestrial animals (Maynord and Loosli, 1979) and fish (NRC, 1973).

Studying on the methionine requirement of chinook salmon, Halver *et al.* (1959) reported it to be 0.5 to 0.6% (1.3 to 1.5% of the dietary protein) in the presence of 1.0% dietary cystine. On the other hand 1.6% of methionine did not produce maximum growth when the cystine content was reduced to 0.05%. Young ones of channel catfish required methionine at 2.34% of dietary protein in the absence of cystine for proper growth, (Harding *et al.*, 1977).

The dietary requirement of eel for methionine did not differ from that of common carp. Nose (1979) observed good growth of common carp with 1.25 of methionine in the diet containing no cystine and on a diet having 0.8% methionine with 2% cystine. Rumsey *et al.* (1983) investigated the methionine requirement of rainbow trout as 1.7% of dietary protein when the diet contained 1.29% of cystine. They concluded that a total sulphur amino acid requirement of rainbow trout was 2.99% of protein. Thebault *et al.* (1985) determined the methionine requirement of sea bass, *Dicentrachus labrax* to be 1% of the dry diet (2% of protein). They opined that 0.6% supplementation of crystalline methionine is the maximum limit, the total methionine in the diet being 1.3%, beyond which growth rate depressed. In another study, the best growth of the same species was obtained when fed with a diet containing 0.9% methionine and 1.2% cystine having a met : cys ratio of 36 : 64 on a sulphur molar basis (Hidalgo *et al.*, 1987).

Robinson *et al.* (1978) opined that channel catfish utilizes DL - methionine as effectively as L – methionine. They observed no significant growth when taurine or inorganic sulphate was added to the basal diet. Rainbow trout also could not utilize taurine and inorganic sulphate as sulphur sources (Page *et al.*, 1978). Kim *et al.* (1983) showed that D – methionine replaces L – methionine on an equal basis in rainbow trout. Poston *et al.* (1977) reported cataract in lake trout fed methionine deficient diet. They fed soyabean protein isolate as the sole protein source which is deficient in methionine (0.36% methionine). On the other hand, fish fed control diet containing 1.2% methionine often results in reduction in sulphydryl group concentrations and lens glutathione synthesis also decreases rapidly during formation of most cataracts. They opined that lens glutathione possibly protects the lens sulphydril groups from oxidation. Walton *et al.* (1982) also observed cataract in rainbow trout when fed methionine deficient diets. Lower levels of

methionine (0.3 – 0.45%) coupled with lower levels of cystine (0.04 – 0.6%) produced varying degrees of cataracts in rainbow trout (Rumsey *et al.*, 1983).

In some of the recent studies, methionine requirement of Nile tilapia was found to be 0.75% of dry diet or 2.68% of the dietary protein, when cystine level was 0.15% in the diet. Total sulphur amino acid requirement (met + cys) would be 0.9% of diet corresponding to 3.21% of protein (Santiago and Lovell, 1988). Liou (1989) reported 2.8% methionine as the dietary requirement for the growth of blue tilapia, *O. aureus*. Moon and Gatlin (1991) determined the methionine requirement for red drum, *Sciaenops ocellatus* as 0.94% of diet or 2.69% of dietary protein in the presence of 0.12% cystine and total sulphur amino acid requirement to be 1.06% of diet (3.03% of protein). They opined that cystine could replace about 40% of the methionine requirement. Methionine requirement of fry of *Catla catla* was found to be 1.5% of diet (Ravi and Devaraj, 1991). In the absence of cystine, the methionine requirement of catla fry was 1.42% of diet or 3.55% of dietary protein. Milkfish requires 2.5% methionine as a percentage of dietary protein for normal growth when cystine level is 0.75% of protein in the diet as suggested by Borlongan and Coloso (1993).

Better growth and food conversion of sea bass, *Dicentrarchus labrax* was obtained by supplementing the diet with methionine and cystine at 0.9% and 1.29% respectively (Hidalgo *et al.*, 1987). Murai *et al.* (1989) reported that supplementation of soya flour basal diet with 0.25% methionine, improved growth, feed efficiency and protein deposition in fingerlings of *C. carpio*. Millamena *et al.* (1996) reported that the requirement of *P. monodon* post-larvae for methionine was 0.89% of diet, while that for valine was 13.5 g/kg of diet. Fernandez and Sukumar (1995) reported that growth rate, FCR, PER, consumption rate, metabolism, SGR and daily growth rate were better with the diet (squid meal) having

the synthetic amino acid mixture incorporated at 0.6% level in *P. indicus*. The lysine requirement of juvenile *P. monodon* was estimated to be 2.08% of the diet, while arginine was 1.85% of the diet (Millamena *et al.*, 1998). Growth rate, feed intake and protein content increased significantly by the addition of increasing dietary methionine in common carp, (*Cyprinus carpio*). The levels tested were 0.49 g, 0.61g, 0.79 g, 1.08 g and 1.34 g methionine/ 100 gm diet (dry matter). Dietary lysine requirement of African catfish, *Clarias gariepinus* was 57 g/kg dietary protein (Rumsey, 1983; Fagbenro *et al.*, 1998; Conceic *et al.*, 2003).

Table 5. Quantitative essential amino acid requirements (percent of dietary protein) of carps, tilapias and channel catfish.

Amino acid	*Cyprinus carpio*	*Catla catla*	*Labeo rohita*	*Oreochromis niloticus*	*Ictalurus punctatus*
Arginine	4.2	4.8	5.8	4.2	4.3
Histidine	2.1	2.5	2.3	1.7	1.5
Isoleucine	2.3	2.4	3.0	3.1	2.6
Leucine	3.4	3.7	4.6	3.4	3.5
Lysine	5.7	6.2	5.6	5.1	5.1
Methionine	3.1	3.6	2.9	2.7	2.3
Phenylalanine	6.5	3.7	4.0	3.8	5.0
Threonine	3.9	5.0	4.3	3.8	2.0
Tryptophan	0.8	1.0	1.1	1.0	0.5
Valine	3.6	3.6	3.8	2.8	3.0

From information summarized by Ravi and Devaraj (1991); NRC (1993); Jantrarotai (1996); Satheesha and Murthy (1999); Satheesha and Murthy (2000); Murthy (2002) and Shiau (2002).

To create an optimum diet, the ratio of protein to energy must be determined separately for each fish species. Excess energy relative to protein content in the diet may result in high lipid deposition. Because fish feed to meet their energy requirements, diets with excessive energy levels may result in decreased feed intake and reduced weight gain. Similarly, a diet with inadequate energy content can result in reduced weight gain because the fish cannot eat enough feed to satisfy their energy requirements for growth. Properly formulated prepared feeds have a well-balanced energy to protein ratio (De Silva and Gunasekara, 1991; Hossein *et al.*, 2002; Shankar, 1988; Shyama and Keshavanath, 1993).

2.4 *Labeo rohita* – the prime fish species

The Indian freshwater aquaculture system is typically characterized by culture of Indian major carps like Catla (*Catla catla*), Rohu (*Labeo rohita*), Mrigal (*Cirrhinus mrigala*) and exotic carps like Silver carp (*Cyprinus carpio*). Among all the six species it is rohu (*Labeo rohita*) is in great demand from the consumer point of view.

2.4.1 Historical background

Rohu (*Labeo rohita*) is one of the most important species among the three Indian major carps that are cultured under carp polyculture systems in India. This graceful Indo-Gangetic riverine species is a natural inhabitant of the riverine system of northern and central India, and the rivers of Pakistan, Bangladesh and Myanmar. In India, it has been transplanted into almost all riverine systems including the freshwaters of Andaman, where its population has been successfully established. The species has also been introduced in

many other countries, including Sri Lanka, the former USSR, Japan, China, Philippines, Malaysia, Nepal and some countries of Africa. The traditional culture of this carp dates back to hundreds of years in the small ponds of the eastern Indian states (Basavaraju and Varghese, 1980; Choudhury, 1995; FAO, 2005; Hayat, 1995; Jayaram, 1981; Khan and Jhingran, 1975; Pathak and Palanisamy, 1995; Somalingam *et al*., 1990).

Information on its culture is availale only from the early part of the 20th century. The compatibility of rohu with other carps like catla (*Catla catla*) and mrigal (*Cirrhinus mrigala*) made it an ideal candidate for carp polyculture systems. While riverine collection of seed was solely meeting the requirement for culture of the species until the first half of the 20th century, the success in induced breeding in 1957 and the assured seed supply thereafter were the major factors for the development of its culture in freshwater ponds and tanks. Its high growth potential, coupled with high consumer preference, have enabled rohu as the most important freshwater species cultured in India, Bangladesh and other adjacent countries in the region. Considering its importance in the culture system, emphasis has also been given to its genetic improvement through selective breeding in India (Ayyappan and Jena, 2003; CIFA. 2004; Gopakumar *et al*., 1999).

2.4.2 Biological features of *Labeo rohita*

The rohu has bilaterally symmetrical body, moderately elongate, its dorsal profile more arched than the ventral profile. The body has cycloid scales, head without scale; snout fairly depressed and projecting beyond mouth, without lateral lobe. The eyes are dorsolateral in position, not visible from outside of the head. The mouth is small and inferior; lips thick and fringed with a distinct inner fold to each lip, lobate or entire. A pair of small maxillary barbels concealed in lateral groove; no teeth on jaws; pharyngeal teeth

in three rows; upper jaw not extending to front edge of eye. The rohu has three or four simple (unbranched) dorsal fin rays with 12 to 14 branched dorsal fin rays. Dorsal fin inserted midway between snout tip and base of caudal fin; pectoral and pelvic fins laterally inserted. The pectoral fin is devoid of any osseous spine and caudal fin deeply forked. The lower lip is usually joined to isthmus by a narrow or broad bridge. Pre-dorsal scales 12-16; lateral line distinct, complete and running along median line of the caudal peduncle. It has 40 to 44 lateral line scales, lateral transverse scale-rows six or six and a half between lateral line and pelvic fin base; snout not truncate, without any lateral lobe; colour bluish on black, silvery on flanks and belly (Jayaram, 1981; Talwar and Jhingran, 1991; Khan and Jhingran, 1975; Ayyappan and Jena, 2001).

2.4.3 Habitat and biology

Rohu in its early stages prefer zooplankton, mainly composed of rotifers and cladocerans, with phytoplankton forming the emergency food. In the fingerling stage, there is a strong positive selection for all the zooplanktonic organisms and for some smaller phytoplankters like desmids, phytoflagellates and algal spores. On the other hand, adults show a strong positive selection for most of the phytoplankton. In the juvenile and adult stages rohu is essentially an herbivorous column feeder, preferring algae and submerged vegetation. Furthermore, the occurrence of decayed organic matter, sand and mud in its gut suggests its bottom feeding habit. The nibbling type of mouth with soft fringed lips, sharp cutting edges and absence of teeth in the bucco-pharyngeal region helps the fish to feed on soft aquatic vegetation which do not require seizure and crushing. The modified thin and hair-like gill rakers also suggest that the fish feed on minute plankton through sieving water. In ponds, the fry and fingerlings exhibit schooling behavior mainly for feeding; however, this habit is not observed in adults (Khoke, 1995). Rohu is a eurythermal species

and however, does not thrive at temperatures below 14°C. it is a fast growing species and attains about 35-45 cm of total length and 700-800 g of weight is one year under normal culture conditions. Generally, in polyculture, its growth rate is higher than that of mrigal but lower than Catla (Pathak and Palanisami, 1995).

The minimum age at first maturity for both the sexes is two years, while complete maturity is reached only after four years in males and five years in females. In nature, spawning occurs in the shallow and marginal areas of flooded rivers. The spawning season of rohu generally coincides with the south-west monsoon, extending from April to September. In captivity with proper feeding the species attains maturity towards the end of second year. However, breeding does not take place in such lentic pond environments; thus induced breeding becomes necessary. The fecundity varies from 2,26,000 to 27,94,000 depending upon fish size and ovary weight; on an average it ranges from 2,00,000-3,00,000 eggs/kg body weight. Rohu is a polygamous fish and also seems to be promiscuous. The optimum temperature for spawning is 22-31°C (Basavaraju and Varghese, 1980 and Jena et al., 1998).

2.4.4 Contribution to fish production system

Rohu is the principal species reared in carp polyculture systems along with the other two Indian major carps viz., catla, *Catla catla* and mrigal, *Cirrhinus mrigala.* Due to its wider feeding niche, which extends from column to bottom, rohu is usually stocked at relatively higher levels than the other two species. In India, the species is also cultured within composite carp culture systems incorporating all three Indian major carps, as well as common carp (*Cyprinus carpio*) and two Chinese carps viz., silver carp (*Hypophthalmichthys molitrix*) and grass carp (*Ctenopharyngodon idella*). However, the

percentage of rohu, even within this six species combination is retained at 35-40 percent in the three-species polyculture system. The higher consumer preference and market demand for rohu during recent years have also led to the practice of two-species culture with catla. The latter type of aquaculture is occurring in over 100000 ha of ponds in the Koleru lake region of Andhra Pradesh, India, in which rohu forms more than 70 percent of the stock. Among the three Indian major carps, rohu being the most important, are also the dominant species cultured in other countries such as Bangladesh, Pakistan, Myanmar, Lao People's Democratic Republic, Vietnam and Nepal. In all these countries, silver carp, grass carp and common carp are the most important species reared with the three Indian major carps in aquaculture (Basavaraju and Varghese, 1980; Jhingran, 1991; Somalingam *et al.*, 1990; Singh, 1995).

Plate 1. Rohu, *Labeo rohita* (Hamilton, 1822)

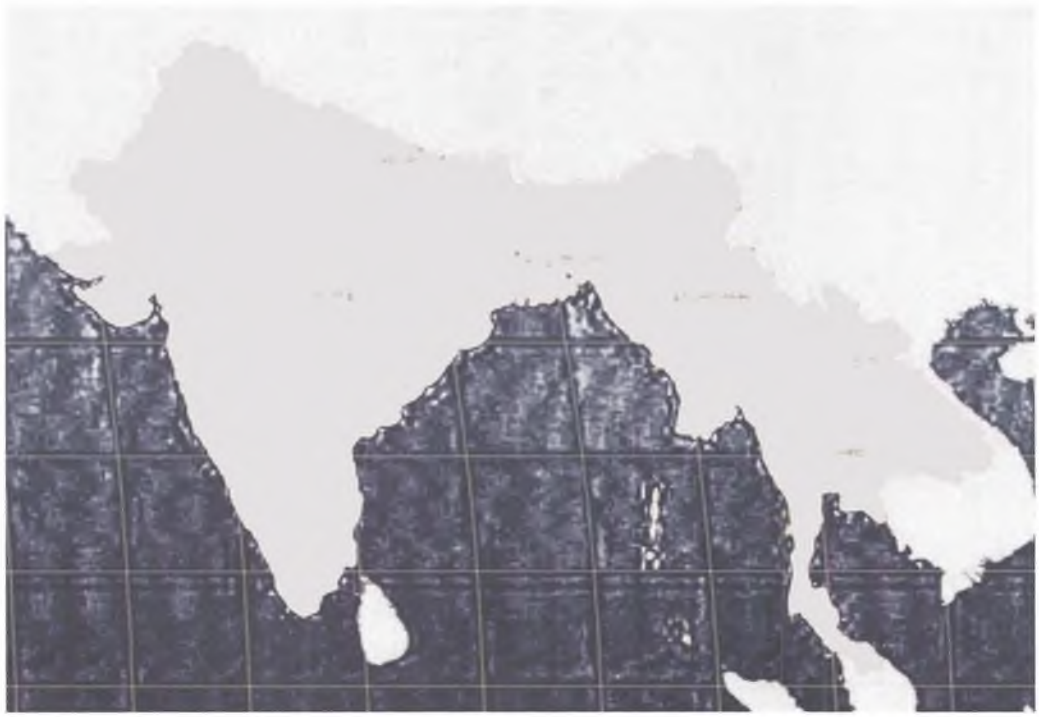

Plate 2. The natural inhabitant rohu (*Labeo rohita*)

Materials and Methods

III. MATERIALS AND METHODS

The present investigation was carried out in cement cisterns at the Research and Instructional Fish Farm of the College of Fisheries, Mangalore to study the effect of dietary supplementation of methionine on growth, survival and immune response of Indian major carp, *Labeo rohita*.

The study was carried out in two experiments. First experiment was conducted for a period of 90 days to study the effect of dietary supplementation of methionine on growth, survival and immune response of rohu fingerlings, whereas the second experiment was conducted for a period of 60 days to study the effect of dietary supplementation of methionine on growth, survival and immune response of rohu juveniles. Both the experiments were divided into three treatments namely T_0, T_1, T_2 and all the treatments were carried out in five replicated groups.

3.1. Feed ingredients, formulation and analysis

The ingredients used in the formulation of different experimental diets were soyabean meal, rice bran, corn flour, cottonseed meal, rapeseed meal, fish meal, sardine oil, vitamin mineral mixture, vit. C, methionine (MetAMINO). All the ingredients were procured from the local market except MetAMINO which was supplied by M/S from Evonik Degussa India Pvt, Mumbai. All the ingredients except methionine were ground and sieved to get particles of uniform size. The sieved ingredients were packed in high density polythene bags and stored in a dry place at ambient temperature. Vitamin and mineral premix in the form of Supplevite-M, a product of M/S Sarabhai Chemicals, Baroda, was obtained locally.

3.2. Proximate composition of feed ingredients

All the ingredients were analyzed for proximate composition prior to preparation of the test diets employing standard methods (AOAC, 1975). Moisture content was estimated by heating samples at 105°C for 30 minutes and then cooling and weighing to a constant weight. Crude protein was analyzed using Kjeltec system (Tecater 1002 Distilling unit), fat content by Soxtech system (Tecater 1043 Extraction Unit), fibre content by using Fibretech system (Tecater 1017 Hot Extractor). Carbohydrate content was calculated as nitrogen free extract (NFE) by the difference method (Hastings, 1976) as given below.

NFE = 100 – (% moisture + % crude protein + % crude fat + % crude fibre + % ash).

The ash content was determined by first charring the sample and then heating it in a muffle furnace at 550 ± 10°C for 6 hours.

3.3. Formulation and preparation of experimental diets

Two test diets namely T_1 and T_2 with 30% protein content were formulated using the square method (Hardy, 1980). Diet T_1 had 0.08% methionine and T_2 had 0.48% methionine and without methionine supplementation served as control (T_0).

The required quantities of ingredients were weighed accurately, mixed and hand kneaded to required consistency with just sufficient quantity of water (1:0.8) to get smooth dough. The dough so obtained was cooked under steam in a pressure cooker at 105^0 C for 20 to 30 minutes. The cooked dough was then cooled to room temperature rapidly by spreading in an enamel tray and required quantity of methionine, sardine oil and vitamin-mineral premix were added, mixed and blended. The dough was extruded through a pelletizer having 3 mm diameter. Extruded pellets were dried in a hot air oven at 60° C till

the moisture content was reduced to less than 10%. Diets were packed separately in high density polythene bags, labelled and stored in a dry place at room temperature for further use.

3.4. Preparation of cement cisterns

Cement cisterns of 25 m^3 size (5×5×1 m) with soil base were used for the growth study. The diets were evaluated in 5 replicate groups for a period of 90 days for the first experiment and 60 days for the second experiment. The cisterns were thoroughly flushed with water and allowed to dry. They were then filled with freshwater drawn from an open well to a depth of 0.7±0.5 m prior to stocking and this level was maintained throughout the experimental period.

3.5. Stocking

Uniform sized fingerlings and juveniles of rohu (*Labeo rohita*) with an average weight of 10.68±0.5 g and 109.07±2.6758 g respectively (reared in a nursery pond of the farm at College of Fisheries, Mangalore) were used. The seeds were stocked at the rate of 20 numbers/cistern and 10 numbers/cistern respectively. The two experimental diets (0.08% and 0.48% methionine) and the control diet were separately fed to fishes in five replicated groups.

3.6. Feeding and rearing

The total daily ration of feed was divided into two equal meals and fed twice daily at the rate of 5% and 2% body weight respectively during the experimental period. After each fish sampling, based on the weight gain the quantity of feed given was adjusted. The feed was hand broadcasted over the pond surface.

3.7. Sampling

Fishes were sampled at every two weeks interval to record growth in terms of weight and length. A minimum of 50% of the population was collected from each tank. The fish were caught by drag net and individual length and weight were recorded.

3.8. Water sampling

Water samples collected from all the cisterns at fortnightly intervals during morning hours were analyzed for important water quality parameters namely, pH, dissolved oxygen, free carbon dioxide, total ammonia and total alkalinity following standard methods (APHA, 1998).

3.9. Harvesting

After a rearing period of 90 and 60 days of the experiments, all the surviving fishes were collected by complete draining of ponds and individual length and weight were recorded.

3.10. Specific growth rate (SGR)

The specific growth of fish was calculated by the formula:

$$SGR(\%) = \frac{\ln \text{final weight (g)} - \ln \text{initial weight (g)}}{\text{No.of days}} \times 100$$

The calculated values give the average percentage increase in body weight of fish per day over the experimental period.

3.11. Food conversion ratio (FCR)

The food conversion ratio was calculated by the formula:

$$FCR = \frac{\text{Dry weight of feed given (g)}}{\text{Gain in wet weight of fish (g)}}$$

3.12. Protein efficiency ratio (PER)

PER of different treatments was calculated as

$$PER = \frac{\text{Increment in body weight}}{\text{Protein intake}}$$

3.13. Survival, weight gain and net production

On termination of the experiment, all surviving fishes were collected and percentage of survival was calculated. Based on the total weight of fish in each treatment net production was worked out.

3.14. Biochemical composition of fish muscle

Fish muscle was collected and analyzed for proximate composition. Muscle was dried at 60°C for 12 hr to obtain dry matter. Then the muscle or whole fish was powdered and stored in airtight bottles for further analysis. The samples were analysed for protein, fat, fibre, moisture and ash following standard procedures as described earlier.

3.15. Laboratory studies

The experiment (Challenge studies) was conducted in a closed recirculatory system (Murthy, 1998) equipped with a three stage biological filter. An air blower was installed for aeration of all the experimental tanks. Twelve fibre glass tanks of 120 l capacity were used for the study.

Biofilter tanks were comprised of fiberglass tanks. Three bigger rectangular tanks each of 1000 liters capacity and one circular tank of 750 l were kept outside the field laboratory. They were covered to avoid direct sunlight and subsequent algal growth. The tanks were connected to one another in such a way that waste water collected from outlets of the 12 experimental tanks was carried by a drain pipe and connected to first circular tank. The first biofilter tank was filled with graded sand and gravel. The water gets filtered in the graded layers of sand and gravel and enters from the bottom of the first tank to the top of second circular tank. The second biofilter tank was filled with dry oyster shells for biological filteration. Water passes through this tank and third tank which was filled with dry clam shells for efficient biofilteration. The filtered water then enters fourth tank which serves as temporary storage tank.

The water was pumped from this tank to an overhead tank. The water flows from over head tank to each culture tank by gravity. The water flow rate in each experimental tank was normally maintained at one liter per minute. Separate control valve was provided for each tank to regulate water flow. A one H.P. air blower was continuously used to provide aeration to the experimental tanks.

3.15.1. Preparation of biofilter and recirculatory culture system to conduct challenge study

All biofilter tanks were cleaned before the start of the experiment and washed thoroughly with detergents and disinfected. The oyster shells were flushed thoroughly, dried and filled back in the respective tanks. Similarly all the culture tanks were thoroughly scrubbed, cleaned and flushed with water. About 100 l water was filled to each culture tanks and aeration tube with air stones were connected.

3.15.2. Stocking

Uniform sized fry of *L. rohita* with an average weight of 95.38±5.1 g were stocked at a rate of 7 fish per tank. They were reared for a period of 2 weeks for adaptation and 2 weeks in this system prior to challenge. The treatments were designed with five replicate groups for each experiment.

3.15.3. Feeding and rearing

The fishes were fed twice daily with the respective treatment diets @ 3% of the body weight. During every sampling, based on fish weight, the quantity of feed to be given was adjusted. The left over feed was removed daily, weighed and feed intake was calculated. Fish faeces were siphoned out daily.

3.15.4. Challenge studies with *Aeromonas hydrophila*

At the end of the feeding trial, 75 fishes from the control were selected and stocked @ 5 fishes per each of 12 aquariums having a capacity of 60 liters. They challenged with different dosage of fresh culture of *A. hydrophila*, after 2 weeks of adaption to aquaria. A virulent strain of *A. hydrophila* was grown for 24 hours on nutrient agar slants, harvested, washed and resuspended in sterile phosphate buffered saline (PBS) having pH 7.2±0.2. Fishes were challenged by injection of 0.1-0.5 ml/fish of PBS. The 75 challenged fishes were kept in 12 aquaria to find LD_{50} after 5 days. Aeration was maintained, with replacement of 50% of the water daily.

3.15.5. LD_{50}

The control fishes were then transferred to aquaria and fed with control diet to determine LD_{50} at 120 days. Five individuals each from control and treatments were

challenged with graded densities (10^4, 10^5, 10^6, 10^7 and 10^8 CFU/ml @ 0.1 ml/fish) of virulent *A. hydrophila* (24 h TSB culture). Specific mortalities were ascertained by re-isolating the pathogen from the kidneys of the diseased fish according to Reed and Muench (1983).

3.15.6. Challenge study

Experimented fish were injected intra muscular with a 24 h culture of *A. hydrophila* (10^6 CFU/fish). A minimum of 10 fish per treatment in triplicate were challenged at 90 days post treatment. Challenged fish were maintained in well aerated aquarium tanks. Dead fish were tested for *A. hydrophila* positivity by re-isolating the pathogen from the kidney on Rimler Shot's medium (Himedia, India).

3.15.7. Relative percent survival (RPS)

Post challenge mortalities were recorded for seven days. Only specific mortalities were used in evaluating the potency of injection (10^6 CFU/fish) challenges. Relative percent survival (RPS) was calculated according to Amend (1981).

$$\text{RPS} = \left(1 - \frac{\%\ \text{mortality in treatment group}}{\%\ \text{mortality in control group}}\right) \times 100$$

3.16. Immune parameters of *Labeo rohita*

3.16.1. Super oxide anion production assay (NBT assay)

The test was performed as described by Anderson and Siwicki (1993) in flat bottomed microtitre plates. The activated phagocytes (neutrophils and macrophages) were characterized by their ability to adhere to glass or plastic and produce oxygen free radicals.

NBT in its reaction with oxygen free radicals was reduced to blue formazan, the extent of which could be determined by spectrophotometrically. The procedure followed was:

1. The blood collected from the caudal vein of fish was centrifuged at 3000 rpm for 6 minutes. 50 µl of buffy coat containing leucocytes was dispensed into a microtitre plate and incubated for 1 hr and 50µl of 0.3% N.B.T. was added to the plate and incubated for 1 hr.

2. The contents of the well were carefully removed and the adhered cells were fixed by adding methanol and incubated for 2-5 minutes. Then, the plates were rinsed 3-4 times with 70% methanol and air dried.

3. The blue formazan was solubulised by adding 60 µl of 2 M KOH. On the top of it, 70µl of Dimethyl Sulphoxide (DMSO) was added.

4. The results were read on an Enzyme Linked Sorbent Assay (ELISA) reader at 620 nm using KOH and DMSO mixture as blank.

3.17. Statistical analysis

The mean and standard error were calculated for each treatment. One-way Analysis of Variance (ANOVA) was employed to test statistical difference between the treatments and Duncan's multiple range tests was carried out to find out the significant difference (Duncan, 1955).

Plate 3. Experimental setup

Plate 4. Drying of ponds

Plate 5. Liming the ponds

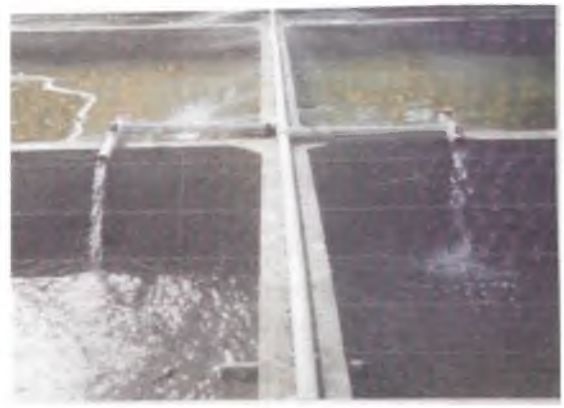

Plate 6. Addition of water in ponds

Plate 7. Initial stocking

Plate 8. Record of individual length during sampling

Plate 9. Fishes were caught by drag

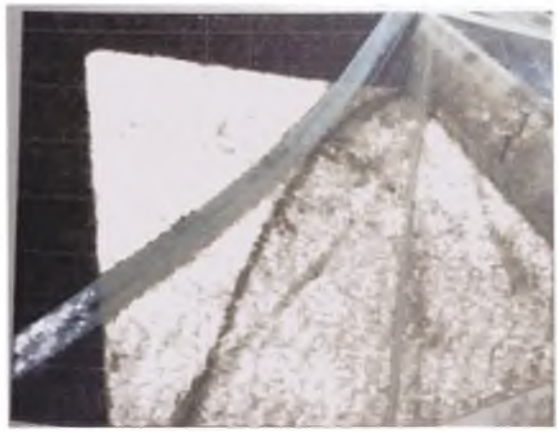

Plate 10. Final harvesting by complete draining

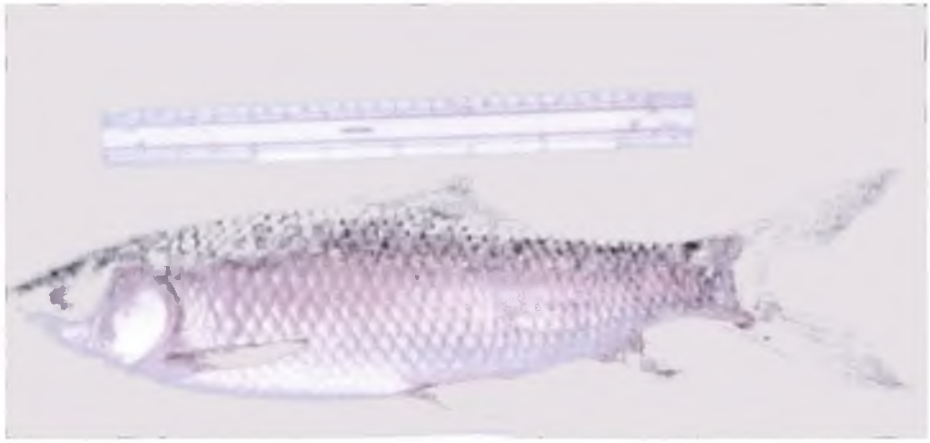

Plate 11. Record of final individual length

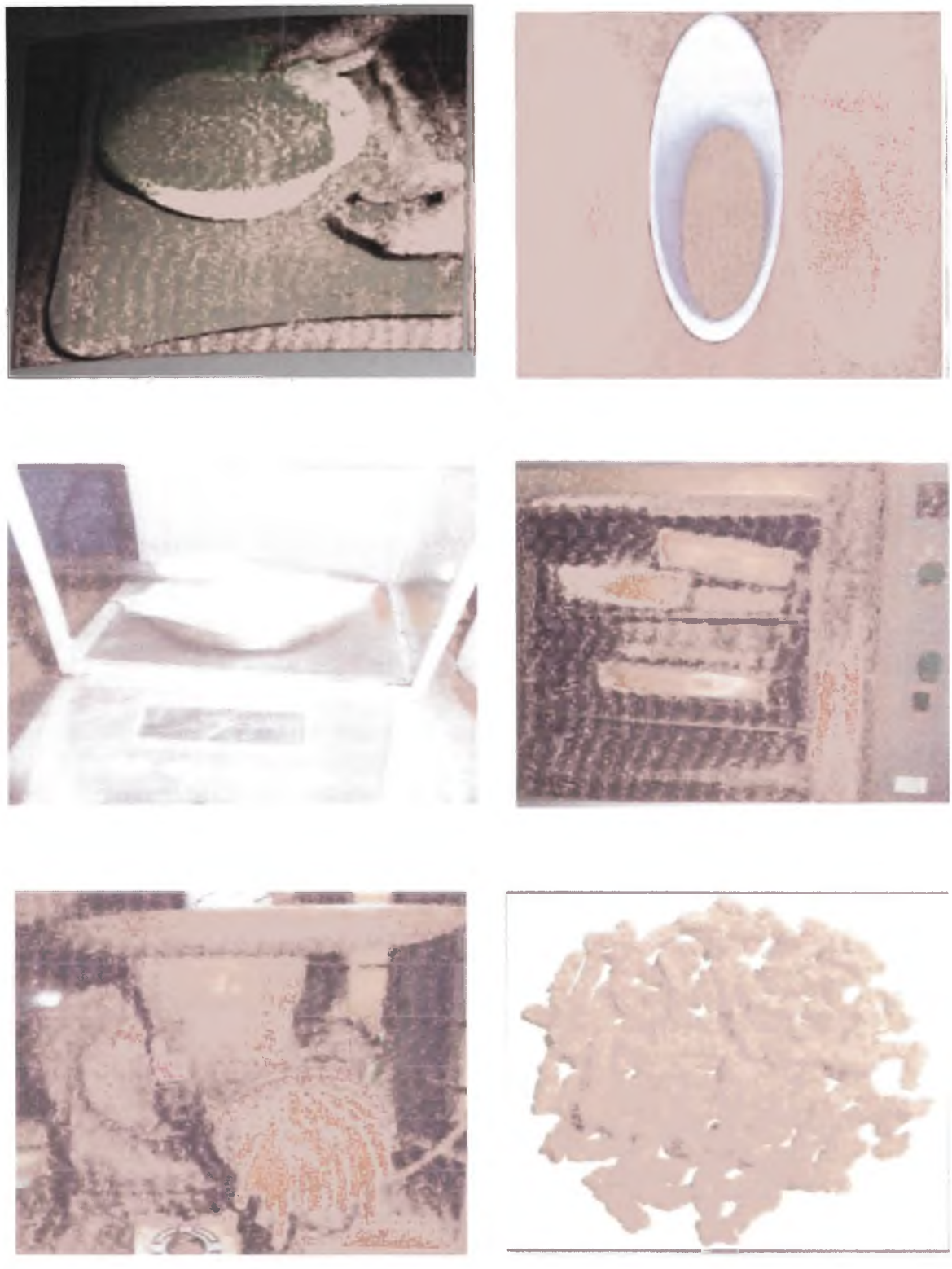

Plate 12. Different steps during feed formulation

Plate 13. Laboratory settings for LD50 and challenge studies of *L. rohita* with *A. hydrophila*

Experimental Results

IV. EXPERIMENTAL RESULTS

4.1. Growth study

The growth trial was conducted to study the effect of graded levels of the amino acid, methionine incorporated diets on growth, survival and carcass composition of rohu, *Labeo rohita.*

4.1.1. Feed ingredients

The feed ingredients were analyzed for proximate composition before formulating the experimental diets. The different ingredients used and their proximate composition are presented in the Table 6. Soyabean meal had the highest moisture content (10.90 %) while corn flour recorded the lowest (6.40 %). Protein content of the ingredients varied from 6.01 % (corn) to 57.85 % (fish meal). Fat content was the highest in rice bran (10.20 %) while corn flour recorded least fat level (5.15 %). Crude fibre in rice bran was the highest (21.60 %) and lowest in corn flour (1.25 %). Ash content was maximum in fish meal (20.12 %), while it was least in corn flour (5.10 %).

4.1.2. Formulated feeds

The proportion of the ingredients used and percent protein contribution of each ingredient is given in Table 7. From the table it is evident that highest contribution of protein was from soyabean meal followed by rice bran and fish meal. Table 8 gives the composition of different ingredients used in each of the test diets with level of incorporation of methionine. The T_1 feed consisted of 0.08% level of methionine, while 0.48% incorporation of methionine in T_2 feed. In feed T_0 no methionine was incorporated and served as a control diet. Proximate composition of formulated feed is presented in

Table 9. Moisture content of diet ranged from 8.89% in T_0 to 8.34% in T_1. Diet T_2 recorded the moisture content of 8.13%. the value of crude protein were 31.95% in T_0, 32.32% in T_1 and 32.82% in T_2. Crude fat content was highest in T_2 (9.28%), followed by T_1 (8.79%) and T_0 (7.39%). The fibre content was highest in T_2 (7.19%) and lowest in T_1 (7.01%). Ash content ranged from 12.63% in T_0 to 10.87% in T_2.

Table 6. Proximate composition of the ingredients used in the formulated diet (%)

Ingredients	Moisture (%)	Dry matter (%)	Crude protein (%)	Crude fat (%)	Crude fibre (%)	Ash (%)	NFE (%)
Soyabean meal	10.90±0.30	88.08±0.15	48.61±0.40	6.95±0.15	4.35±0.15	7.25±0.35	20.92±0.11
Rice bran	10.27±0.03	89.72±0.03	10.32±0.09	10.20±0.03	21.60±0.12	16.35±0.07	30.35±0.17
Corn	6.40±0.05	93.48±0.77	6.01±0.20	5.15±0.55	1.25±0.05	5.10±0.20	75.95±0.15
Cotton seed meal	9.43±0.10	89.78±1.01	34.50±0.42	7.70±0.10	9.35±0.45	10.21±0.09	28.03±0.79
Rape seed meal	10.61±0.52	88.62±0.07	34.57±0.49	6.70±0.10	7.89±0.46	6.20±0.30	33.26±0.17
Fish meal	8.13±0.06	91.87±0.05	57.85±0.56	6.14±0.07	1.75±0.08	20.12±0.32	6.01±0.71

Table 7. Ingredients and their protein contribution

Ingredient	Percent composition in feed	Protein contribution (%)
Soyabean meal	38	18.478
Rice bran	33	3.41
Corn	13	0.781
Cotton seed meal	05	1.725
Rape seed meal	05	1.728
Fish meal	03	1.735
Sardine oil	01	-
Vitamin-mineral mixture	01	-
Vit C	01	-
Total	100	27.857

Table 8. Composition of different ingredients used in the experimental diets

Treatment / Ingredient	T_0	T_1	T_2
Soyabean meal	38	38	38
Rice bran	33	32.92	32.52
Corn	13	13	13
Cotton seed meal	05	05	05
Rape seed meal	05	05	05
Fish meal	03	03	03
Sardine oil	01	01	01
Vitamin-mineral mixture	01	01	01
Vit C	01	01	01
Methionine	-	0.08	0.48
Total	100	100	100

Table 9. Proximate composition of the formulated diets (%)

Treatments	Parameters							
		Moisture	Dry matter	Crude protein	Crude fat	Fibre	Ash	NFE
T_0	Mean±SE	8.89±0.04	91.02±0.04	31.95±0.00	7.39±0.00	7.09±0.03	12.63±0.02	31.96±0.08
T_1	Mean±SE	8.34±0.04	91.38±0.04	32.32±0.05	8.79±0.06	7.01±0.04	11.19±0.10	32.07±0.26
T_2	Mean±SE	8.13±0.01	91.33±0.01	32.82±0.08	9.28±0.01	7.19±0.14	10.87±0.02	31.17±0.06

Experiment-1

4.2. Growth and length trial

The average weight and length of rohu fingerlings over the experimental period of 90 days are given in Table 10 and 12 respectively. The best growth was obtained in rohu fed 0.48% methionine diet, followed by 0.08% methionine treated group and control. The final mean weights were 90.28 g, 91.64 g, 100.48 g in rohu receiving the control diet, 0.08% and 0.48% methionine diet respectively, and the corresponding final mean lengths were 19.64 cm, 19.67 cm and 19.72 cm.

4.3. Specific growth rate

Specific growth rate (%/day) of fish is presented in Table 14. There was significant difference ($P<0.05$) in specific growth rate of fish in the study (Table 15). The highest SGR was 2.44% per day in T_2 with 0.48% methionine incorporated diet followed by 2.43% in T_1 (0.08% methionine) diet and 2.42% in T_0 without methionine incorporation as a control diet.

4.4. Feed conversion ratio

The values of food conversion rate are presented in Table 16. The lowest and best FCR was observed in diet containing 0.48% methionine in T_2 (1.35) followed by T_1 (1.44) containing 0.08% methionine and in T_0 (1.52) which served as control.

4.5. Survival rate, weight gain and net production

The data on survival, weight gain and net production is given in Table 18. The average survival rate was highest in T_2 (92.8) followed by T_1 (90.4) and control (89.6)

treatment. Net production was highest in T_2 treatment where the growth was also maximum.

The average net productions were 1792.32 g, 1867.88 g and 2061.91 g in control, 0.08% and 0.48% of methionine supplemented diets respectively. The corresponding mean weight gains were 80.10 g, 82.59 g and 89.30 g.

4.6. Proximate composition of fish muscle

The result of proximate composition of rohu at the end of the experiment is given in Table 22. Both protein and fat compositions of fish muscle were significant ($P<0.05$) compare to control group (Table 23 and 24).

Maximum moisture content was recorded in T_1 (77.23%) followed by T_0 (76.95%) and minimum in T_2 (76.88%). The maximum protein content of fish muscle of 17.24% was observed in T_2 followed by 17.04% in T_1 and 16.45% in T_0. The crude fat value of fish muscle was 2.45% in T_2 followed by 2.37% in T_0 and 2.29% in T_1. Mean ash levels in fish muscle were 1.41% in T_0 followed by 1.37% in T_2 and 1.25% in T_1. The highest mean NFE was recorded in T_0 (2.83%) followed by T_1 (2.19%) and T_2 (1.88%).

4.7. Immune response of *Labeo rohita* fed methionine supplemented diets

4.7.1. Super oxide anion production (NBT assay)

NBT was carried out at a wave length of 650 nm and the results are presented in Tables 25 and 26. Super oxide anion production of fish fed with 0.08% and 0.48% methionine incorporated diets was higher than that of fish fed control diet. The highest super oxide anion production was recorded in T_2 followed by T_1 and T_0. Super oxide anion

production of fish recorded in different treatments were significantly ($P > 0.05$) higher than that of control group.

4.8. Challenge studies

The results of experimental fish injected intra muscularly with a 24 h culture of *A. hydrophila* (10^6 CFU/fish) are presented in Table 27 and 28. One-way analysis of variance technique was carried out to find out test significant difference in the mean survival of challenged fish among different treatments. ANOVA and Duncan's multiple range test results are presented in Table 29 and Fig 9.

Table 10. Weight (g) attained by *Labeo rohita* fingerlings in different treatments

Treatments	Replications	Days						
		0	15	30	45	60	75	90
T_0	1	9.5	19.75	33.13	48.00	65.55	78.38	91.20
	2	9.8	17.13	31.69	44.32	61.85	76.64	89.42
	3	10.5	18.15	35.62	50.19	66.30	80.72	92.54
	4	10.6	18.34	33.34	47.50	64.53	77.53	90.17
	5	10.5	19.00	35.00	48.50	60.20	75.14	88.08
	Mean±SE	**10.18±0.22**	**18.47±0.44**	**33.76±0.70**	**47.70±0.96**	**63.69±1.15**	**77.68±0.93**	**90.28±0.76**
T_1	1	9.5	16.68	33.34	67.00	76.52	85.76	95.00
	2	9.8	19.50	33.50	45.44	71.25	79.30	90.85
	3	10.7	18.44	33.44	47.60	64.65	78.18	91.97
	4	10.6	18.63	39.85	52.53	69.32	81.39	93.45
	5	11.6	18.86	40.11	56.95	74.44	82.57	93.89
	Mean±SE	**10.44±0.37**	**18.42±0.47**	**36.05±1.61**	**53.90±3.83**	**71.24±2.06**	**81.44±1.33**	**93.03±0.73**
T_2	1	11.0	19.66	38.20	46.66	73.33	89.13	103.09
	2	11.5	17.71	35.40	63.00	79.00	88.85	98.16
	3	12.5	19.38	49.50	63.40	70.00	81.02	91.20
	4	10.5	18.91	46.36	57.55	74.11	85.45	96.72
	5	10.4	17.66	35.20	66.66	72.22	93.57	113.23
	Mean±SE	**11.18±0.38**	**18.66±0.42**	**40.93±2.95**	**59.45±3.52**	**73.73±1.49**	**87.60±2.09**	**100.48±3.71**

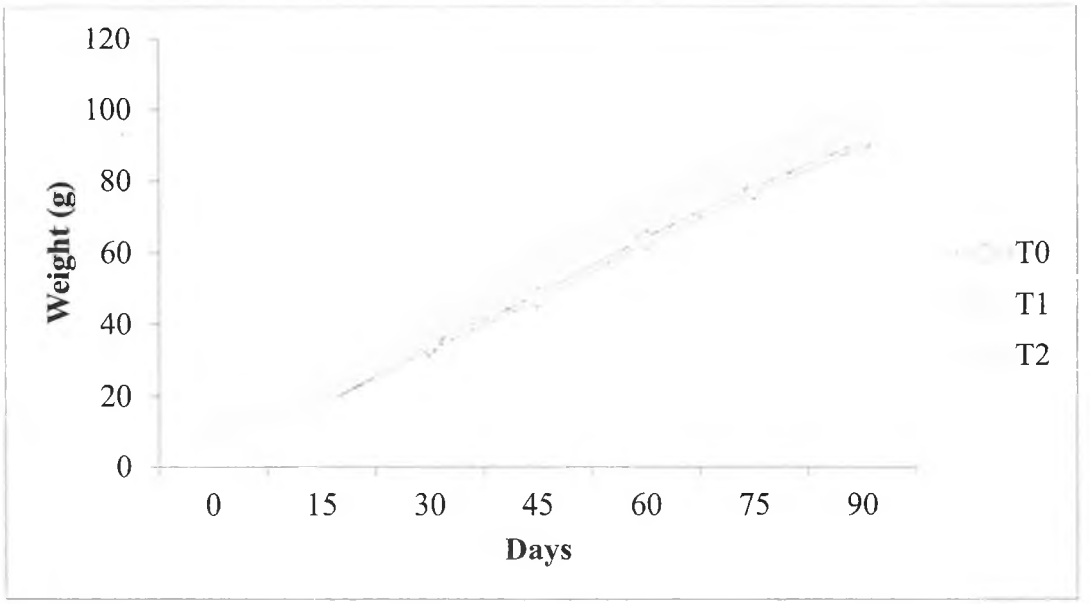

Fig 1: Mean weight (g) of *Labeo rohita* in different treatments

Table 11 (a). Analysis of variance of final mean weights (g) of *L. rohita* from different treatments

Source of variation	Sum of squares (SSQ)	Degree of freedom (df)	Mean sum of square (MSSQ)	F- ratio
Between Groups	278.391	2	139.195	5.620*
Within Groups	297.232	12	24.769	
Total	575.622	14		

*Significant at 5 % level

Table 11 (b). Duncan's multiple range test

Treatments	Mean
T_0	90.28^a
T_1	93.03^a
T_2	100.48^b

Superscripts indicate significant difference among treatments at 5% level.

Table 12. Length (cm) attained by *Labeo rohita* fingerlings in different treatments

Treatment	Replication	Days						
		0	15	30	45	60	75	90
T_0	1	10.3	12.42	14.68	15.65	17.0	18.84	19.8
	2	10.8	12.02	14.32	15.69	16.8	18.99	19.4
	3	10.5	12.36	14.98	16.10	17.2	19.54	20.1
	4	10.8	12.26	14.66	15.81	17.0	18.84	19.76
	5	10.8	12.45	15.10	16.00	16.1	18.36	19.16
	Mean±SE	**10.64 ±0.10**	**12.30 ±0.08**	**14.75 ±0.14**	**15.85 ±0.09**	**16.82 ±0.19**	**18.91 ±0.19**	**19.64 ±0.16**
T_1	1	10.00	10.50	12.00	16.00	17.80	18.70	19.60
	2	11.20	13.20	14.80	15.75	17.10	18.10	19.00
	3	10.9	12.32	14.68	16.16	17.23	18.58	19.72
	4	10.81	11.34	11.98	15.25	16.74	18.91	20.18
	5	11.9	12.52	14.23	15.46	16.75	18.96	19.87
	Mean±SE	**10.96 ±0.31**	**11.98 ±0.47**	**13.54 ±0.64**	**15.72 ±0.17**	**17.12 ±0.19**	**18.65 ±0.15**	**19.67 ±0.19**
T_2	1	11.6	12.41	14.2	16.8	17.0	19.41	20.0
	2	12.2	12.87	13.2	14.7	18.0	19.18	19.5
	3	12.8	13.39	14.1	14.9	17.5	19.89	18.6
	4	11.7	12.22	13.83	15.46	17.5	18.96	19.70
	5	10.8	11.40	12.50	15.50	17.3	19.10	20.80
	Mean±SE	**11.82 ±0.33**	**12.46 ±0.33**	**13.57 ±0.32**	**15.47 ±0.37**	**17.46 ±0.16**	**19.31 ±0.16**	**19.72 ±0.36**

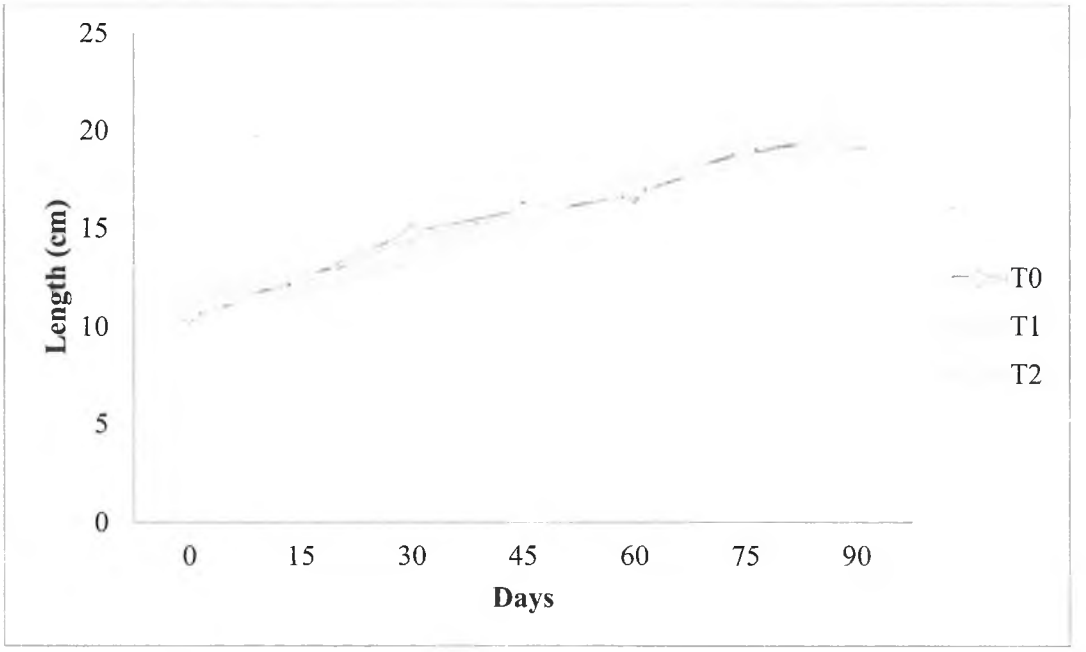

Fig 2. Mean length (cm) of *Labeo rohita* in different treatments

Table 13 (a). Analysis of variance of final average length (g) of *L. rohita* from different treatments

Source of variation	Sum of squares (SSQ)	Degree of freedom (df)	Mean sum of square (MSSQ)	F- ratio
Between Groups	0.015	2	0.007	0.023*
Within Groups	3.844	12	0.320	
Total	3.858	14		

*Significant at 5 % level

Table 13 (b). Duncan's multiple range test

Treatments	Mean
T_0	19.64^a
T_1	19.67^a
T_2	19.72^a

Unlike superscripts indicate significant difference among treatments at 5% level.

Table 14. Specific growth rate (SGR) of fish recorded after 90 days feeding trial

Treatment	Replication	SGR (%)
T_0	1	2.51
	2	2.46
	3	2.42
	4	2.38
	5	2.36
	Mean±SE	**2.42±0.02**
T_1	1	2.56
	2	2.47
	3	2.39
	4	2.42
	5	2.32
	Mean±SE	**2.43±0.04**
T_2	1	2.50
	2	2.38
	3	2.21
	4	2.47
	5	2.65
	Mean±SE	**2.44±0.07**

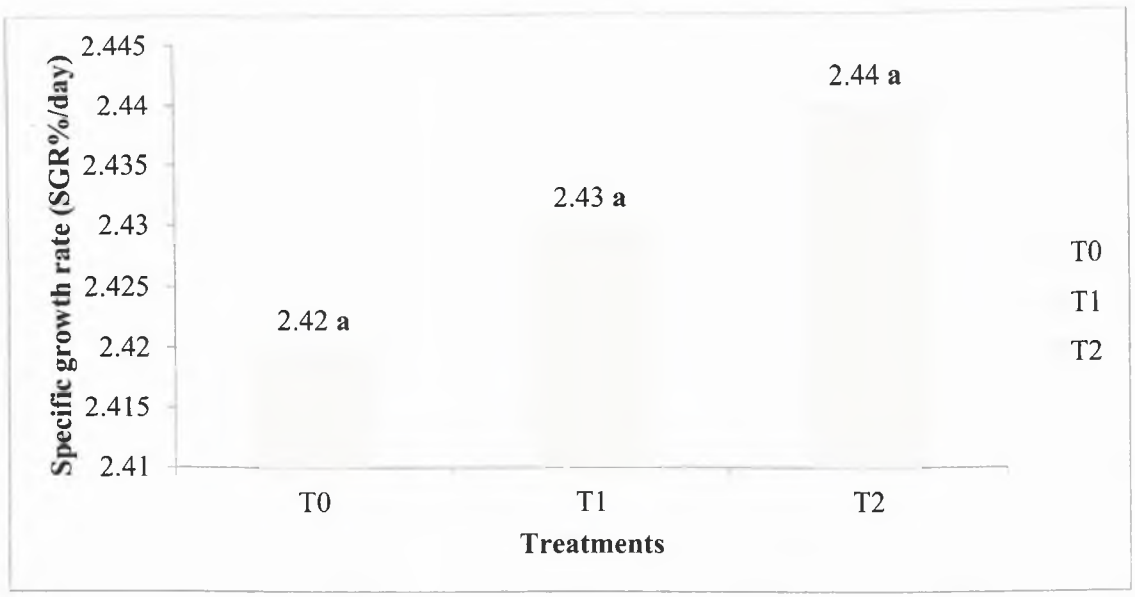

Fig 3: Specific growth rate (SGR) of fish recorded under different treatments and control group

Table 15 (a). Analysis of variance of mean SGR values among different treatments

Source of variation	Sum of squares (SSQ)	Degree of freedom (df)	Mean sum of square (MSSQ)	F- ratio
Between Groups	0.001	2	0.000	0.026*
Within Groups	0.152	12	0.013	
Total	0.153	14		

*Significant at 5 % level

Table 15 (b). Duncan's multiple range test

Treatments	Mean
T_0	2.43^a
T_1	2.43^a
T_2	2.44^a

Unlike superscripts indicate significant difference among treatments at 5% level.

Table 16. Feed conversion rate (FCR) of fish recorded after 90 days feeding trial

Treatment	Replication	FCR %
T_0	1	1.53
	2	1.49
	3	1.48
	4	1.52
	5	1.57
	Mean±SE	**1.52±0.02**
T_1	1	1.53
	2	1.36
	3	1.38
	4	1.45
	5	1.48
	Mean±SE	**1.44±0.03**
T_2	1	1.42
	2	1.22
	3	1.27
	4	1.38
	5	1.48
	Mean±SE	**1.35±0.05**

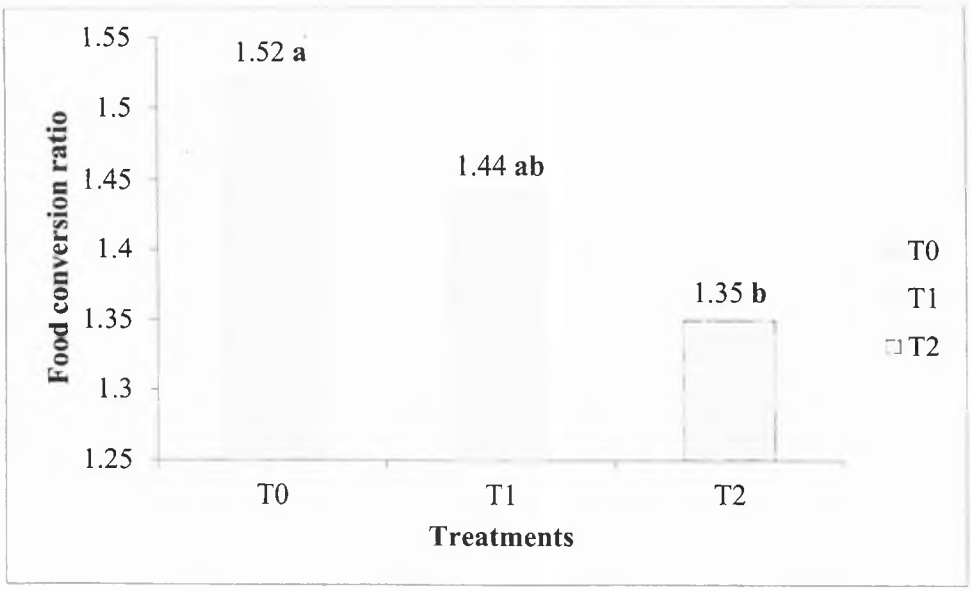

Fig 4: Feed conversion ratio (FCR) of fish recorded under different treatment and control group

Table 17 (a). Anaysis of variance of mean FCR values

Source of variation	Sum of squares (SSQ)	Degree of freedom (df)	Mean sum of square (MSSQ)	F- ratio
Between Groups	0.067	2	0.034	5.703*
Within Groups	0.071	12	0.006	
Total	0.138	14		

*Significant at 5 % level

Table 17 (b). Duncan's multiple range test

Treatments	Mean
T_0	1.52^{a}
T_1	1.44^{ab}
T_2	1.35^{b}

Superscripts indicate significant difference among treatments at 5% level.

Table 18. Survival (%), weight gain and net production of *Labeo rohita* fingerlings in different treatments

Treatment	Replication	No. survived	Survival %	Weight gain (g)	Net production (g/90 days)
T_0	1	23	92	81.70	1879.10
	2	22	88	79.62	1751.64
	3	20	80	82.04	1640.80
	4	22	88	79.57	1750.54
	5	25	100	77.58	1939.50
	Mean±SE	**22.4±0.81**	**89.6±3.25**	**80.10±0.81**	**1792.32±52.70**
T_1	1	25	100	85.50	2137.50
	2	25	100	81.05	2026.25
	3	20	80	81.27	1625.40
	4	21	84	82.85	1739.85
	5	22	88	82.29	1810.38
	Mean±SE	**22.6±1.03**	**90.4±4.12**	**82.59±0.80**	**1867.88±93.88**
T_2	1	22	88	92.09	2025.98
	2	24	96	86.66	2079.84
	3	24	96	78.70	1888.80
	4	25	100	86.22	2155.50
	5	21	84	102.83	2159.43
	Mean±SE	**23.2±0.73**	**92.8±2.94**	**89.30±4.00**	**2061.91±49.92**

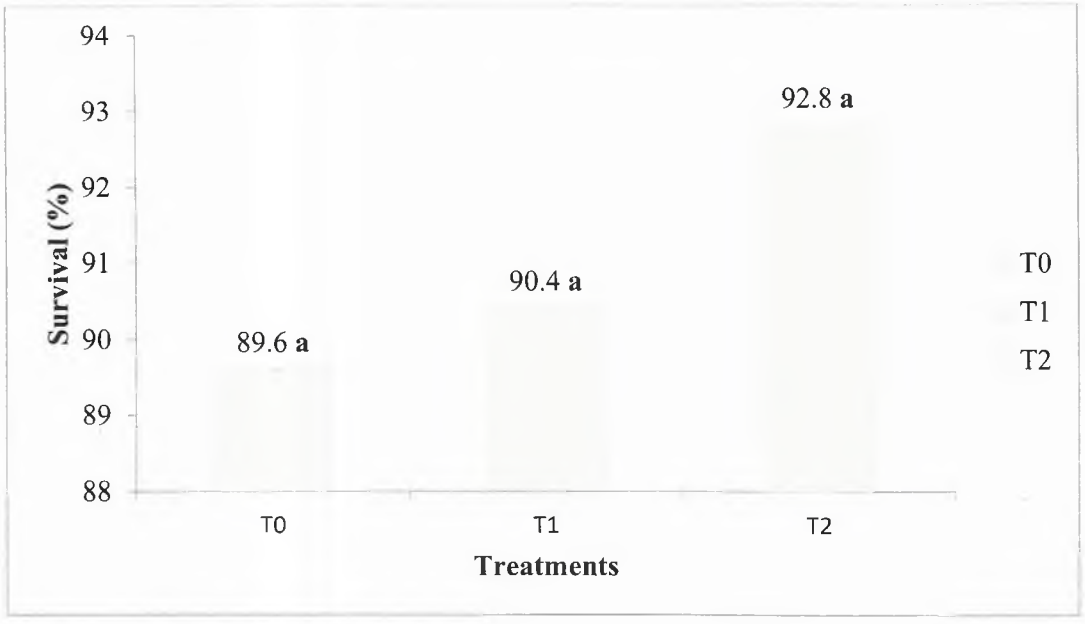

Fig 5: Survival (%) of *Labeo rohita* after 90 days feeding under different treatments and control group

Table 19 (a). Analysis of variance of mean survival from different treatments

Source of variation	Sum of squares (SSQ)	Degree of freedom (df)	Mean sum of square (MSSQ)	F- ratio
Between Groups	27.733	2	13.867	0.230*
Within Groups	723.200	12	60.267	
Total	750.933	14		

*Significant at 5 % level

Table 19 (b). Duncan's multiple range test

Treatments	Mean
T_0	89.60^a
T_1	90.40^a
T_2	92.80^a

Unlike superscripts indicate significant difference among treatments at 5% level.

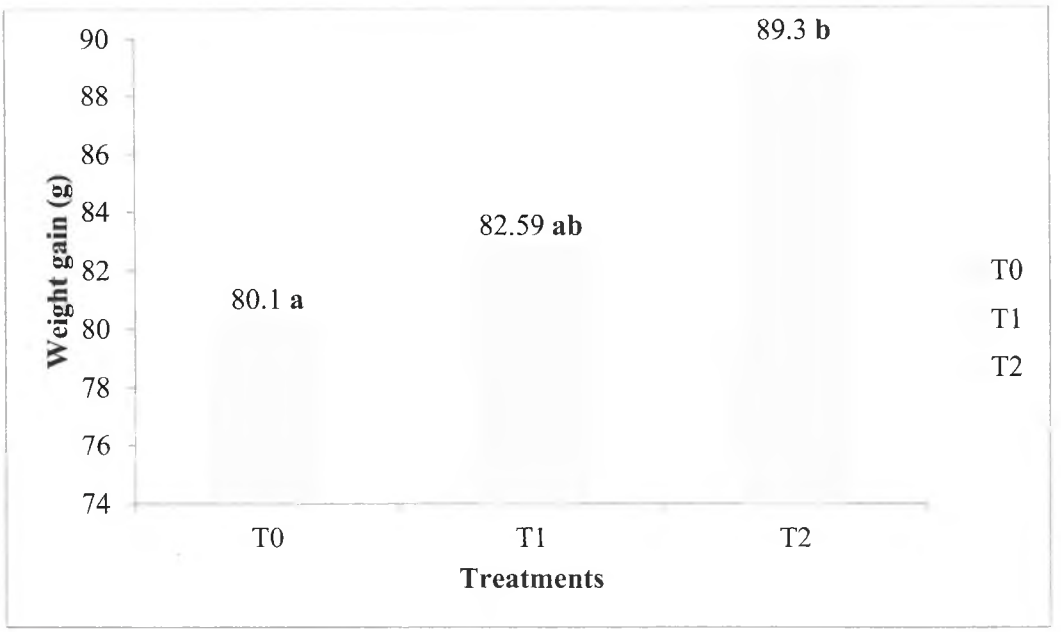

Fig 6: Mean weight gain of *Labeo rohita*

Table 20 (a). Analysis of variance of mean weight gain from different treatments

Source of variance	Sum of squares (SSQ)	Degree of freedom (df)	Mean sum of square (MSSQ)	F- ratio
Between Groups	226.334	2	113.167	3.930*
Within Groups	345.586	12	28.799	
Total	571.920	14		

*Significant at 5 % level

Table 20 (b). Duncan's multiple range test

Treatments	Mean
T_0	80.10^{a}
T_1	82.59^{ab}
T_2	89.30^{b}

Superscripts indicate significant difference among treatments at 5% level.

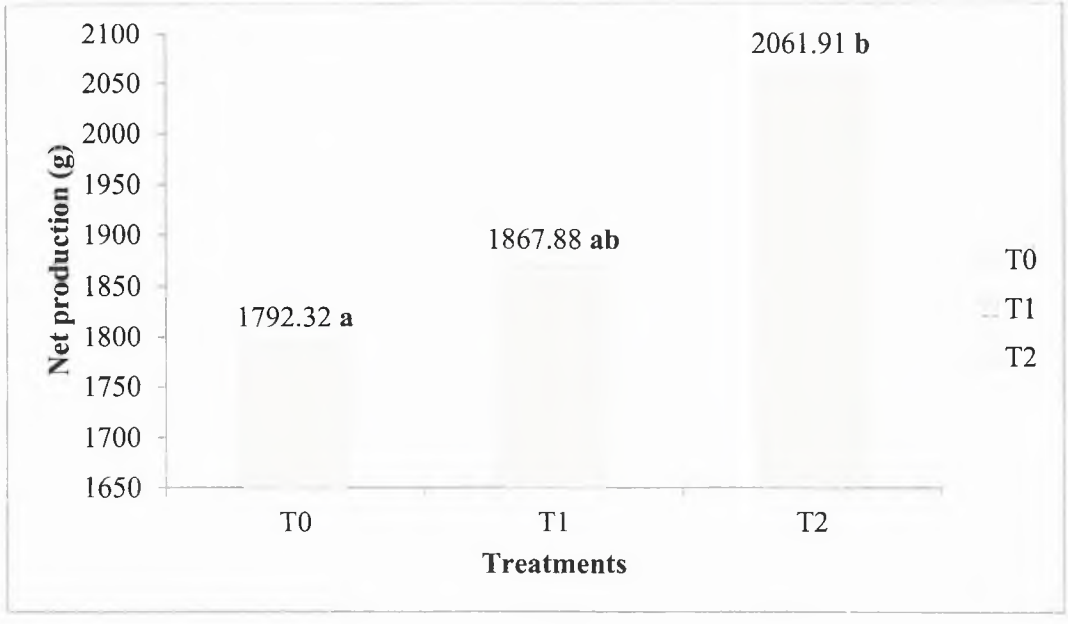

Fig 7: Mean net production of *Labeo rohita*

Table 21 (a). Analysis of variance of mean net production from different treatments

Source of variance	Sum of squares (SSQ)	Degree of freedom (df)	Mean sum of square (MSSQ)	F- ratio
Between Groups	193399.053	2	96699.526	4.120*
Within Groups	281670.705	12	23472.559	
Total	475069.757	14		

*Significant at 5 % level

Table 21 (b). Duncan's multiple range test

Treatments	Mean
T_0	1792.32^{a}
T_1	1867.88^{ab}
T_2	2061.91^{b}

Superscripts indicate significant difference among treatments at 5% level.

Table 22. Mean percentage of proximate composition of *L. rohita* in different treatments

Treatments	Replications	Parameter					
		Moisture	Dry matter	Crude protein	Crude fat	Ash	NFE
T_0	1	76.58	23.42	16.40	2.44	1.53	3.05
	2	77.10	22.90	16.22	2.38	1.30	3.00
	3	76.72	23.28	16.38	2.40	1.51	2.99
	4	76.79	23.21	16.33	2.40	1.47	3.01
	5	77.56	22.44	16.90	2.22	1.23	2.09
	Mean±SE	**76.95±0.17**	**23.05±0.17**	**16.45±0.12**	**2.37±0.04**	**1.41±0.06**	**2.83±0.18**
T_1	1	77.44	22.56	16.88	2.24	1.30	2.14
	2	77.40	22.60	17.02	2.18	1.26	2.14
	3	77.46	22.53	16.93	2.21	1.26	2.12
	4	76.98	23.02	17.15	2.38	1.31	2.18
	5	76.88	23.12	17.22	2.42	1.13	2.35
	Mean±SE	**77.23±0.12**	**22.77±0.13**	**17.04±0.06**	**2.29±0.05**	**1.25±0.03**	**2.19±0.04**
T_2	1	76.99	23.01	17.08	2.26	1.41	2.26
	2	76.95	23.05	17.15	2.35	1.28	2.26
	3	77.05	22.95	17.08	2.48	1.10	1.38
	4	76.75	23.25	17.42	2.50	1.51	1.82
	5	76.68	23.32	17.45	2.65	1.54	1.68
	Mean±SE	**76.88±0.07**	**23.12±0.07**	**17.24±0.08**	**2.45±0.07**	**1.37±0.08**	**1.88±0.17**

Table 23 (a). Analysis of variance of mean crude protein from different treatments

Source of variation	Sum of squares (SSQ)	Degree of freedom (df)	Mean sum of square (MSSQ)	F- ratio
Between Groups	1.692	2	0.846	20.494*
Within Groups	0.495	12	0.041	
Total	2.188	14		

*Significant at 5 % level

Table 23 (b). Duncan's multiple range test

Treatments	Mean
T_0	16.45^a
T_1	17.04^b
T_2	17.24^b

Superscripts indicate significant difference among treatments at 5% level.

Table 24 (a). Analysis of variance of mean crude fat from different treatments

Source of variation	Sum of squares (SSQ)	Degree of freedom (df)	Mean sum of square (MSSQ)	F- ratio
Between Groups	0.066	2	0.033	2.391*
Within Groups	0.165	12	0.014	
Total	0.230	14		

*Significant at 5 % level

Table 24 (b). Duncan's multiple range test

Treatments	Mean
T_0	2.37^a
T_1	2.29^a
T_2	2.45^a

Unlike superscripts indicate significant difference among treatments at 5% level.

Table 25. NBT-test of *Labeo rohita* in different treatments

Treatment	Replication	NBT-test
T_0	1	0.510
	2	0.531
	3	0.529
	4	0.523
	5	0.535
	Mean±SE	**0.53±0.0044**
T_1	1	0.725
	2	0.613
	3	0.624
	4	0.872
	5	0.654
	Mean±SE	**0.70±0.048**
T_2	1	1.132
	2	1.235
	3	0.897
	4	0.956
	5	1.141
	Mean±SE	**1.07±0.063**

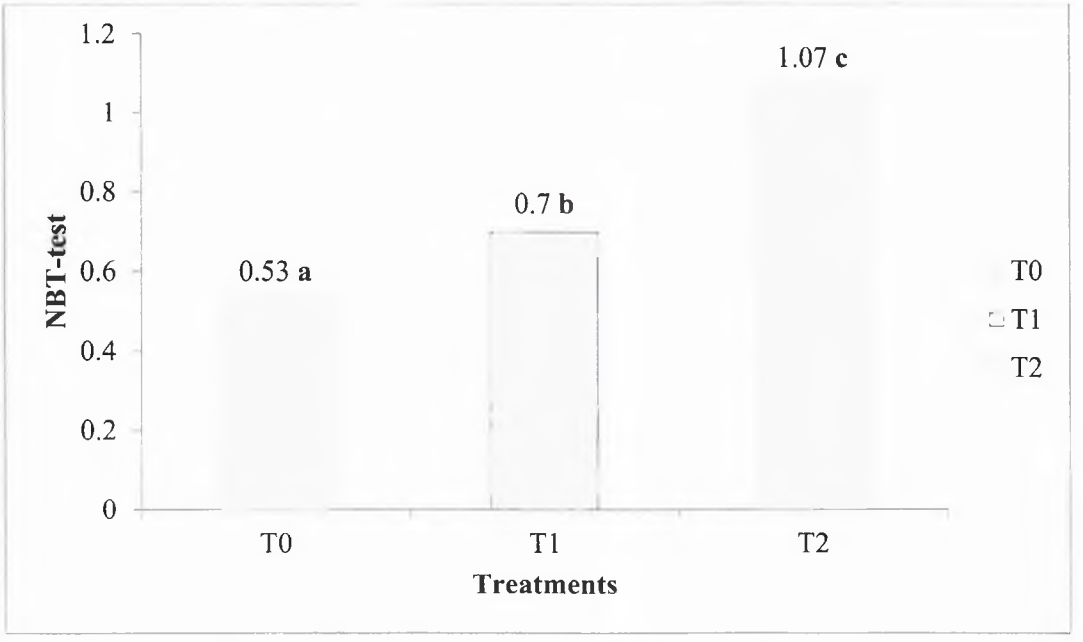

Fig 8. Super oxide anion production of *Labeo rohita* in different treatments and control group

Table 26 (a). Analysis of variance of NBT-test of *Labeo rohita* in different treatments

Source of variation	Sum of squares (SSQ)	Degree of freedom (df)	Mean sum of square (MSSQ)	F- ratio
Between Groups	0.781	2	0.391	37.485*
Within Groups	0.125	12	0.010	
Total	0.906	14		

*Significant at 5 % level

Table 26 (b). Duncan's multiple range test

Treatments	Mean
T_0	0.5256^a
T_1	0.6976^b
T_2	1.0722^c

Superscripts indicate significant difference among treatments at 5% level.

Table 27. Challenge studies of *Labeo rohita* with *A. hydrophila*

Mortality per day / Treatments	Replication	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	Total live after 7th day	Percentage survival after 7th day challenge
T_0	1	1	0	2	0	0	2	0	2/7	28.57
	2	1	0	1	0	0	1	1	3/7	42.85
	3	1	2	1	0	0	0	1	2/7	28.57
	Mean±SE	**1±0.00**	**0.67±0.67**	**1.33±0.33**	**0.00±0.00**	**0.00±0.00**	**1.00±0.58**	**0.67±0.33**	**2.33±0.33**	**33.33±4.76**
T_1	1	1	0	0	1	0	1	0	4/7	57.14
	2	0	1	0	1	0	0	1	4/7	57.14
	3	0	0	1	0	1	1	0	4/7	57.14
	Mean±SE	**0.33±0.33**	**0.33±0.33**	**0.33±0.33**	**0.67±0.33**	**0.33±0.33**	**0.67±0.33**	**0.33±0.33**	**4.00±0.00**	**57.14±0.00**
T_2	1	1	0	0	1	0	0	1	4/7	57.14
	2	0	1	0	1	0	0	0	5/7	71.42
	3	0	0	0	2	0	0	1	4/7	57.14
	Mean±SE	**0.33±0.33**	**0.33±0.33**	**0.00±0.00**	**1.33±0.33**	**0.00±0.00**	**0.00±0.00**	**0.67±0.33**	**4.33±0.33**	**61.91±4.76**

Table 28. Relative percent survival (RPS)

RPS / Treatments	Relative percent survival against control
T_1	35.71
T_2	42.86

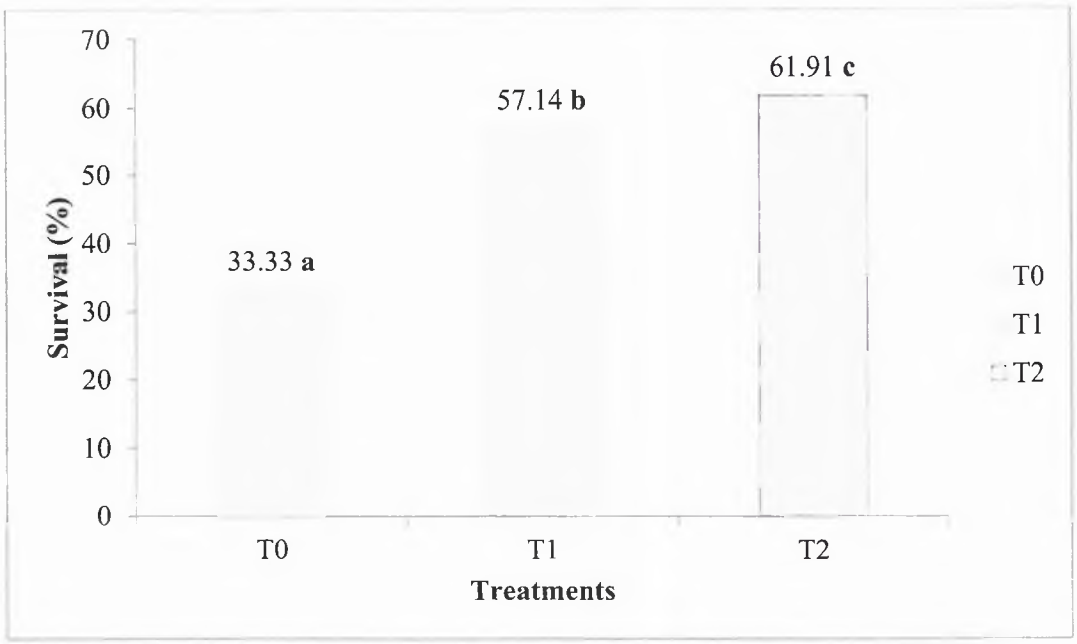

Fig 9. Cumulative percentage of survival of *Labeo rohita* recorded in different treatments and control group after challenging with *Aeromonas hydrophila*.

Table 29 (a). Analysis of variance of average survival (%) of Labeo rohita after challenge with *A. hydrophila* from different treatments

Source of variation	Sum of squares (SSQ)	Degree of freedom (df)	Mean sum of square (MSSQ)	F- ratio
Between Groups	1405.819	2	702.909	15.512*
Within Groups	271.891	6	45.315	
Total	1677.710	8		

*Significant at 5 % level

Table 29 (b). Duncan's multiple range test

Treatments	Mean
T_0	33.33^a
T_1	57.14^b
T_2	61.90^c

Superscripts indicate significant difference among treatments at 5% level.

4.9. Water quality parameters

4.9.1. Temperature

The fluctuation in air and water temperatures recorded during the experimental period are presented in the Table 30. Air temperature varied from 29.1 to 32.9°C and water temperature from 27.2 to 31.5°C.

4.9.2. pH

The pH was slightly alkaline throughout the study period in all the experimental tanks and ranging from a mean value of 7.44 to 7.64 in T_0, 7.52 to 7.92 in T_1 and 7.82 to 8.18 in T_2 (Table 31).

4.9.3. Dissolved oxygen

The dissolved oxygen content recorded on different sampling days is tabulated in Table 32 and average values are depicted in Fig 12. The average values of dissolved oxygen were 6.63 to 7.71 mg l^{-1} in T_0, 6.72 to 7.60 mg l^{-1} in T_1 and 6.72 to 8.11 mg l^{-1} in T_2.

4.9.4. Free carbon dioxide

The values of free carbon dioxide recorded over the experimental period are shown in Table 33 and Fig 13. The average values of free carbon dioxide were 0.15 to 0.42 mg l^{-1} in T_0, 0.24 to 0.61 mg l^{-1} in T_1 and 0.23 to 0.82 mg l^{-1} in T_2.

4.9.5. Ammonia-Nitrogen

The ammonia-nitrogen estimated during the experimental period is presented in Table 34 and the mean values presented in Fig 14. The average values of ammonia-

nitrogen ranged from 0.063 to 0.264 μg l^{-1} in T_0, 0.058 to 0.245 μg l^{-1} in T_1 and 0.049 to 0.327 μg l^{-1} in T_2.

4.9.6. Total alkalinity

The total alkalinity values recorded in the different tanks during the experimental period are presented in Table 35 and the average values are presented in Fig 15. The total alkalinity value recorded during the experimental period were 36.86 to 39.87 mg l^{-1} of $CaCO_3$ in treatment T_0, 37.77 to 45.85 mg l^{-1} of $CaCO_3$ in treatment T_1 and 44.74 to 52.14 mg l^{-1} of $CaCO_3$ in treatment T_2.

Table 30. Mean values of air and water temperature recorded during the experiment.

Days after stocking	Air temperature (°C)	Water temperature (°C)
0	29.5	28.5
15	32.4	31.0
30	29.1	27.2
45	32.9	31.5
60	31.4	29.9
75	29.5	29.0
90	32.2	31.5

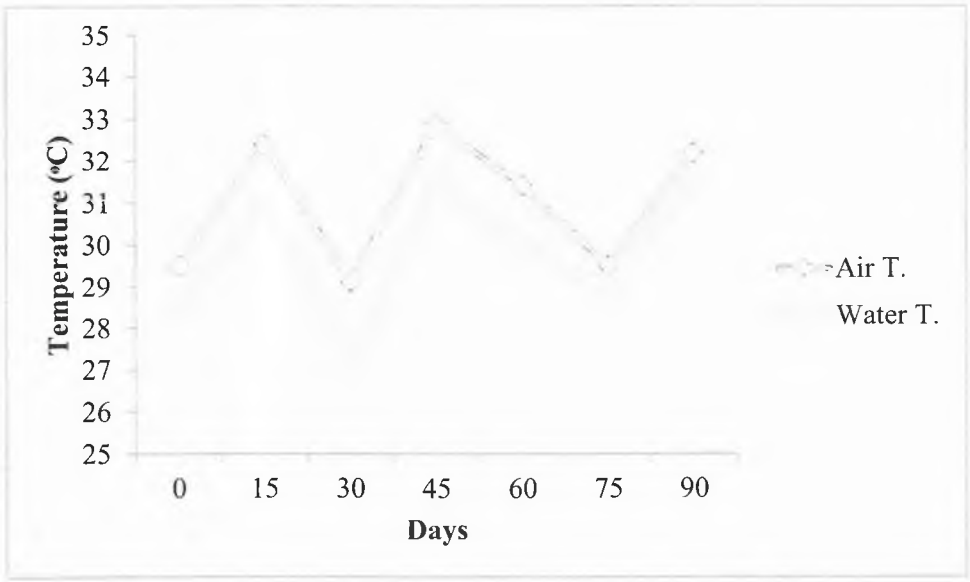

Fig 10. Profile of water and air temperatures recorded during different sampling days

Table 31. The pH of water recorded during different sampling days of the experimental period.

Treatment	Replication	Days						
		0	15	30	45	60	75	90
T_0	1	7.4	7.6	7.7	7.4	8.1	7.8	7.7
	2	7.5	7.3	7.3	7.4	7.6	7.5	7.5
	3	7.5	7.4	7.5	7.6	7.6	7.7	7.6
	4	7.4	7.4	7.5	7.4	7.7	7.6	7.6
	5	7.6	7.5	7.4	7.6	7.9	7.5	7.8
	Mean±SE	**7.48± 0.04**	**7.44± 0.05**	**7.48± 0.07**	**7.48± 0.05**	**7.78± 0.10**	**7.62± 0.06**	**7.64± 0.05**
T_1	1	7.6	7.7	7.9	7.5	7.7	7.6	8.1
	2	7.7	7.8	8.1	8.1	7.8	7.5	7.9
	3	7.6	7.7	7.7	7.8	7.6	7.4	7.6
	4	7.6	7.8	8.0	7.8	7.5	7.5	7.8
	5	7.5	7.8	7.9	7.7	7.6	7.6	7.9
	Mean±SE	**7.6±0 .03**	**7.76± 0.02**	**7.92± 0.07**	**7.78± 0.10**	**7.64± 0.05**	**7.52± 0.04**	**7.86± 0.08**
T_2	1	7.7	8.1	8.0	8.1	7.9	8.2	8.2
	2	7.9	7.9	8.3	8.0	8.4	8.2	8.0
	3	7.8	8.1	7.9	7.9	8.3	8.3	8.1
	4	7.8	8.0	8.1	8.2	8.0	8.0	8.1
	5	7.9	7.8	8.3	8.3	8.3	8.2	8.3
	Mean±SE	**7.82± 0.04**	**7.98± 0.06**	**8.12± 0.08**	**8.10± 0.08**	**8.18± 0.10**	**8.18± 0.05**	**8.14± 0.05**

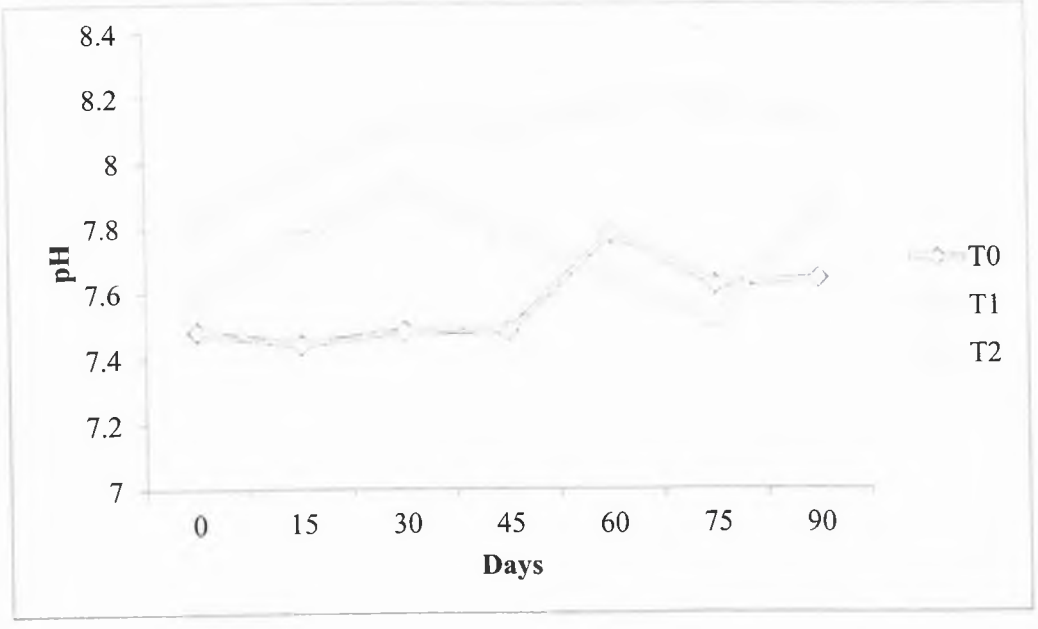

Fig 11. Fluctuations in pH in different treatments

Table 32. Dissolved oxygen (mg/l) level of water recorded during different sampling days.

Treatment	Replication	Days						
		0	15	30	45	60	75	90
T_0	1	6.80	7.20	7.80	7.06	7.20	7.50	7.80
	2	6.50	8.00	7.67	7.10	6.66	7.31	7.50
	3	6.00	7.20	7.66	7.46	7.40	7.80	6.80
	4	6.75	8.10	8.10	8.90	7.50	7.50	7.20
	5	7.10	7.80	7.30	7.70	6.80	7.30	7.20
	Mean±SE	**6.63± 0.18**	**7.66± 0.19**	**7.71± 0.13**	**7.64± 0.34**	**7.11± 0.16**	**7.48± 0.09**	**7.30± 0.17**
T_1	1	7.50	7.81	9.15	7.50	7.61	7.48	7.53
	2	6.40	7.70	6.50	6.80	7.80	7.70	7.80
	3	6.85	7.80	6.80	7.70	7.80	6.80	7.30
	4	6.15	5.81	7.15	7.80	7.40	7.80	6.80
	5	6.70	7.05	7.30	7.86	7.42	7.31	7.62
	Mean±SE	**6.72± 0.23**	**7.23± 0.38**	**7.38± 0.46**	**7.53± 0.19**	**7.60± 0.09**	**7.42± 0.18**	**7.41± 0.17**
T_2	1	6.80	7.83	7.61	8.80	7.31	7.20	7.20
	2	6.80	6.93	7.46	8.10	7.80	7.50	7.60
	3	6.98	7.55	7.20	7.86	7.90	7.40	7.80
	4	6.50	6.80	7.80	5.81	7.55	8.98	8.80
	5	6.50	6.83	7.53	8.49	7.56	8.01	9.15
	Mean±SE	**6.72± 0.09**	**7.19± 0.21**	**7.52± 0.10**	**7.81± 0.53**	**7.62± 0.10**	**7.82± 0.32**	**8.11± 0.37**

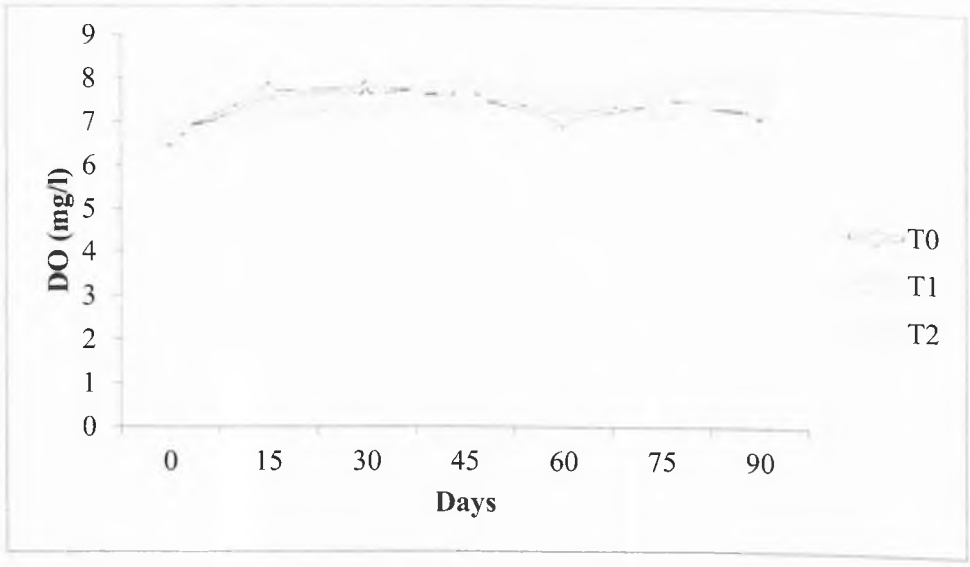

Fig 12. Dissolved oxygen (mg/l) recorded on different sampling days during the experimental period.

Table 33. Free carbon dioxide content of water (mg/l) recorded during different sampling days of the experiment.

Treatment	Replication	Days						
		0	15	30	45	60	75	90
T_0	1	0.15	0.27	0.25	0.65	0.15	0.17	0.34
	2	0.00	0.10	0.18	0.80	0.43	0.24	0.53
	3	0.20	0.25	0.10	0.10	0.50	0.32	0.28
	4	0.20	0.15	0.40	0.20	0.11	0.43	0.50
	5	1.00	0.00	0.50	0.35	0.25	0.32	0.35
	Mean±SE	**0.31± 0.18**	**0.15± 0.05**	**0.29± 0.07**	**0.42± 0.13**	**0.29± 0.08**	**0.30± 0.04**	**0.40± 0.05**
T_1	1	0.20	0.30	0.25	0.65	0.51	0.56	0.64
	2	0.00	0.22	0.12	0.13	0.10	0.07	0.82
	3	0.40	0.10	0.30	0.75	0.40	0.76	0.43
	4	0.35	0.15	0.45	1.00	0.12	0.59	0.37
	5	0.25	0.70	0.40	0.50	0.75	0.62	0.58
	Mean±SE	**0.24± 0.07**	**0.29± 0.11**	**0.30± 0.06**	**0.61± 0.14**	**0.38± 0.12**	**0.52± 0.12**	**0.57± 0.08**
T_2	1	0.50	1.00	0.25	0.14	0.19	0.89	0.51
	2	0.30	0.00	0.10	0.10	0.75	0.95	0.56
	3	0.08	0.30	0.18	0.35	0.50	0.82	0.49
	4	0.24	0.78	0.28	0.38	1.20	0.76	0.32
	5	0.41	0.67	0.32	0.56	0.20	0.69	0.87
	Mean±SE	**0.31± 0.07**	**0.55± 0.18**	**0.23± 0.04**	**0.31± 0.08**	**0.57± 0.19**	**0.82± 0.05**	**0.55± 0.09**

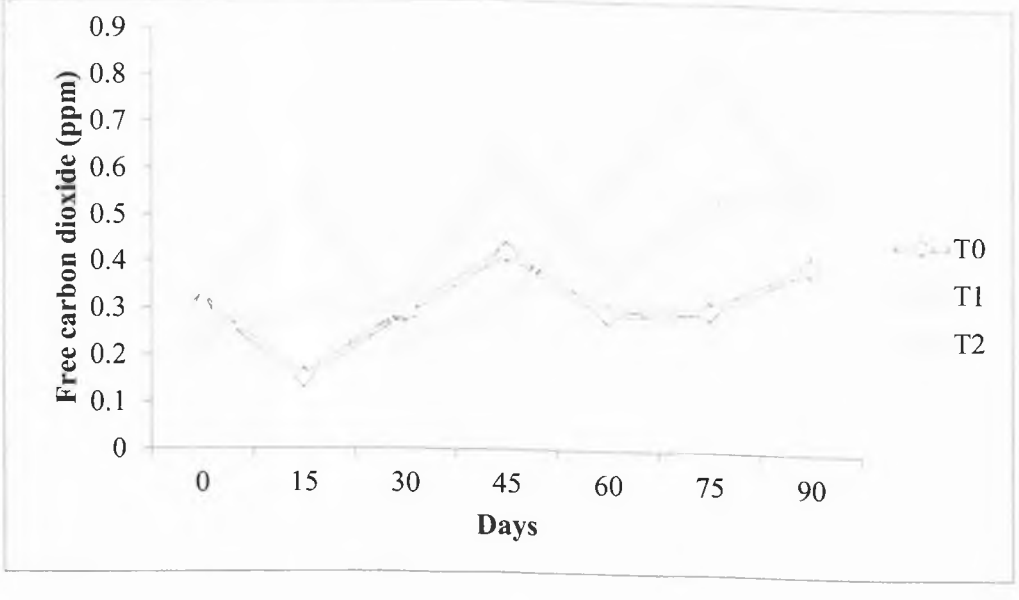

Fig 13. Free Carbon Dioxide profile of water (mg/l) recorded during different sampling days

Table 34. Ammonia (µg-at NH_3-N/l) of water recorded during different sampling days of the experiment.

Treatment	Replication	Days						
		0	15	30	45	60	75	90
T_0	1	0.052	0.360	0.234	0.167	0.265	0.249	0.174
	2	0.047	0.122	0.178	0.125	0.290	0.312	0.236
	3	0.062	0.471	0.185	0.173	0.201	0.270	0.198
	4	0.053	0.317	0.252	0.176	0.202	0.215	0.197
	5	0.100	0.050	0.234	0.023	0.160	0.120	0.160
	Mean±SE	**0.063± 0.010**	**0.264± 0.078**	**0.217± 0.014**	**0.133± 0.029**	**0.224± 0.024**	**0.233± 0.032**	**0.193± 0.013**
T_1	1	0.087	0.176	0.189	0.231	0.062	0.304	0.181
	2	0.046	0.138	0.102	0.146	0.227	0.287	0.242
	3	0.053	0.231	0.572	0.121	0.166	0.242	0.161
	4	0.072	0.190	0.154	0.260	0.280	0.167	0.138
	5	0.030	0.150	0.195	0.220	0.240	0.224	0.143
	Mean±SE	**0.058± 0.010**	**0.177± 0.016**	**0.242± 0.084**	**0.196± 0.026**	**0.195± 0.038**	**0.245± 0.024**	**0.173± 0.019**
T_2	1	0.074	0.238	0.276	0.177	0.204	0.270	0.435
	2	0.052	0.189	0.232	0.135	0.160	0.206	0.211
	3	0.043	0.101	0.197	0.121	0.220	0.256	0.374
	4	0.018	0.193	0.244	0.131	0.178	0.281	0.274
	5	0.056	0.176	0.235	0.145	0.197	0.263	0.340
	Mean±SE	**0.049± 0.009**	**0.179± 0.022**	**0.237± 0.013**	**0.141± 0.010**	**0.192± 0.010**	**0.255± 0.013**	**0.327± 0.039**

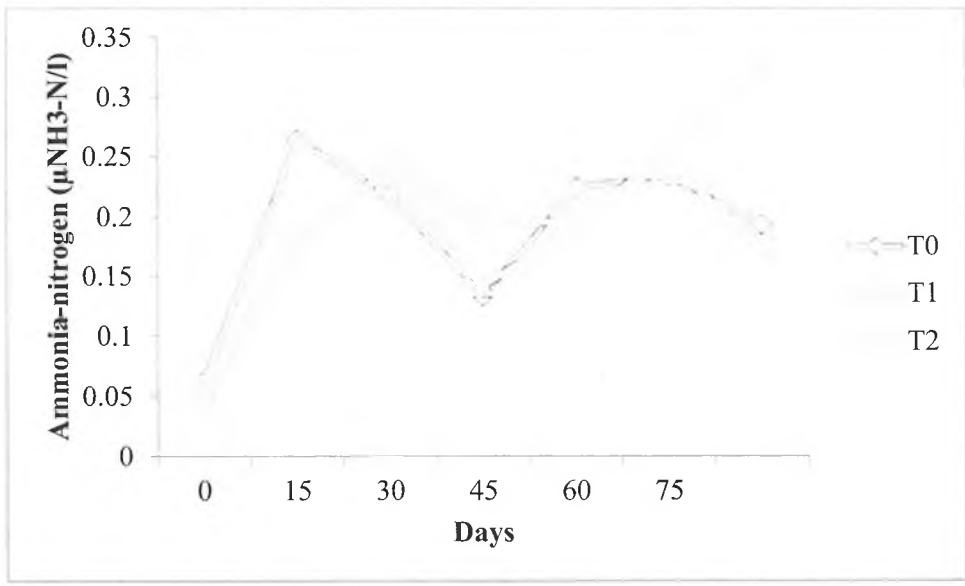

Fig 14. Ammonia (µg-at NH_3-N/l) of water recorded during different sampling days

Table 35. Total alkalinity of water (mg/l) recorded during different sampling days of the experiment.

Treatment	Replication	Days						
		0	15	30	45	60	75	90
T_0	1	35.60	40.00	37.82	38.75	38.55	40.00	36.40
	2	38.00	38.45	40.50	36.52	32.66	39.40	44.00
	3	42.00	37.82	35.00	40.58	38.69	40.50	39.60
	4	37.00	36.00	39.00	38.50	38.40	39.97	32.66
	5	46.75	39.87	42.50	40.75	36.00	36.15	38.15
	Mean±SE	**39.87 ±2.02**	**38.43 ±0.74**	**38.96 ±1.26**	**39.02 ±0.78**	**36.86 ±1.16**	**39.20 ±0.78**	**38.16 ±1.86**
T_1	1	36.65	39.25	37.00	36.00	38.40	39.87	48.86
	2	38.98	41.26	40.50	30.00	36.40	45.28	42.21
	3	42.45	40.12	38.50	45.08	44.00	43.07	43.65
	4	45.72	38.32	32.00	42.25	39.60	43.45	43.21
	5	41.00	45.35	40.83	43.50	41.36	49.12	51.32
	Mean±SE	**40.96 ±1.54**	**40.86 ±1.22**	**37.77 ±1.60**	**39.37 ±2.80**	**39.95 ±1.29**	**44.16 ±1.52**	**45.85 ±1.79**
T_2	1	50.26	49.50	38.15	51.00	53.05	50.25	47.43
	2	45.89	47.10	36.52	38.75	49.23	51.12	47.85
	3	41.70	45.33	52.23	52.83	52.46	50.00	49.50
	4	41.20	46.50	52.48	54.32	57.48	49.63	54.16
	5	44.65	48.00	53.25	50.16	48.50	48.40	50.38
	Mean±SE	**44.74 ±1.64**	**47.29 ±0.70**	**46.53 ±3.76**	**49.41 ±2.76**	**52.14 ±1.60**	**49.88 ±0.44**	**49.86 ±1.20**

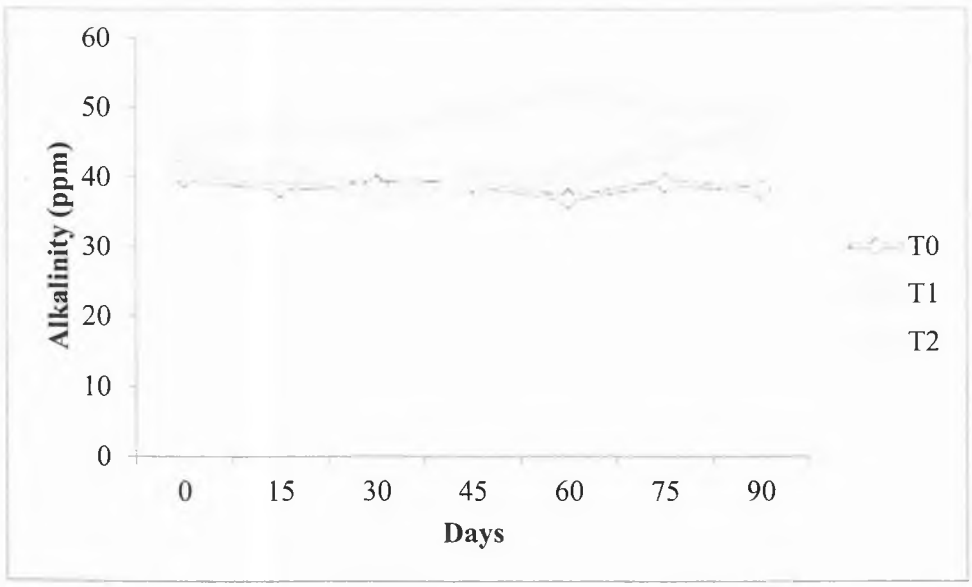

Fig 15. Total alkalinity Profile of water (mg/l) recorded during different sampling days

Experiment-2

4.10. Growth and length trial

The average weight and length of rohu juveniles over the experimental period of 60 days are given in Table 36 and 38 respectively. The best growth was obtained in rohu fed 0.48% methionine diet, followed by 0.08% methionine feed group and control. The final mean weights were 314.00 g, 372.00 g, 412.00 g in rohu receiving the control diet, 0.08% and 0.48% methionine diet respectively, and the corresponding final mean lengths were 29.08cm, 30.81cm and 30.62cm.

4.11. Specific growth rate

Specific growth rate (%/day) of fish is presented in Table 40. There was significant difference ($P<0.05$) in specific growth rate of fish in the study (Table 41). The highest SGR of 2.25% per day in T_2 (0.48% methionine incorporated diet) followed by 2.10% in T_1 (0.08% methionine) diet and 1.69% in T_0 (control diet).

4.12. Feed conversion ratio

The values of food conversion ratio are presented in Table 42. The lowest and best FCR was observed in diet containing 0.48% methionine in T_2 (1.01) followed by T_1 (1.03) containing 0.08% methionine and in T_0 (1.20) which served as control.

4.13. Survival rate, weight gain and net production

The data on survival, weight gain and net production is given in Table 44. Net production was highest in T_2 treatment where the growth was also maximum.

The average net productions were 1995.80 g, 2658.00 g and 3054.00 g with control, 0.08% and 0.48% of methionine diets respectively. The corresponding in mean weight gain were 199.58 g, 265.80 g and 305.40 g.

4.14. Proximate composition of fish muscle

The result of proximate composition of rohu muscle carried out at the end of the experiment is given in Table 47. Both protein and fat compositions of fish muscle were significant ($P<0.05$) in treatment groups compare to control group (Table 48 and 49).

Maximum mean moisture content was recorded in T_0 (75.79%) followed by T_1 (74.26%) and minimum in T_2 (73.01%). The maximum mean protein content of fish muscle of 14.77% was observed in T_2 followed by 14.37% in T_1 and 13.61% in T_0. The crude fat value of fish muscle was 3.10% in T_2 followed by 2.73% in T_1 and 2.66% in T_0. Mean ash levels in fish muscle were 2.22% in T_2 followed by 2.21% in T_1 and 2.10% in T_0. The highest mean NFE was recorded in T_2 (6.89%) followed by T_1 (6.43%) and T_0 (5.84%).

4.15. Super oxide anion production (NBT assay)

NBT was carried out at a wave length of 650 nm and the results are presented in Table 50 and 51. Super oxide anion production of fish fed 0.08% and 0.48% methionine supplemented diets was higher than that of fish fed control diet. The highest super oxide anion production of was recorded in T_2 followed by in T_1 and T_0. Super oxide anion production of fish recorded in different treatments were significantly ($P > 0.05$) higher than that of control group.

Table 36. Weight attained by *Labeo rohita* juveniles in different treatments

Treatment	Replication	Days				
		0	15	30	45	60
T_0	1	121.60	202.69	289.00	300.00	340.00
	2	117.80	184.61	250.40	280.00	320.00
	3	115.00	182.67	250.00	280.00	310.00
	4	119.70	160.00	230.00	250.00	310.00
	5	98.00	110.00	182.70	240.00	290.00
	Mean±SE	**114.42 ±4.25**	**167.99 ±16.00**	**240.42 ±17.31**	**270.00 ±10.95**	**314.00 ±8.12**
T_1	1	109.00	190.00	241.00	310.00	360.00
	2	120.00	200.00	276.00	327.00	370.00
	3	112.00	230.00	290.00	335.00	380.00
	4	80.00	190.00	214.00	287.00	340.00
	5	110.00	250.00	300.00	359.00	410.00
	Mean±SE	**106.20 ±6.83**	**212.00 ±12.00**	**264.20 ±16.04**	**323.60 ±12.08**	**372.00 ±11.58**
T_2	1	110.00	200.00	318.00	350.00	380.00
	2	98.00	190.00	248.00	310.00	370.00
	3	110.00	270.00	320.00	365.00	410.00
	4	98.00	230.00	310.00	360.00	400.00
	5	117.00	280.00	350.00	430.00	500.00
	Mean±SE	**106.60 ±3.74**	**234.00 ±40.37**	**309.20 ±16.74**	**363.00 ±19.34**	**412.00 ±23.10**

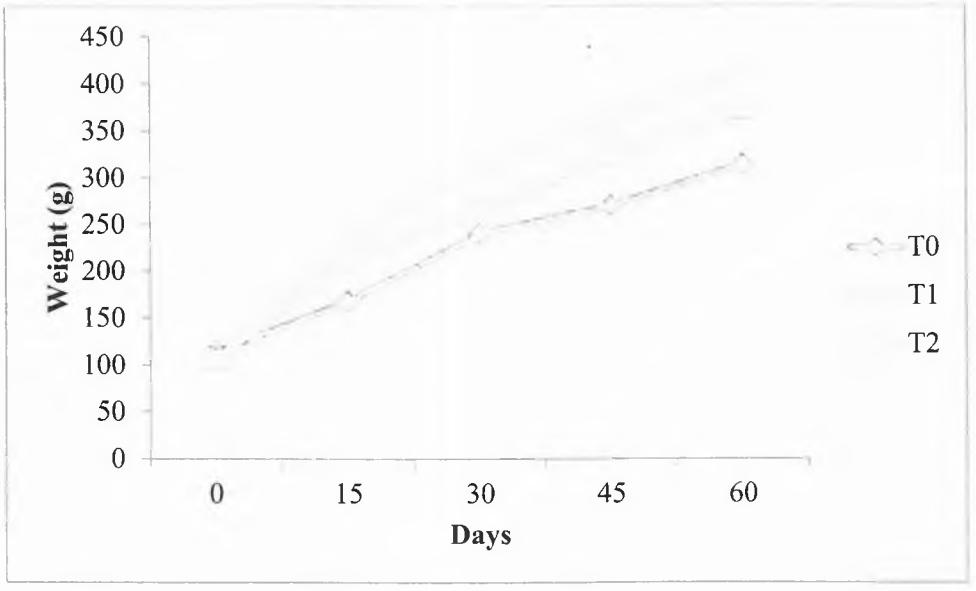

Fig 16. Mean weight (g) of *Labeo rohita* in different treatments

Table 37 (a). Analysis of variance of final mean weight (g) of *L. rohita* from different treatments

Source of variation	Sum of squares (SSQ)	Degree of freedom (df)	Mean sum of square (MSSQ)	F- ratio
Between Groups	24280.000	2	12140.000	9.924*
Within Groups	14680.000	12	1223.333	
Total	38960.000	14		

*Significant at 5 % level

Table 37 (b). Duncan's multiple range test

Treatments	Mean
T_0	314.00^a
T_1	372.00^b
T_2	412.00^b

Superscripts indicate significant difference among treatments at 5% level.

Table 38. Length (cm) of *Labeo rohita* juveniles in the different treatments

Treatment	Replication	Days				
		0	15	30	45	60
T_0	1	22.72	25.09	27.40	28.80	30.60
	2	22.66	24.30	25.88	26.90	29.40
	3	22.40	23.89	25.58	26.20	28.60
	4	22.69	22.84	25.02	27.20	29.00
	5	21.60	22.40	24.00	25.20	27.80
	Mean±SE	**22.41±0.21**	**23.70±0.49**	**25.58±0.56**	**26.86±0.59**	**29.08±0.46**
T_1	1	21.90	23.96	27.48	29.55	31.23
	2	22.30	23.94	26.48	28.96	30.90
	3	22.70	23.96	25.98	28.25	30.43
	4	20.70	23.42	24.54	27.43	30.14
	5	22.40	26.35	27.67	29.87	31.35
	Mean±SE	**22.00±0.35**	**24.33±0.52**	**26.43±0.57**	**28.81±0.44**	**30.81±0.23**
T_2	1	21.50	24.25	27.40	28.75	30.10
	2	18.90	22.12	23.54	26.96	29.70
	3	22.30	25.84	26.87	29.35	31.40
	4	21.54	25.46	27.76	29.85	31.20
	5	23.30	25.67	27.64	29.77	30.70
	Mean±SE	**21.51±0.73**	**24.67±0.70**	**26.64±0.79**	**28.94±0.53**	**30.62±0.32**

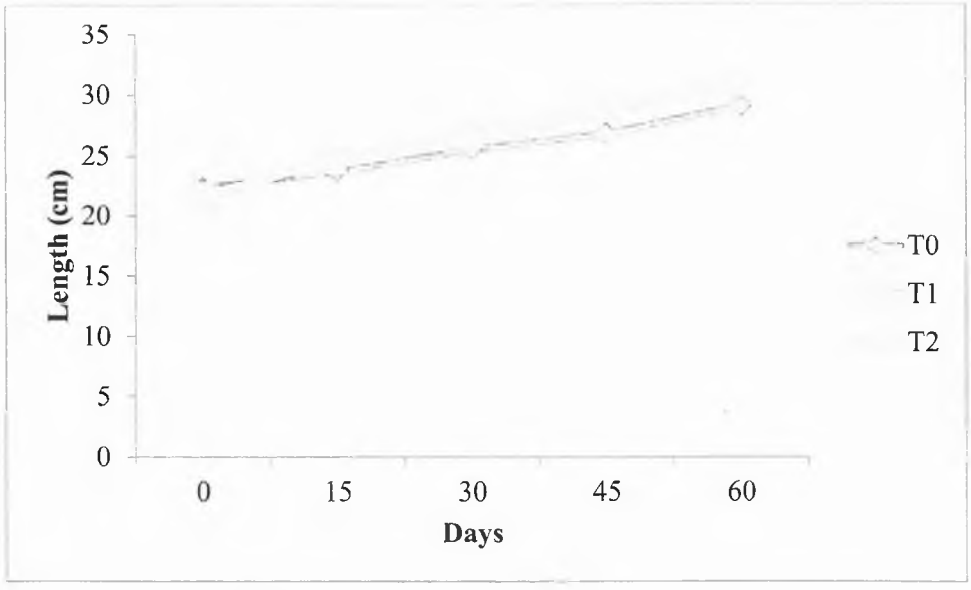

Fig 17. Mean length (cm) of *Labeo rohita* in different treatments

Table 39 (a). Analysis of variance of final average length (cm) of *L. rohita* from different treatments

Source of variation	Sum of squares (SSQ)	Degree of freedom (df)	Mean sum of square (MSSQ)	F- ratio
Between Groups	9.196	2	4.598	7.255*
Within Groups	7.605	12	0.634	
Total	16.801	14		

*Significant at 5 % level

Table 39 (b). Duncan's multiple range test

Treatments	Mean
T_0	29.08^a
T_1	30.62^b
T_2	30.84^b

Superscripts indicate significant difference among treatments at 5% level.

Table 40. Specific growth rate (%/day) of *Labeo rohita* in different treatments.

Treatment	Replication	SGR (%)
T_0	1	1.71
	2	1.67
	3	1.65
	4	1.59
	5	1.81
	Mean±SE	**1.69±0.04**
T_1	1	1.99
	2	1.88
	3	2.04
	4	2.41
	5	2.19
	Mean±SE	**2.10±0.09**
T_2	1	2.07
	2	2.21
	3	2.19
	4	2.34
	5	2.42
	Mean±SE	**2.25±0.06**

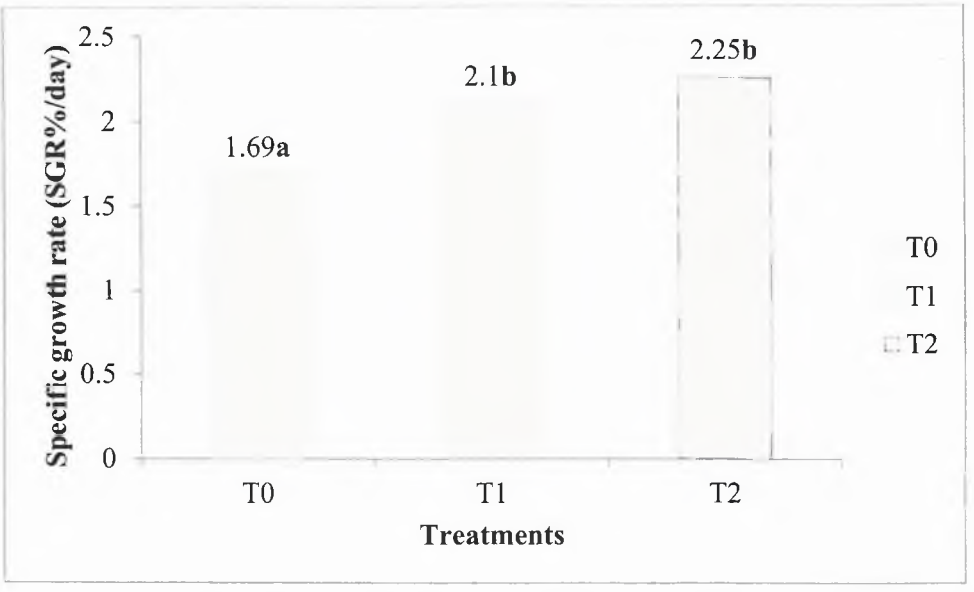

Fig 18. Specific growth rate (SGR) of fish recorded under different treatments and control group

Table 41 (a). Analysis of variance of mean SGR values from different treatments

Source of variation	Sum of squares (SSQ)	Degree of freedom (df)	Mean sum of square (MSSQ)	F- ratio
Between Groups	0.846	2	0.423	18.826*
Within Groups	0.270	12	0.022	
Total	1.115	14		

*Significant at 5 % level

Table 41 (b). Duncan's multiple range test

Treatments	Mean
T_0	1.69^a
T_1	2.10^b
T_2	2.25^b

Superscripts indicate significant difference among treatments at 5% level.

Table 42. Feed conversion ratio (FCR) of fish recorded after 60 days feeding trial in different treatments and control group

Treatment	Replication	FCR %
T_0	1	1.27
	2	1.25
	3	1.28
	4	1.21
	5	1.00
	Mean±SE	**1.20±0.05**
T_1	1	1.03
	2	1.12
	3	1.09
	4	0.90
	5	1.03
	Mean±SE	**1.03±0.03**
T_2	1	1.10
	2	0.94
	3	1.08
	4	1.00
	5	0.93
	Mean±SE	**1.01±0.03**

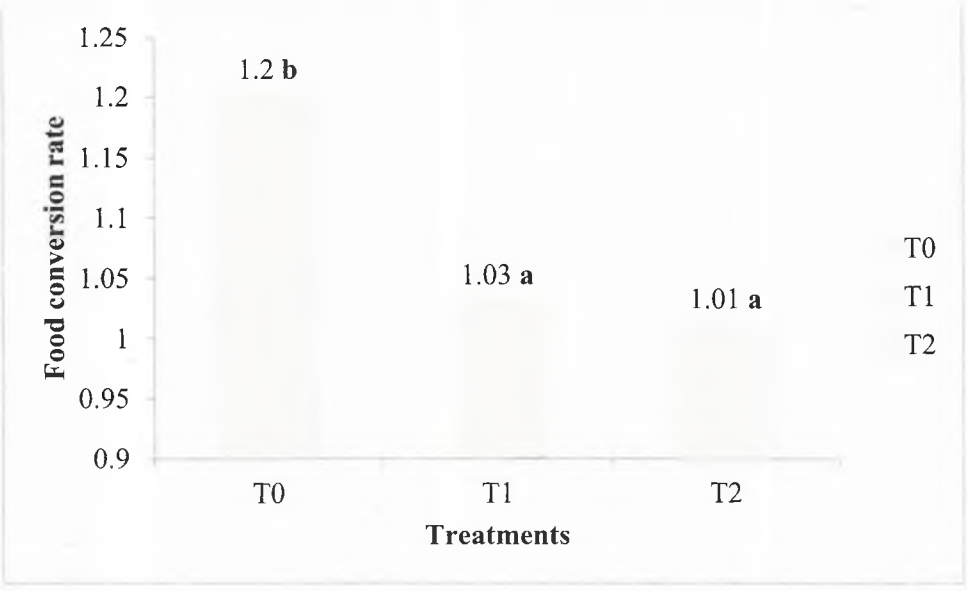

Fig 19. Feed conversion rate (FCR) of fish recorded under different treatments and control group

Table 43 (a). Anaysis of variance of mean FCR values from different treatments

Source of variation	Sum of squares (SSQ)	Degree of freedom (df)	Mean sum of square (MSSQ)	F- ratio
Between Groups	0.109	2	0.055	6.148*
Within Groups	0.107	12	0.009	
Total	0.216	14		

*Significant at 5 % level

Table 43 (b). Duncan's multiple range test

Treatments	Mean
T_0	1.20^b
T_1	1.03^a
T_2	1.01^a

Superscripts indicate significant difference among treatments at 5% level.

Table 44. Survival (%), weight gain and net production of *Labeo rohita* juveniles in different treatments

Treatment	Replication	No. survived	Survival %	Weight gain (g)	Net production (g/60 days)
T_0	1	10	100	218.40	2184.00
	2	10	100	202.20	2022.00
	3	10	100	195.00	1950.00
	4	10	100	190.30	1903.00
	5	10	100	192.00	1920.00
	Mean±SE	**10±0**	**100±0**	**199.58±5.13**	**1995.80±51.26**
T_1	1	10	100	251.00	2510.00
	2	10	100	250.00	2500.00
	3	10	100	268.00	2680.00
	4	10	100	260.00	2600.00
	5	10	100	300.00	3000.00
	Mean±SE	**10±0**	**100±0**	**265.80±9.16**	**2658±91.56**
T_2	1	10	100	270.00	2700.00
	2	10	100	272.00	2720.00
	3	10	100	300.00	3000.00
	4	10	100	302.00	3020.00
	5	10	100	383.00	3830.00
	Mean±SE	**10±0**	**100±0**	**305.40±20.53**	**3054±205.32**

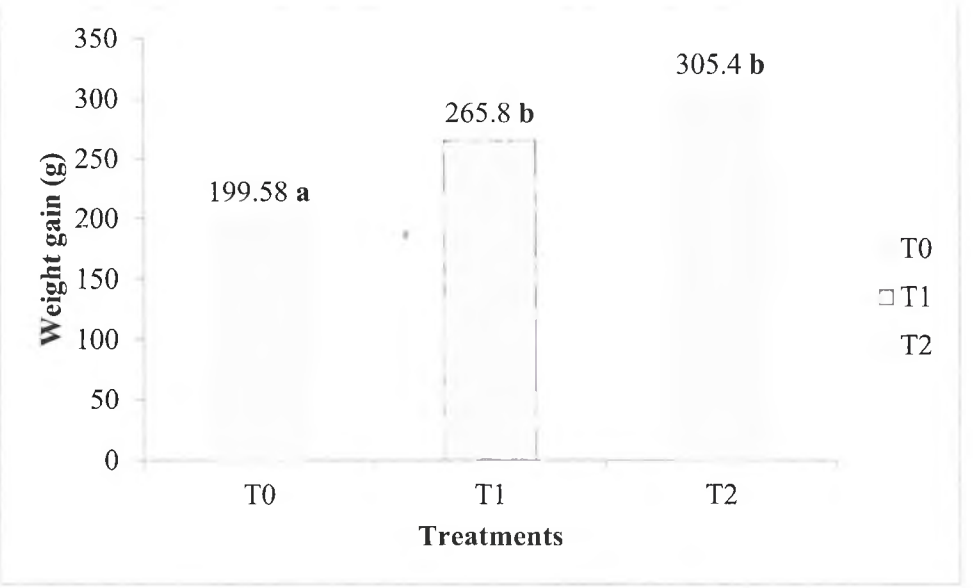

Fig 20. Mean weight gain of *Labeo rohita*

Table 45 (a). Analysis of variance of mean weight gain from different treatments

Source of variation	Sum of squares (SSQ)	Degree of freedom (df)	Mean sum of square (MSSQ)	F- ratio
Between Groups	28585.201	2	14292.601	16.129*
Within Groups	10633.608	12	886.134	
Total	39218.809	14		

*Significant at 5 % level

Table 45 (b). Duncan's multiple range tests

Treatments	Mean
T_0	199.58^a
T_1	265.80^b
T_2	305.40^b

Superscripts indicate significant difference among treatments at 5% level.

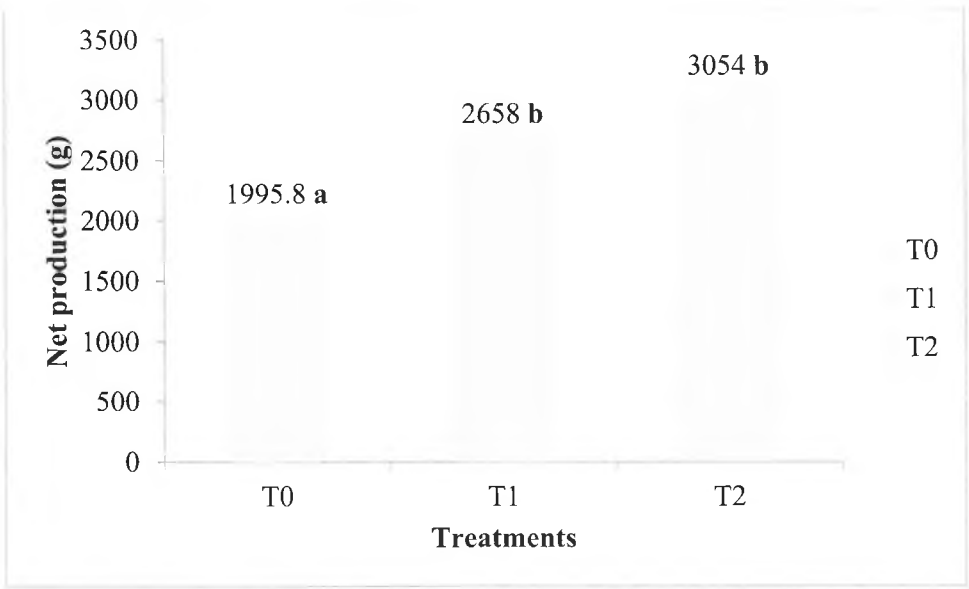

Fig 21. Mean net production of *Labeo rohita*

Table 46 (a). Analysis of variance of mean net production from different treatments

Source of variation	Sum of squares (SSQ)	Degree of freedom (df)	Mean sum of square (MSSQ)	F- ratio
Between Groups	2858520.133	2	1429260.067	16.129*
Within Groups	1063360.800	12	88613.400	
Total	3921880.933	14		

*Significant at 5 % level

Table 46 (b). Duncan's multiple range tests

Treatments	Mean
T_0	1995.80^a
T_1	2658.00^b
T_2	3054.00^b

Superscripts indicate significant difference among treatments at 5% level.

Table 47. Mean muscle proximate composition (%) of *L. rohita* in different treatments

Treatments	Replications	Parameter					
		Moisture	Dry matter	Crude protein	Crude fat	Ash	NFE
T_0	1	75.73	24.27	13.15	2.79	2.06	6.27
	2	76.17	23.83	13.91	2.63	2.03	5.26
	3	75.77	24.23	13.33	2.69	2.05	6.16
	4	75.89	24.11	13.46	2.71	2.05	5.89
	5	75.39	24.61	14.22	2.47	2.31	5.61
	Mean±SE	**75.79±0.13**	**24.21±0.13**	**13.61±0.20**	**2.66±0.05**	**2.10±0.05**	**5.84±0.18**
T_1	1	74.79	25.21	14.29	2.55	2.27	6.10
	2	73.91	26.09	14.61	2.97	2.17	6.34
	3	74.25	25.75	14.37	2.85	2.11	6.42
	4	73.68	26.32	14.44	2.79	2.19	6.90
	5	74.66	25.34	14.15	2.51	2.29	6.39
	Mean±SE	**74.26±0.21**	**25.74±0.21**	**14.37±0.08**	**2.73±0.09**	**2.21±0.03**	**6.43±0.13**
T_2	1	73.12	26.88	14.92	3.17	2.24	6.55
	2	72.50	27.50	14.74	3.19	2.21	7.36
	3	72.74	27.26	14.88	3.12	2.25	7.01
	4	72.80	27.20	14.84	3.16	2.23	6.97
	5	73.93	26.07	14.49	2.87	2.15	6.56
	Mean±SE	**73.01±0.25**	**26.98±0.25**	**14.77±0.08**	**3.10±0.06**	**2.22±0.02**	**6.89±0.15**

Table 48 (a). Analysis of variance of mean crude protein from different treatments

Source of variation	Sum of squares (SSQ)	Degree of freedom (df)	Mean sum of square (MSSQ)	F- ratio
[illegible]	3.470	2	1.735	20.601*

Table 50. NBT-test of *Labeo rohita* in different treatments

Treatment	Replication	NBT-test
	1	0.531
	2	0.510
		0.523
		0.513

Table 50. NBT-test of *Labeo rohita* in different treatments

Treatment	Replication	NBT-test
T_0	1	0.531
	2	0.510
	3	0.523
	4	0.613
	5	0.529
	Mean±SE	**0.54±0.01**
T_1	1	0.624
	2	1.056
	3	1.235
	4	1.141
	5	1.523
	Mean±SE	**1.12±0.15**
T_2	1	1.872
	2	1.654
	3	1.568
	4	1.698
	5	1.545
	Mean±SE	**1.67±0.06**

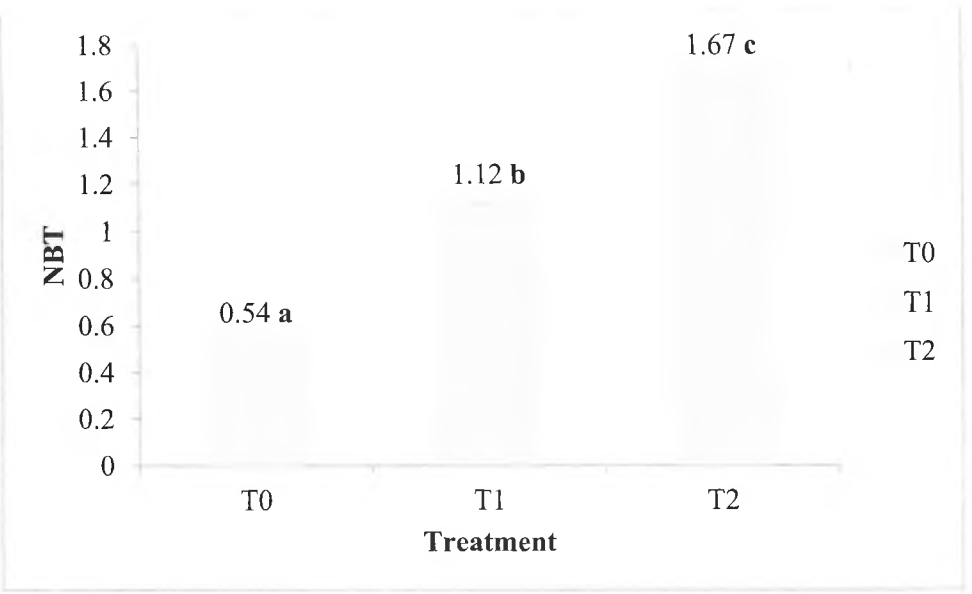

Fig 22. Super oxide anion production of *Labeo rohita* in different treatments and control group

Table 51 (a). Analysis of variance of NBT from different treatments

Source of variation	Sum of squares (SSQ)	Degree of freedom (df)	Mean sum of square (MSSQ)	F- ratio
Between Groups	3.171	2	1.586	38.006*
Within Groups	0.501	12	0.042	
Total	3.672	14		

*Significant at 5 % level

Table 51 (b). Duncan's multiple range test

Treatments	Mean
T_0	0.54^a
T_1	1.12^b
T_2	1.67^c

Superscripts indicate significant difference among treatments at 5% level.

4.16. Water quality parameters

4.16.1. Temperature

The fluctuation in air and water temperatures recorded during the experimental period are presented in the Table 52. Air temperature varied from 29.1 to 32.9°C and water temperature from 28.5 to 31.5°C.

4.16.2. pH

The pH was slightly alkaline throughout the study period in all the experimental tanks and ranging from a mean value of 7.40 to 7.62 in T_0, 7.52 to 7.92 in T_1 and 7.82 to 8.18 in T_2 (Table 53).

4.16.3. Dissolved oxygen

The dissolved oxygen content recorded on different sampling days is tabulated in Table 54 and average values are presented in Fig 25. The average values of dissolved oxygen were 6.68 to 7.70 mg l^{-1} in T_0, 6.67 to 7.89 mg l^{-1} in T_1 and 6.80 to 7.94 mg l^{-1} in T_2.

4.16.4. Free carbon dioxide

The values of free carbon dioxide recorded over the experimental period are shown in Table 55 and Fig 26. The average values of free carbon dioxide were 0.18 to 0.30 mg l^{-1} in T_0, 0.33 to 0.49 mg l^{-1} in T_1 and 0.23 to 0.49 mg l^{-1} in T_2.

4.16.5. Ammonia-Nitrogen

The ammonia-nitrogen estimated during the experimental period is presented in Table 56 and the mean values are depicted in Fig 27. The average values of ammonia-

nitrogen ranged from 0.063 to 0.264 μg l^{-1} in T_0, 0.058 to 0.238 μg l^{-1} in T_1 and 0.049 to 0.187 μg l^{-1} in T_2.

4.16.6. Total alkalinity

The total alkalinity values recorded in the different tanks during the experimental period are presented in Table 57 and the average values are presented in Fig 28. The total alkalinity value recorded during the experimental period were 39.03 to 42.27 mg l^{-1} of $CaCO_3$ in treatment T_0, 41.66 to 48.81 mg l^{-1} of $CaCO_3$ in treatment T_1 and 40.03 to 49.05 mg l^{-1} of $CaCO_3$ in treatment T_2.

Table 52. Mean values of air and water temperature recorded during the experimental period.

Days after stocking	Air temperature (°C)	Water temperature (°C)
0	30.4	28.5
15	32.9	31.5
30	32.2	31.4
45	31.2	30.1
60	30.5	29.5

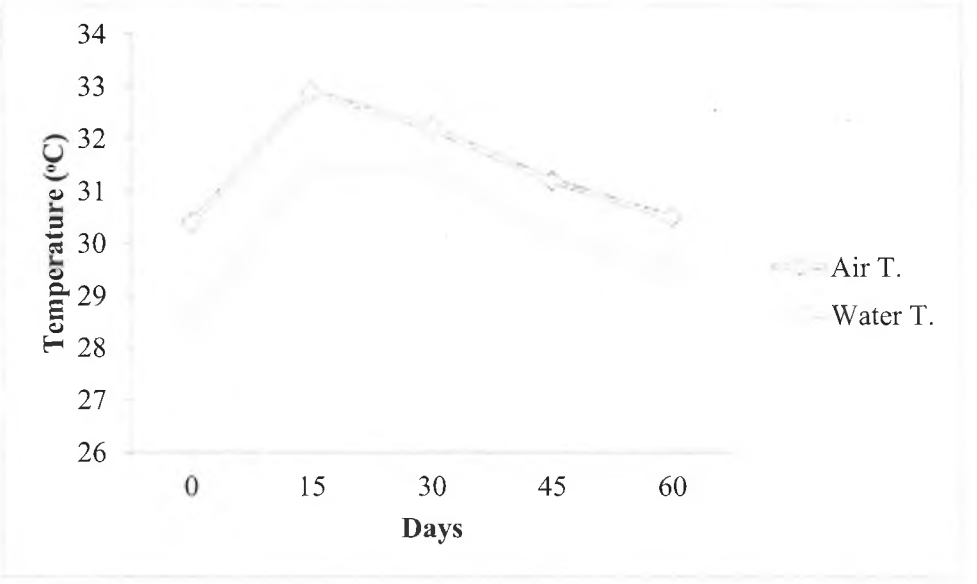

Fig 23. Profile of water and air temperatures recorded during different sampling days of the experimental period.

Table 53. The pH of water recorded during different sampling days of the experiment.

Treatment	Replication	Days				
		0	15	30	45	60
T_0	1	7.6	7.4	7.7	7.4	7.8
	2	7.4	7.4	7.3	7.5	7.5
	3	7.3	7.6	7.5	7.5	7.7
	4	7.3	7.4	7.5	7.4	7.6
	5	7.4	7.6	7.4	7.6	7.5
	Mean±SE	**7.4±0.05**	**7.48±0.05**	**7.48±0.07**	**7.48±0.04**	**7.62±0.06**
T_1	1	7.8	7.5	7.9	7.6	7.6
	2	7.7	8.1	8.1	7.7	7.5
	3	7.7	7.8	7.7	7.6	7.4
	4	7.6	7.8	8.0	7.6	7.5
	5	7.8	7.7	7.9	7.5	7.6
	Mean±SE	**7.72±0.04**	**7.78±0.10**	**7.92±0.07**	**7.60±0.03**	**7.52±0.04**
T_2	1	7.7	8.1	8.0	7.7	8.2
	2	7.9	8.0	8.3	7.9	8.2
	3	7.7	7.9	7.9	7.8	8.3
	4	8.1	8.2	8.1	7.8	8.0
	5	7.8	8.3	8.3	7.9	8.2
	Mean±SE	**7.84±0.07**	**8.10±0.07**	**8.12±0.08**	**7.82±0.04**	**8.18±0.05**

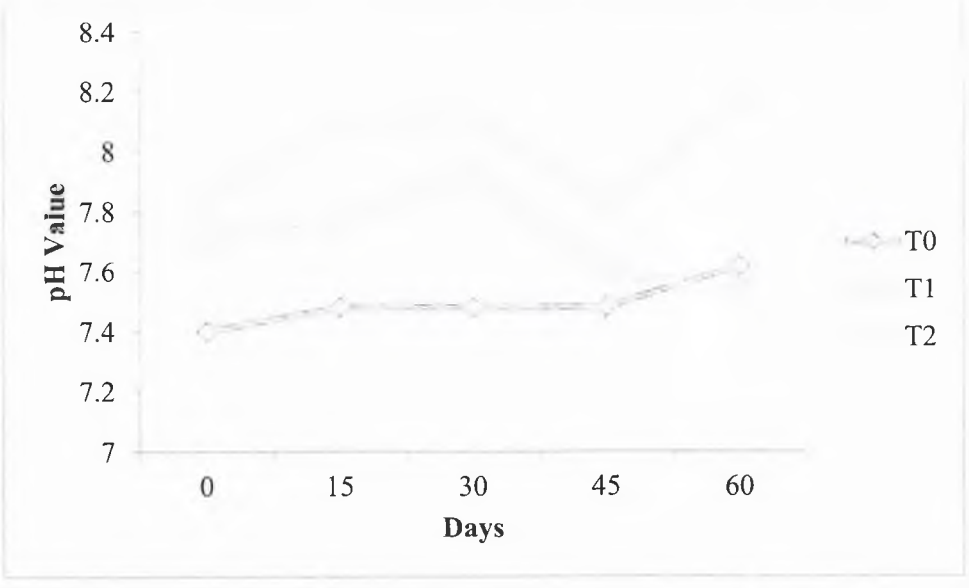

Fig 24. The pH of water recorded during different sampling days of the experimental period.

Table 54. Dissolved oxygen (mg l^{-1}) content of water recorded during different sampling days of the experiment.

Treatment	Replication	Days				
		0	15	30	45	60
T_0	1	6.50	6.90	7.70	7.70	7.70
	2	7.20	6.81	7.70	7.86	7.50
	3	6.40	7.30	7.60	7.66	7.90
	4	6.50	7.80	7.80	8.00	7.60
	5	6.80	7.50	7.20	7.20	7.80
	Mean±SE	**6.68±0.15**	**7.26±0.18**	**7.60±0.10**	**7.68±0.14**	**7.70±0.07**
T_1	1	6.80	7.60	6.80	7.50	7.80
	2	6.61	7.80	6.50	7.15	8.10
	3	6.57	6.50	6.80	7.80	7.90
	4	6.56	6.93	7.15	7.50	7.90
	5	6.80	8.60	7.30	7.60	7.77
	Mean±SE	**6.67±0.05**	**7.49±0.36**	**6.91±0.14**	**7.51±0.11**	**7.89±0.06**
T_2	1	6.70	7.90	7.61	6.80	7.86
	2	6.90	8.49	7.46	7.62	7.70
	3	7.01	7.36	7.20	7.70	8.21
	4	6.50	5.79	7.80	9.45	7.65
	5	6.90	7.20	7.53	7.88	8.29
	Mean±SE	**6.80±0.09**	**7.35±0.45**	**7.52±0.10**	**7.89±0.43**	**7.94±0.13**

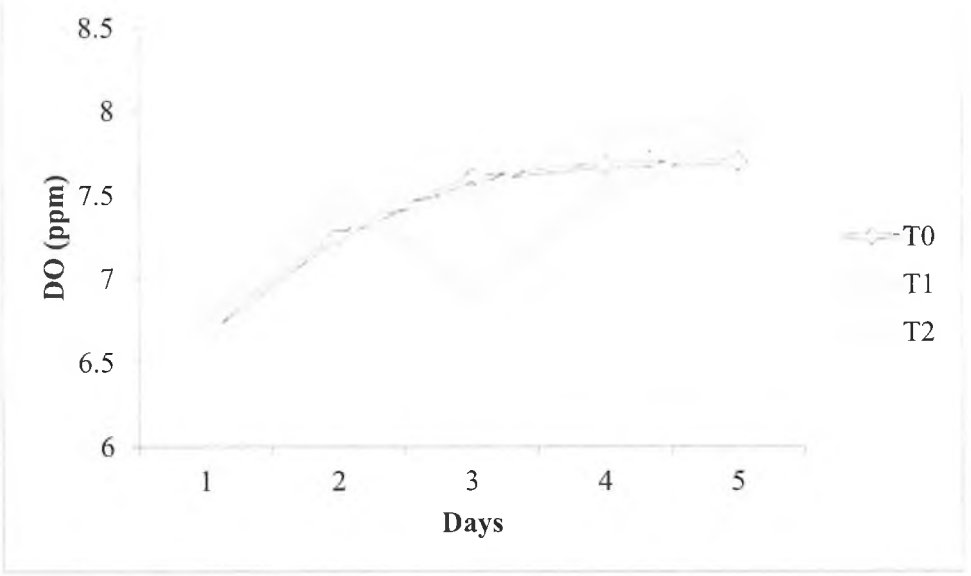

Fig 25. Dissolved oxygen (mg l^{-1}) of water recorded during different sampling days.

Table 55. Free Carbon Dioxide content of water (mg l^{-1}) recorded during different sampling days of the experiment.

Treatment	Replication	Days				
		0	15	30	45	60
T_0	1	0.15	0.10	0.18	0.26	0.25
	2	0.40	0.00	0.25	0.00	0.38
	3	0.31	0.15	0.40	0.00	0.10
	4	0.35	0.25	0.10	0.60	0.20
	5	0.28	0.40	0.25	0.40	0.35
	Mean±SE	**0.30±0.04**	**0.18±0.07**	**0.24±0.05**	**0.25±0.12**	**0.26±0.05**
T_1	1	0.60	0.90	0.50	1.00	0.14
	2	1.00	0.25	0.12	0.18	0.20
	3	0.19	0.25	0.30	0.20	0.35
	4	0.25	0.08	0.45	0.10	0.38
	5	0.40	0.75	0.40	0.20	0.56
	Mean±SE	**0.49±0.15**	**0.45±0.16**	**0.35±0.07**	**0.34±0.17**	**0.33±0.07**
T_2	1	0.40	0.67	0.25	0.11	0.26
	2	0.20	0.53	0.10	0.10	0.30
	3	1.05	0.31	0.18	0.42	0.14
	4	0.26	0.35	0.28	0.32	0.60
	5	0.56	0.41	0.32	0.51	0.40
	Mean±SE	**0.49±0.15**	**0.45±0.07**	**0.23±0.04**	**0.29±0.08**	**0.34±0.08**

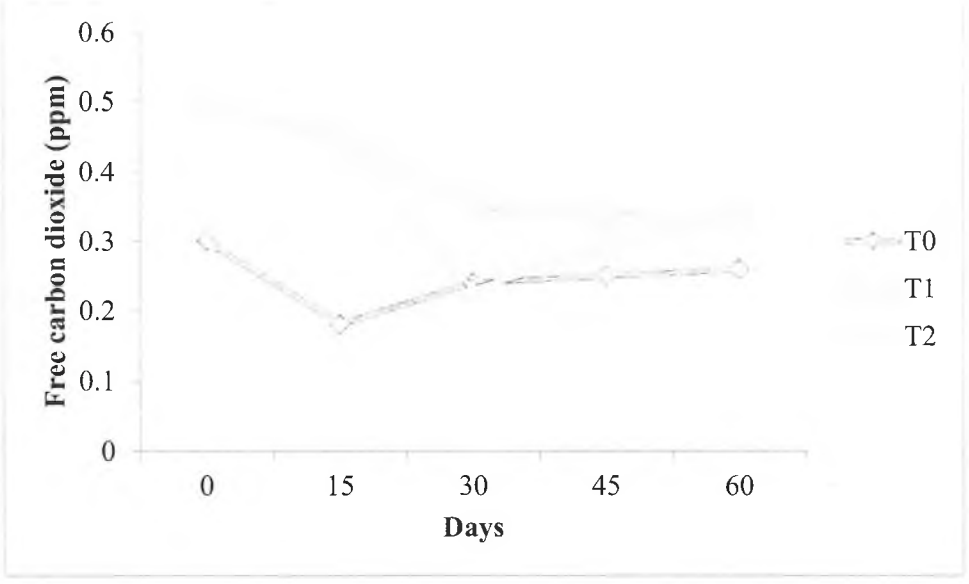

Fig 26. Free Carbon Dioxide of water (mg l^{-1}) recorded during different sampling days of the experiment.

Table 56. Ammonia (µg-at NH_3-N l^{-1}) of water recorded during different sampling days of the experiment.

Treatment	Replication	Days				
		0	15	30	45	60
T_0	1	0.100	0.052	0.154	0.360	0.197
	2	0.050	0.047	0.189	0.122	0.231
	3	0.250	0.062	0.176	0.471	0.215
	4	0.199	0.053	0.155	0.317	0.277
	5	0.100	0.100	0.151	0.050	0.200
	Mean±SE	**0.140±0.037**	**0.063±0.010**	**0.165±0.007**	**0.264±0.078**	**0.224±0.015**
T_1	1	0.475	0.087	0.154	0.176	0.197
	2	0.162	0.046	0.312	0.138	0.322
	3	0.276	0.053	0.215	0.231	0.209
	4	0.110	0.072	0.217	0.190	0.170
	5	0.070	0.030	0.290	0.150	0.251
	Mean±SE	**0.219±0.073**	**0.058±0.010**	**0.238±0.028**	**0.177±0.016**	**0.230±0.026**
T_2	1	0.190	0.074	0.196	0.238	0.165
	2	0.137	0.052	0.256	0.189	0.179
	3	0.090	0.043	0.124	0.101	0.226
	4	0.022	0.018	0.146	0.193	0.176
	5	0.035	0.056	0.192	0.176	0.190
	Mean±SE	**0.095±0.031**	**0.049±0.009**	**0.183±0.023**	**0.179±0.022**	**0.187±0.010**

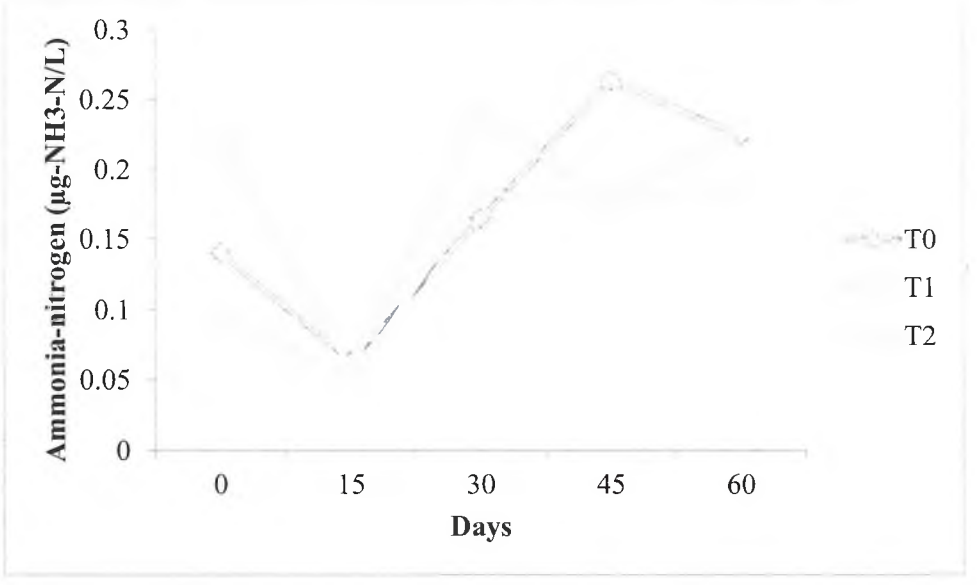

Fig 27. Ammonia (µg-at NH_3-N l^{-1}) of water recorded during different sampling days of the experiment.

Table 57. Total alkalinity of water (mg l^{-1}) recorded during different sampling days of the experiment.

Treatment	Replication	Days				
		0	15	30	45	60
T_0	1	38.00	38.05	44.00	37.50	38.50
	2	42.00	40.00	39.50	41.93	48.50
	3	46.75	40.15	38.00	38.00	40.00
	4	42.25	39.40	40.50	45.00	42.33
	5	38.11	37.54	38.40	42.78	42.00
	Mean±SE	**41.42±1.61**	**39.03±0.53**	**40.08±1.07**	**41.04±1.44**	**42.27±1.71**
T_1	1	35.60	41.50	36.40	41.93	48.30
	2	37.00	48.30	44.00	49.62	44.30
	3	36.90	42.45	39.60	51.24	44.25
	4	48.35	43.00	48.30	50.12	42.24
	5	51.00	44.00	40.00	51.16	44.46
	Mean±SE	**41.77±3.26**	**43.85±1.18**	**41.66±2.05**	**48.81±1.75**	**44.71±0.99**
T_2	1	50.00	44.00	41.36	48.69	49.50
	2	49.78	43.67	48.50	52.32	47.35
	3	39.53	39.00	46.26	52.63	51.15
	4	40.80	36.00	47.25	37.50	45.88
	5	43.75	37.50	47.33	51.21	51.35
	Mean±SE	**44.77±2.20**	**40.03±1.62**	**46.14±1.25**	**48.47±2.83**	**49.05±1.07**

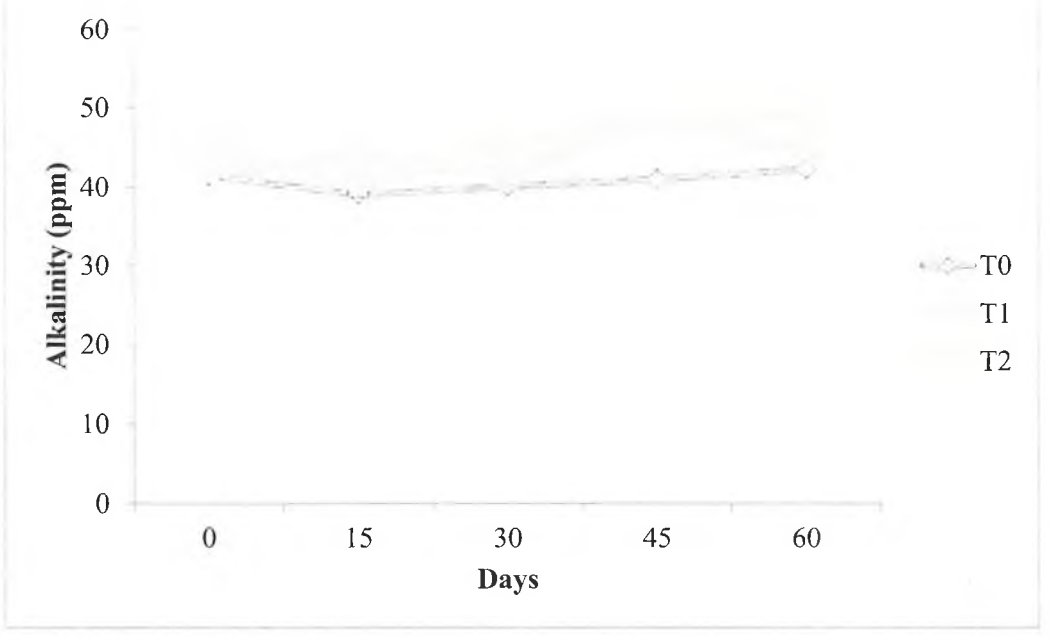

Fig 28. Total alkalinity Profile of water (mg l^{-1}) recorded during different sampling days.

Discussion

V. DISCUSSION

5.1. Proximate composition of feed ingredients and diets

To develop nutritionally balanced diet for fish culture, it is essential to determine the dietary protein requirement within acceptable limits. Nutritionally complete diets supply all the ingredients (proteins, carbohydrates, fats, vitamins and minerals) necessary for the optimal growth and health of the fish. All the ingredients used for the preparation of experimental diets namely soyabean meal, rice bran, corn flour, cotton seed meal, rape seed meal, fish meal were analysed for proximate composition before formulation of diets. In the present study, the highest protein level was found in fish meal (57.85 %). Fish meal with protein content above 50% is considered as of good quality meal by National Research Council (1977). The lowest protein level was found in corn flour (6.01%). The highest fat content was found in rice bran (10.20 %). Fibre content was highest in rice bran (21.60 %). Highest ash content was recorded in fish meal (20.12 %).

The carps require 30% dietary protein for proper growth and survival. Fingerlings and fry of these carps require 35% and 40% dietary protein, respectively for good growth (Renukaradhya and Varghese, 1986). Rajabamshi and Mumtazuddin (1989) reported that protein requirement of rohu fingerlings was 35% for optimum growth. Varghese *et al.* (1976), Venugopal (1980) and Keshavanath *et al.* (1991) reported that carps can grow better with diets containing 30% protein. In the present study, the feed that was formulated for *Labeo rohita* ranged from 31.95% to 32.82% indicating close proximity to published reports. Thus the quality of all the ingredients used in the present investigation for feed formulation was nutritionally adequate and the dietary protein content of the formulated feeds was ideal for optimum growth of the fish.

5.2. Growth studies

5.2.1. Effect of methionine on growth of *Labeo rohita*

Methionine has been much investigated as growth and health promoting feed additive. It is a sulfur containing essential amino acid and was first isolated in 1922 from casein and belongs to a group of compounds called lipotropics - the others in this group include choline, inositol, and betaine. It is important in the process of methylation where methyl is added to compounds as well as being a precursor to the amino acids cystine and cysteine. Severe deficiency of methionine may manifest in dementia, while lesser deficiencies may be known by symptoms like fatty liver, slow growth, weakness, edema and skin lesions. Young ones of channel catfish required methionine at 2.34% of dietary protein in the absence of cystine for proper growth, (Harding *et al.*, 1977). Phillips *et al.* (1965) observed that brook trout supplemented with methionine had improved growth rates. Mc Cartney (1967), however, found that brook trout supplemented with 2% methionine showed improved growth and higher body protein when a high protein, high calorie diet was fed, but lower growth and body protein when fed a low protein and lower calorie diet. Similar results were obtained by Blaza *et al.* (1982) in growing dogs where digestibility of energy improved significantly when methionine supplementation was increased in the diet. The required amount of threonine has been investigated in common carp (Nose, 1979), Japanese eel (Arai *et al.*, 1972), channel catfish (Wilson *et al.*, 1978), Nile tilapia (Santiago and Lovell, 1988), milkfish (Borlongan, 1991), catla (Ravi and Devaraj, 1991) and rohu (Murthy and Varghese, 1996). Toxic and adverse effects of excessive amino acids on growth have been attributed to the fact that disproportional intake of amino acids affects the absorption and utilization of the amino acids (Harper *et al.*, 1970; Austic, 1978; Borlongan and Coloso, 1993; Murthy and Varghese, 1995). Choo

et al. (1991) reported the toxic effect of excess dietary leucine in rainbow trout: growth decreased when the essential amino acids exceeded the requirements. This decrease is attributed to the use of energy for nitrogenous excretion, because amino acids are deaminated and excreted in the form of ammonia (Walton, 1985).

5.2.2. Specific growth rate (SGR)

Specific growth rate (SGR% per day) can be taken as an index of growth in the evaluation of diets. In the present experiment, SGR was higher in all the diets which had methionine as compared to the control. The highest SGR was recorded with the diet having 0.48% methionine in *Labeo rohita*. Hossain and Dewan (1997) obtained the highest growth in *L. rohita* using inorganic fertilizer and supplementary feed consisting of mustard oil cake and rice bran (1:1). SGR value indicates the nutritional value of a diet and also defines the relationship between growth and feeding rate.

5.2.3. Food conversion ratio (FCR)

FCR of an animal is the ratio at which it can convert food eaten into flesh. It was higher in *Labeo rohita* fed with the diet containing 0.48% methionine. The addition and improving the lysine and methionine in the fish diet improve feed intake, feed conversion ratio, body weight, body weight gain, yield and immunity of the fish against different fish diseases (Eduardo *et al.*, 2009). These results agreed with those of (Lee *et al.,*2007*)* where they reported that, the clinical signs due to methionine and/or lysine deficiency attributed to the poor feed utilization and efficiency appear in the form of emaciation, nervous manifestation and haemorrhages on the body surface. FCR and PER are known to be decrease with increasing dietary protein content (Jauncey, 1982). Mondal *et al.* (2000) obtained higher food conversion when fish were fed a diet containing a 39% protein level. In striped bass, feed conversion improved in animals fed a fishmeal-based diet

supplemented with a feeding stimulant mixture of several amino acids and betaine at a rate of 2.7% of the diet (Papartyphon and Soares, 2001).

5.2.4. Survival

The average survival of fish was highest in the diet with 0.48% methionine followed by 0.08% methionine and control in experiment 1, whereas in experiment 2, 100% survival was seen in all the three treatments. Mondal *et al.* (2000) obtained higher survival when fish were fed a diet containing a 39% protein level. *Penaeus monodon* fed a diet containing 1.55% of taurine and amino acid mixture had better growth, feed intake, assimilation, FCR, and survival (Hartati and Briggs, 1976). Hossain and Dewan (1997) obtained 38.4% survival rate in *L. rohita* spawn using mustard oil cake and rice bran (1:1) as supplementary feed.

5.2.5. Net production

The average of net production was the highest in fish fed with 0.48% methionine, wherein growth was also maximum in both the experiments. Khan *et al.* (2012) obtained satisfactory result using 35% protein in terms of weight gain.

5.3. Proximate composition of the muscle

Knowledge on the proximate composition of an animal chosen for aquaculture is essential for not only selecting an ideal stock but also to formulate an ideal diet (Davis and Gatlin, 1996). Carcass composition is known to be influenced by several factors such as geographical location, age, sex, maturity and feeding conditions. Among these factors, the type and nature of feed ingested are considered to be most important (Parova, 1976; Reimers and Meske, 1977; Srikar *et al.*, 1979). Zitler *et al.* (1984) noticed that the body composition of fish is much influenced by the chemical composition of the diet. Insufficient dietary protein levels resulted in poor growth performance in many fish species

(Yang *et al.*, 2002; Giri *et al.*, 2003; Kim and Lee, 2005) due to insufficiency of amino acids supplied to maintain the body composition (Halver and Hardy, 2002).

5.3.1. Moisture

The body fluid in every animal act as the medium of transport of nutrients, metabolites etc. The proportion of water in the flush widely varies between 65% and 90%. Moisture content of fish muscle has an inverse relationship with lipids and proteins (Lone and Matty, 1980; Winfre and Stickney, 1981).

5.3.2. Protein

Maximum growth is associated with the highest deposition of protein in the muscle of fish (Lovell, 1970). In the present experiment, the highest growth was recorded with 0.48% methionine based diet. This is attributed to higher protein deposition in the muscle of fish fed with methionine incorporated diets. In the presence of 0.29% cystine the value (1.44% of diet, 3.34% of dietary protein) of methionine requirement for large yellow croaker based on growth is similar to those previously reported for other fish species, such as Chinook salmon (1.6% of diet, 4.0% of dietary protein) (Halver *et al.*, 1959), gilthead sea bream (1.4% of diet, 4.0% of dietary protein) (Luquet and Sabaut, 1974), Japanese eel (1.2% of diet, 3.2% of dietary protein) (NRC, 1993) and Japanese flounder (1.49% of diet, 3.1% of dietary protein) (Alam *et al.*, 2001). Moreover, methionine is the key amino acid required for the start of protein synthesis and thus, in its absence, protein synthesis does not proceed (Stryer, 1998).

5.3.3. Fat

Fat is stored in the form of energy and also a part of the cell structure. During feeding season, the animal accumulates fat in certain tissues. Fat content varies between species as also within species. Increased dietary lipid was found to enhance deposition of

body fat (Das, 1990). Hanumanthappa (1998) opined that the deposition of fat in the body is an indication of growth. He observed increased fat content in *catla* fed with diets containing increased nutripro-aqua incorporation. Using high energy feed (excess lipid content) showed adverse effects on the carcass fat content (Samantary and Mohanty, 1997) and resulted in off-flavour in fish products (Ng *et al.*, 2003).

5.3.4. Ash

Vinogradov, 1953; Love, 1961; Natarajan and Srinivasan, 1961 reported that ash content represents the total inorganic matter as mineral constituents in the tissue. Muscle ash content is known to be influenced by sex, season, food and fertility of water. When the ash level is lower then the growth will be higher. A high level of ash indicates greater mineral requirements (Khawaja, 1966).

5.4. Immunoassay

5.4.1. NBT test

Ali (2006) observed that, deficiency of methionine and lysine causes of all the blood parameters examined, the red blood cell count and mean cell volume showed significant decrease than the control. Other haematological values were lower than that of the normal values. The RBCs and WBCs counts showed lower than the the control, also, Chaiyapoom *et al.* (2006) noticed that, hematocrit value, Hb % were lower than the control with deficiency of methionine and/or lysine or with each other. In human, an amino acid mixture containing methionine was shown to increase plasma concentration of cholecystokinin and bile salt and lipase output (Colombel *et al.*, 1988).

5.5. Challenge studies

5.5.1. LD_{50}

Fishes taken from control treatment tank were stocked in 40 l capacity aquaria and were fed for one week. Five fishes stocked per each aquarium for determining LD_{50} for control. Five individuals each from control challenged with graded densities (10^3, 10^4,10^5,10^6,10^7,10^8 CFU/ml) of virulent *A. hydrophila* (24 h TSB culture). Specific mortalities were ascertained by reisolating the pathogen from kidney of the diseased fish. Specific mortalities were used for calculating LD_{50} according to Reed and Muench (1938). A density of 10^6 showed LD_{50}, and then it was injected to fishes for challenge studies.

5.5.2. Challenge with *A. hydrophila*

Fishes grown in different treatments were challenged with a 24 h culture *A. hydrophila* (106 CFU/fish). A minimum of 21 fish per treatment used in triplicate groups and challenged after 7 days adaptation. Challenged fish were maintained in well aerated tanks. Post challenge mortalities were recorded for seven days. Only specific mortalities were used in evaluating the potency of injection (10^6 CFU/fish). In the present study there was significant difference ($P<0.05$) in % survival after challenge with *A. hydrophila* in various dietary treatments. Highest survival of recorded 61.91 in T_2 followed by 57.14 in T_1 and 33.33 in T_0. Highest relative percentage survival of 42.86% was recorded in T_2 followed by 35.71% in T_1. Thus the results in the present study shows that incorporation of methionine at 0.48% level can increase resistance to *A. hydrophila* infection. Oral administration of nucleotide enhanced the immune responses and/or disease resistance in several fish species, including *Labeo rohita* (Burrells *et al.*, 2001; Sakai *et al.*, 2001). Swain *et al.*, (1996) recorded the better performance of mrigal, *Cirrhinus mrigala*, fry in

terms of growth and disease resistance with a diet, supplemented with 0.15% of a probiotic.

Supplementation feeds responsible for returning haematological and biochemical parameters to hear normal values and triggering the immune system of the specific and innate immunity of goldfish against *A. hydrophila* when treated with 400 mg/kg or 800 mg/kg of mixed herbal supplementation feeds, and, indicated that the ethanol of triherbal solvent extract seems to be a better immunostimulancy, which can have a promising role in aquaculture to prevent diseases and infectious outbreaks (Harikrishnan *et al* ., 2010). Sahu *et al.* (2007) reported that long term dietary administration of mango kernel led to considerably increases immunity and survival of fingerlings of rohu. Burgents *et al.* (2004) evaluated the effect of yeast supplement on disease resistance in the pacific white shrimp, *Litopenaeus vannamei.* Earlier studies in this line also revealed that dietary supplementation of *O. sanctum* intraperitoneal injection of water soluble and hexane soluble fraction of *Eclipta alba* have enhanced the disease resistance against *A. hydrophila* in *O. mossambicus* (Logambal and Azadirachtin, 2001). Similarly the disease resistance against *A. hydrophila* was also enhanced in *L. rohita* fed with 0.5% of *Achyranthes* (Joseph and Carnahan, 1994).

5.6. Water quality

Water quality is simply defined as the degree of excellence that given water possesses for the propagation of desirable aquatic organisms to achieve high survival, growth and reduction (Deo, 2006). A complete understanding of the relationship between water quality and aquatic productivity is a pre-requisite for optimum growth and survival (Boyd, 1982). Water quality management is an ongoing and never-ending process. An

analysis of physical, chemical and biological properties of the proposed source of water must be conducted. In the present study, important water quality parameters such as temperature, pH, dissolved oxygen, free carbon dioxide, total alkalinity and ammonia-nitrogen were measured throughout the experimental period. The water quality parameters measured in different treatments thought the experimental period were found to be well within the acceptable range for culture of *Labeo rohita*.

5.6.1. Temperature

Manifestation or measurement of heat energy is termed as temperature. Water temperature is related to solar radiation and air temperature. Temperature is one of the most important environmental variables for aquatic organisms, because it influences the oxygen content of water, and also it plays a major role in fish physiology. It affects the growth, food intake, metabolic rate and enzyme activity in fish. Temperature maintains the rate of metabolism by controlling molecular dynamics and biochemical reaction (Deo, 2006). Food intake increases with the increase in temperature up to optimum levels as the energy requirement for maintenance increases (Cho and Slinger, 1980). Optimum temperature for metabolism varies from species to species. Optimum temperature range for many cold water and warm water species are 14-18^{0}C and 24-30^{0}C respectively. Indian major carps can tolerate temperature ranging from 10 to 37.8^{0}C (Jhingran, 1982). Water and air temperature recorded during the present study ranged from 27.2 to 31.5°C and 29.1 to 32.9°C respectively, which is within the tolerance limit of *L. rohita*. Goolish and Adelman (1984) obtained the maximum growth rate at 30°C in case of juvenile common carp.

5.6.2. pH

The pH is an expression of hydrogen ion concentration in water which serves as an indicator of acidity and alkalinity. If the water is too acidic it decreases fish appetite, eventually growth and immunity. The water having a pH range of 6.5-9.0 is more suitable for fish culture and values above 9.5 are unsuitable as carbon dioxide becomes unavailable at higher pH (Das et al., 1995). It influences toxicity of ammonia and hydrogen sulphide and solubility of nutrients in water (Deo, 2006). Fish dies at a pH above 11.0. Das *et al.* (1995) suggested that a pH range of 6.12-8.6 is most suitable for survival of the Indian major carp fry. Variation in pH affects metabolism and other physiological processes. (Swingle, 1961; Banerjee, 1967) Neutral to slightly alkaline pH has been found to be most favourable for fish ponds. The pH in the present study ranged between 7.40 and 8.18, which is in the desirable limits for the optimum growth of *Labeo rohita.*

5.6.3. Dissolved oxygen

Dissolved oxygen (DO) is the most important environmental factor influencing the health condition of fish in an aquaculture systems. As the temperature increases, the solubility of oxygen in the water decreases. Dissolved oxygen in ponds is very crucial for aquatic life and survival of fishes (Boyd 1982). Oxygen consumption varies with species, size, activity, season and temperature. According to Banerjee (1967), cyprinids require 6-7 ppm oxygen for good growth, but they can tolerate levels as low as 3 ppm for short periods. DO value higher than $5mgl^{-1}$ have often been recommended for intensive culture practices (Cheng *et al.*, 2003). Banerjee (1967) reported that oxygen concentration above 5ppm is indicative of productivity, but dissolved oxygen below that level indicates that the water is unproductive. The dissolved oxygen content observed in the present study was

6.63 to 8.11 mg l^{-1}. It is in acceptable limit and suitable for the optimum growth of *Labeo rohita*.

5.6.4 Free carbon dioxide

The main source of carbon dioxide (CO_2) in pond water is through absorption from the atmosphere, decomposition of organic matter and respiration of aquatic animals. Aquatic animals are affected by carbon dioxide depending on the oxygen level of water. Inspite of its high solubility in water, its concentration in most water bodies is low. High concentration of CO_2 reduces the capacity of blood to transport oxygen. If there is sufficient oxygen in water fish can survive level of CO_2 as high as 60 ppm (Hart, 1944). The CO_2 concentrations in intensively managed aquaculture waters normally fluctuate between 0 to >20 ppm free CO_2 in a 24 hour cycle with the lowest concentrations during the hours of photosynthesis (Schmittou, 1998). The recorded free carbon dioxide in the present study ranged from 0.15 to 0.82 mg l^{-1} and considered as acceptable for optimum growth of rohu.

5.6.5. Ammonia-nitrogen

The major end product of protein catabolism is ammonia which is excreted primarily as un-ionized ammonia by fish. The source of ammonia-nitrogen in water is excreta of cultured animals and microbial decay of nitrogenous compounds. Ammonia occurs in both ionized (NH_4) and unionized (NH_3) forms. Tucker and Boyd (1982) opined that the amount of ammonia reaching pond water through fish metabolite is proportional to the feeding rate. The total ammonia recorded during the present investigation was ranged from 0.049 to 0.327 µg l^{-1} and it was below the tolerance limit of the carps (Das *et al.*, 2004; Jena *et al.*, 2007a).

5.6.6. Total alkalinity

Alkalinity refers to the concentration of bases in water and the capacity of water to accept acidity i.e. the debuffering capacity. Water with a low alkalinity i.e. total alkalinity less than 20 mg l^{-1} has low buffering capacity, shows wide fluctuation of pH (Boyd, 1982). According to Adhikari (2000) Ponds with alkalinity greater than 300 mg l^{-1} may be unproductive because of limitation to carbon dioxide availability at such high concentration. Total alkalinity value range between 20 to 300 mg l^{-1} is ideal for fish and less than 20 mg l^{-1} alkalinity create stress in fish. The total alkalinity values in this study fluctuated between 36.86 to 52.14 mg l^{-1} and it was in the acceptable range for culture of *Labeo rohita* Boyd (1982).

Summary

VI. SUMMARY

The main objectives of the present study were to evaluate the effect of methionine as a feed additive, on growth, survival and immune response of Indian major carp, *Labeo rohita*.

1. The methionine was incorporated in the diet at an inclusion levels of 0.08% and 0.48% and the feeding was carried out for a period of 60 days for *Labeo rohita* fingerlings and 90 days for *Labeo rohita* juveniles respectively.
2. Uniform sized fingerlings of *Labeo rohita* of an average length and weight of 11.14 cm and 10.6g respectively were stocked @ 20 numbers per cistern for first experiment and uniform sized juveniles of *Labeo rohita* of average length and weight of 21.97 cm and 109.07g respectively were stocked @ 10 numbers per tank.
3. The feed were formulated using soyabean meal, rice bran, corn, cottonseed meal, rapeseed meal, fish meal, sardine oil, vitamin and mineral mixture, vit. C and methionine having 30% protein. The formulated feeds were designated as T_0 (0% methionine and served as control diet) and T_1 (0.08% methionine), T_2 (0.48% methionine). The feeding was done twice a day @ 5% and 2% body weight respectively.
4. Growth assessment of fish and water sampling was carried out after every 15 days. Water quality parameters were found to be suitable for the growth of the fish in all the treatments.
5. In experiment 1 results showed significant difference in methionine incorporated diets and control diet with regard to weight after 90 days of feeding so also in experiment 2 after 60 days of feeding.

6. The highest mean weight recorded after 90 days of feeding in T_2 was 100.48g followed by 93.03g in T_1 and 90.28g in T_0 respectively in experiment1. Similarly the highest mean weight recorded after 60 days of feeding in T_2 was 412g followed by 372g in T_1 and 314g T_0 respectively in experiment 2.
7. The highest percentage of weight gain was noticed in fish fed with 0.48% methionine diet. The highest specific growth rate (SGR) per day and also the lowest and best feed conversion ratio (FCR) were noticed in fish fed with diet containing 0.48% methionine (T_2).
8. The highest survival rate was recorded in T_2 followed by T_1 and T_0 in experiment 1 whereas in experiment 2,100% survival rate was recorded in fish fed with 0, 0.08 and 0.48% methionine supplemented diets.
9. After 90 and 60 days of feeding immune parameters of fish were measured. The respiratory burst activity i.e. super oxide anion production of fish fed with methionine incorporated diets increased significantly. The highest super oxide anion production was recorded in T_2 followed by T_1 and T_0.
10. Both protein and fat composition of fish muscle varied significantly between the treatments. The maximum mean protein and crude fat content of fish muscle was observed in T_2 followed by T_1 and T_0.
11. In conclusion, the present study established that *Labeo rohita* fed with diet containing supplemented methionine showed a significant difference in growth and immune response. The growth, survival and immune response in *Labeo rohita* was higher in fish fed with methionine incorporated diets than in the control diet fed fish. Among the two levels, 0.48% methionine showed better performance than 0.08% methionine.

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VIII. ABSTRACT

Effect of dietary supplementation of methionine on growth, survival and immune response of Indian major carp, *Labeo rohita*

Protein is a major constituent of animal body. A liberal and continuous supply of protein is needed throughout life. The primary aim of fish culture is to efficiently transform dietary protein into tissue protein. The amount of dietary protein required by fishes is directly influenced by the indispensable amino acid pattern in the diet. Thus topic mainly focuses on the amount of methionine needed to produce maximum growth which has been investigated. A well balanced feed not only results in higher fish production, but also provides the nutrients necessary to prevent occurrence of disease, besides helping the fish in withstanding the environmental stress. The study on the role of methionine as a feed additives and its influence on growth and health in rohu fingerlings and juveniles were investigated for 90 and 60 days respectively. The basal diet in all five replications contained 30% protein with different doses of methionine at control, 0.08% and 0.48% and a control diet. Significant differences were evident between treatment groups ($P<0.05$) in growth parameters, SGR, FCR. Among the tested doses 0.48% methionine showed better results than 0.08% methionine inclusion in the fish diet. Results of this study indicate that the best overall growth, survival and feed utilization of rohu fingerlings and juveniles were obtained with 0.48% methionine supplementation.