SURVIVAL, GROWTH, COLOURATION AND STRESS RESPONSES OF FRESHWATER GOLD FISH, *CARASSIUS AURATUS* (Linn.) IN INLAND SALINE WATER

Thesis

Submitted to the Guru Angad Dev Veterinary and Animal Sciences University in partial fulfillment of the requirements for the degree of

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in

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(Minor subject: Aquatic Environment Management)

By

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CERTIFICATE-I

This is to certify that the thesis entitled, **"SURVIVAL, GROWTH, COLOURATION AND STRESS RESPONSES OF FRESHWATER GOLD FISH, *CARASSIUS AURATUS* (Linn.) IN INLAND SALINE WATER"** submitted for the degree of M.F.Sc., in the subject of **AQUACULTURE** (Minor Subject: **AQUATIC ENVIRONMENT MANAGEMENT)** of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Ms. DEEPA BHATT (L-2016-F-02-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE-II

This is to certify that the thesis entitled, **"SURVIVAL, GROWTH, COLOURATION AND STRESS RESPONSES OF FRESHWATER GOLD FISH, *CARASSIUS AURATUS* (Linn.) IN INLAND SALINE WATER"** submitted by **Ms. DEEPA BHATT (L-2016-F-02-M)** to the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, in partial fulfillment of the requirements for the degree of M.F.Sc. in the subject of **AQUACULTURE** (Minor Subject: **AQUATIC ENVIRONMENT MANAGEMENT**) has been approved by the student's Advisory Committee after an oral examination on the same, in collaboration with an external examiner.

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ABSTRACT

Short term salinity tolerance test (experiment I for 10 days) and long term rearing experiment (experiment II for 120 days) was conducted to assess the effect of salinity on survival, growth, colouration and stress responses of freshwater shubunkin gold fish, *Carassius auratus* (L.). Fish (length 6.5-8.5 cm and weight 4.5-8.5 g) was exposed to different salinity levels (prepared by dilution of inland saline water) viz., 2, 4, 6, 8, and 10 ppt, after gradual acclimatization (@1ppt hr^{-1}). Survival and colouration of fish was not affected significantly during salinity tolerance test for 10 days, however, during 120 days of salinity exposure, survival and fish growth (negative) was severely affected beyond 4 ppt. Fish behavior was also deviated from normal responses beyond 4 ppt in both the experiments. Significant alterations in haematological (increased levels of RBC, WBC and PCV up to 10 ppt; decreased levels of Hb, MCV, MCH and MCHC up to 10 ppt); biochemical (decreased levels of glucose and total protein up to 8 ppt and abrupt increase in 10 ppt, decrease and increased levels of albumin and globulins in all the salinity treatments, with decreased Alb/Glb ratio up to 4 ppt, increase in 6 ppt and then decrease up to 10 ppt) and antioxidant parameters (increased levels of SOD, LPO and GR up to 10 ppt) were indicative of adaptive behaviour of the fish, towards increasing salinity. Histological alteration in gills, kidney and liver were observed even at salinity level of 2 ppt. Salinity stress also resulted in dull colouration beyond 2 ppt observed in terms of caroteniod content and digital colour analysis. Results of the present study are indicative of conclusion, that the freshwater shubunkin gold fish, *C. auratus* (L.) can be reared in inland saline water by maintaining the salinity $\leq$ 2 ppt.

Key Words: salinity stress, inland saline water, gold fish, physiological changes, colouration

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Signature of Major Advisor **Signature of the Student**

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LIST OF ABBREVIATIONS

A	-	Active
a*	-	Balance between red/green
Alb	-	Albumin
ANOVA	-	Analysis of variance
APHA	-	American Public Health Association
Av	-	Average
b*	-	Balance between yellow/blue
BC	-	Blood congestion
BCG	-	Bromo cresol green
BS	-	Blood sinusoid
BWG	-	Body weight gain
Ca^{2+}	-	Calcium
$CaCl_2$	-	Calcium chloride
$CaCO_3$	-	Calcium carbonate
CAT	-	Catalase
CC	-	Chloride cells
CDGBC	-	Complete degeneration of glomerulus and emptying of Bowman's capsule
CDSL	-	Complete degeneration of secondary lamellae
CIFE	-	Central Institute of Fresh Water Education
Cl^-	-	Chloride
cm	-	Centimeter
CO_3^{2-}	-	Carbonate
CS	-	Cloudy swelling in lumen

CSSRI	-	Central Soil Salinity Research Institute
CV	-	Central vein
D	-	Dilution factor
DCT	-	Distal convoluted tubule
DG	-	Degeneration of Glomerulus
DGR	-	Daily growth rate
DHC	-	Disorganization of hepatic chords
dl	-	Deciliter
DL	-	Dilation of tubules in lumen
E	-	Epithelial cells
EC	-	Electrical conductivity
EDTA	-	Ethylenediamine tetraacetic acid
EF	-	Edematous fluid
EL	-	Epithelial lifting
FC	-	Decrease in the Fibrous component
FW	-	Fresh water
G	-	Glomerulus
g	-	Gram
GCH	-	Granulated cytoplasm of Hepatocytes
GFC	-	Gapping in the Fibrous component
Glb	-	Globulin
GPX	-	Glutathione peroxidise
GR	-	Glutathione reductase
H	-	Haemorrhages
Ha	-	Hactare

HAp	-	High appetite
Hb	-	Haemoglobulin
Hct	-	Hematocrit
Hp	-	Hepatocytes
HP	-	Hyperplasia
HPV	-	Hepatopancreas in portal vein
Hr	-	Hour
HT	-	Hypertrophy
HY	-	Hyaline cartilage
IAB	-	Indian Agriculture in Brief
ICAR	-	Indian Council of Agricultural Research
ICS	-	Increased capsular space filled with fluid
ISW	-	Inland saline water
K^+	-	Potassium
L	-	Lightness
l	-	Litre
LA	-	Less active
LAp	-	Less appetite
LB	-	Lamellar bending
LC_{50}	-	Lethal concentration
LF	-	Lamellar fusion
LPO	-	Lipid peroxidisation
m	-	Meter
m ha	-	Million hectare
MCH	-	Mean corpuscular haemoglobin

MCHC	-	Mean corpuscular haemoglobin concentration
MCI	-	Mononuclear cellular infiltration
MCV	-	Mean corpuscular volume
MDA	-	Malondialdehyde
mg	-	Milligram
mg l^{-1}	-	Milligram per litre
Mg^{2+}	-	Magnesium
ml	-	Millilitre
mm	-	Millimeter
mmole	-	Millimole
MSH	-	Melanocyte stimulating hormone
N	-	Necrosis
Na^{+}	-	Sodium
NaCl	-	Sodium chloride
NADH	-	Nicotinamide adenine dinucleotide
NBT	-	Nitroblue tetrazolium chloride
NH_3-N	-	Ammonical nitrogen
NH_4^{+}	-	Ammonical ion
NKA	-	Na^{+}-K^{+}-ATPase
NO_3^{-}	-	Nitrate
NWG	-	Net weight gain
P	-	Primary cells
PA	-	Pancreatic acini
PC	-	Pillar cells
PCT	-	Proximal convulated tubule

PCV	-	Packed cell volume
pg	-	Picogram
PL	-	Primary lamellae
PMS	-	Phenazine methosulphate
PO_4^-	-	Phosphate
ppm	-	Parts per million
ppt	-	Parts per thousand
PSU	-	Practical salinity unit
PUFA	-	Polyunsaturated fatty acid
PV	-	Portal vein
RBC	-	Red blood cell
RCV	-	Rupturing of central vein
ROS	-	Reactive oxygen species
RPM	-	Revolutions per minute
S	-	Sluggish
SB	-	Swim bladder
SGR	-	Specific growth rate
SL	-	Secondary lamellae
SO_4^{2-}	-	Sulphate
SOD	-	Super oxide dismutase
SPSS	-	Statistical Package for the Social Sciences
SrL	-	Shrunken lumen
ST	-	Salt treatment
SW	-	Sea water
T	-	Talengiectasia

TA	-	Total alkalinity
TBA	-	Thiobarbituric acid
TH	-	Total hardness
TLG	-	Total weight gain
TNB	-	Thionitrobenzoic acid
TP	-	Total protein
TPV	-	Thickening of portal vein
U	-	Unit
UV/VIS	-	Ultraviolet–visible spectroscopy
VH	-	Vacoulisation of Hepatocytes
WBC	-	White blood cell
Yr	-	Year

CHAPTER - I

INTRODUCTION

Ornamental fish keeping is one of the vibrant sectors in the international market due to its significant contribution towards export value along with rural development in many developing countries including India. Since 1969, India is exporting unique varieties of wild caught ornamental fish to many developed countries of the world (Jain *et al* 2016). Further, the export value of these fishes in international trade has increased with approximately 14% at an average annual growth rate. The ornamental fish trade in India is dominated by freshwater ornamental fishes (90%) with 98% contribution from culture sector and only 2% of the trade is being contributed by wild fish captured from natural resources. In Punjab, Ornamental fish industry is mainly a trading activity and dependent upon freshwater fish procured from other states viz. West Bengal, Tamil Nadu, Maharashtra and Karnataka. Since few years, it is also emerging as an aquaculture activity and technologies for culture, breeding and seed production of some commercially important freshwater ornamental fish species [Gold fish (*Carassius auratus* var. Comet, Shubunkin, Red head), Koi Carp (*Cyprinus carpio*), Molly (*Poecilia latipinna),* Guppy (*P. reticulata),* Platy (*Xiphophorus maculatus*) and Sword tail (*X. helleri*)] are also available for commercial adoption in the State. Further, some of the previous reports (Luz *et al* 2008, Küçük 2013) also indicated possibilities of rearing these freshwater ornamental fish species in saline waters.

South-west districts of Punjab (Sri Mukatsar Sahib, Ferozepur, Fazilka, Faridkot and Bathinda) are facing the dual problem of underground salinity and water logging (1.51 lakh ha), which has devastated agricultural productivity in the region. Among these districts, Fazilka is severely affected due to water logging with very less agricultural activity (Kumari *et al* 2017). Salinity of underground water in these districts varied between 10-15 ppt, whereas salinity of water logged areas varied from 1-47 ppt. Further, water quality parameters (pH, conductivity, total alkalinity and total hardness) of water samples collected from different areas varied within wide range even at same salinities. Calcium hardness found to be many folds higher than even sea water. High salinities with high Ca^{2+} and Mg^{2+} resulted in very high total water hardness (Jana *et al* 2004) and have destroyed the productive agricultural land. Further, surface water salinity in water logged area is highest (1-165 ppt) as compared to village pond water (0-29 ppt) and underground water (0-15 ppt) (Dhawan *et al* 2009). For economic utilization of these

degraded lands having abundantly available saline water, 'Aquaculture' is the most viable option. Extensive efforts have been made to reclaim and utilize these lands through aquaculture, with respect to both brackish and freshwater finfish/shellfish species like mullets, milk fish, pearl spot, sea bass, white legged shrimp (vannamei shrimp), tiger prawn, freshwater carps etc. (Dhawan *et al* 2009, 2010, 2016, Ansal *et al* 2013, 2016 and Kumari *et al* 2017).

Physico-chemical or abiotic parameters of water (temperature, pH, salinity, dissolved oxygen, alkalinity, ammonia etc.) play an important role in the biology, physiology and other metabolic activities of the fish and their optimal limits are important for achieving maximum survival and growth. Among, various abiotic factors, salinity is one of the critical parameter for the overall well-being of the freshwater species, as it determines the level of osmoregulatory stress. Preliminary effect of salinity on fish physiology can be studied in terms of osmoregulation process as an effect of intake or loss of ions in high or low salinity in order to maintain ionic concentration of the body through major organs (gills, kidney and intestine) responsible for osmoregulation (Al-Hilali and Al-Khshali 2016). Although saline water tolerance in freshwater fishes appeared to vary between the species as well as within the species having different size (Islam *et al* 2014), however, growth of freshwater fishes has been reported to decline with increase in salinity level, due to their stenohaline nature resulting in imbalance of homeostasis or internal stability (Enayati *et al* 2013). Stress due to salinity chages has also been reported to alter the haematological characteristics, associated with the immune responses governing the physiological status and pathologies (Akinrotimi *et al* 2012 and Al-Hilali and Al-Khshali 2016). These alterations could be explained due to loss of water from blood owing to increased osmotic difference between the blood and external environment. Further, ionic composition of inland saline water not only differs from sea water entirely (Dhawan *et al* 2010), but also varies with location and depth. In this regard, aquaculture technologies for freshwater carps have been developed for low saline zones of south-west districts of Punjab (Dhawan *et al* 2009, 2010, 2016 and Ansal *et al* 2013, 2016). Further, experimental trials for rearing freshwater koi carp in inland saline waters of Punjab have also been conducted successfully (Sharma *et al* 2017) w.r.to stress responses in terms of physiological changes.

Teleost fish are able to maintain the ionic and osmotic homeostasis of their body fluids under varied environmental salinities by using energy demanding osmoregulatory mechanisms,

(Sampaio and Bianchini 2002). Considering the osmoregulatory cost proportional to the osmotic gradient existing between the fish body fluids and the external medium (Handerland *et al* 2000), it is rational to expect the maximum growth, if the fish is reared in water of salinity near the isosmotic condition. In teleosts, the plasma isosmotic point generally corresponds to the water salinity of 9 ppt (Boeuf and Payan 2001, Tsuzuki *et al* 2007, Herrera *et al* 2009 and Nordlie 2009). However, the significance and fate of the energy spared under isosmotic conditions remain controversial (Boeuf and Payan 2001 and Kidder *et al* 2006). In addition, optimal salinities for growth and metabolic rates are influenced by other factors, including species and developmental stage (Morgan and Iwama 1991).

Among freshwater ornamental fishes, Goldfish (*Carassius auratus*) having its origin in central Asia, China and Japan and is one of the earliest bred and highly domesticated. It was first reared in China in (1000 AD) followed by, Europe (1611) and America (1876 AD). Of the cyprinids domesticated, goldfish remains the most prominent and commonly used as ornamental and kept as expendable pet at home, offices and public places such as hotels, parks and tourist centers etc. Its aesthetic nature is as a result of its beautiful yellowish orange golden color. Over the centuries, goldfish has evolved into several varieties and Oranda, Lion head, Comet, Hibuna, Shubunkin, Black moor etc. are few of them (Matsui 1972). Goldfish varieties vary greatly in size, body shape, fin configuration and colouration (various combinations of white, yellow, orange, red, brown and black). Among major gold fish varieties, shubunkin is of much importance due to its unique body colouration and fin pattern along with advantage of hardy nature. Robust nature of shubunkin variety of gold fish can be considered advantageous for its culture in inland salt affected areas of Punjab.

Many reports are available regarding rearing of freshwater ornamental fish (gold fish, crucian carp and molly) in saline water, but all those studies pertain to natural/artificial sea water (Vasagam *et al* 2005, Schofield *et al* 2006 a, b and Küçük 2013). Further, due to varied ionic composition of inland saline water from sea water (Dhawan *et al* 2010), adaptability and tolerance of fish including goldfish to physical and chemical changes in water vary greatly. Most of the freshwater fish are adapted and can only survive in the freshwater; however some have great adaptations for saline water. Some are described as euryhaline having developed certain adaptive features that make them suitable in brackish water (Nikolsky 1963, Matsui 1972, Olanyian 1975, Hunner and Dupree 1984 and Boyd 1990). Schofield *et al* (2006 a, b) reported

that goldfish is able to persist in low salinity environments of <10 ppt for long period of time and higher salinities for short period. Wang *et al* (1997) reported salinity tolerance of gold fish similar to that of common carp (*Cyprinus carpio*), but higher than that of silver carp (*Hypophthalmicthys molitrix)* and lower than those of *Tilapia zillii*. Goldfish has been described as hardy to an extent of tolerating drastic changes in both physical and chemical qualities of water (Matsui 1972) and in general, described as purely freshwater fish. However, of recent, its culture in brackish water and marine environment had received much attention.

Most of the studies have been conducted to assess the tolerance and stress response of freshwater carps to either natural/ artificial sea water or salt solution, but no comprehensive study has been conducted so far in inland saline water. Several studies indicated that oligohaline water (<5 ppt) for freshwater fish species and osmotic water (10-15 ppt) for marine fish cause rapid growth. With changing environmental salinity, fish encounter morphological, biochemical and endocrinological modifications during acclimatization period, resulting in adjustments in oxygen consumption and energy demands. Although, both marine (<5ppt) and freshwater (10-15 ppt) fish demonstrated satisfactory growth performance in intermediate salinities, yet, every fish species has its optimum salinity range for growth. Further, the results of previous studies (Kasim 1983, Dhawan *et al* 2010, Ansal *et al* 2013 and Sharma *et al* 2017) conducted on salinity tolerance in common carp and ornamental koi carp (family Cyprinidae) indicates possibility of similar tolerance level in gold fish belonging to same family.

Among various ornamental fish species tested for salinity tolerance, gold fish and koi have been described as hardy to an extent of tolerating drastic changes in both physical and chemical qualities of water (Vasagam *et al* 2005, Luz *et al* 2008 and Küçük 2013). Stress due to salinity changes has also been reported to alter the haematological characteristics, associated with the immune responses governing the physiological status and pathologies (Poland and Kujawa 2011 and Akinrotimi *et al* 2012). It is pertinent to mention that all these studies carried out so far were either with artificial/natural sea water and practically no reports w.r.t. effect of inland saline water on survival, growth, colour development, stress responses and reproductive performance of ornamental fish is available.

Hence for optimizing the rearing technology of freshwater ornamental fish, gold fish var. shubunkin (*Carassius auraus* L.) in inland saline water areas, it is vital to study the stress response of this species at different salinity levels with tolerance range, so as to reach at the best

possible salinity level of inland saline water. Further, the difference in the ionic composition of the inland saline water and sea water (natural/artificial) along with compositional variability w.r.t. different sites or within the site, demands to carry out these kinds of research trials, so as to understand the optimum salinity level for particular fish species.

In view of the above background, the present research plan has been designed to study the performance of ornamental gold fish, *Carassius auratus* (Linn.) var. Shubunkin in inland saline water with the following objectives

1. To evaluate the salinity tolerance level of freshwater gold fish with respect to natural inland saline waters.
2. To assess the survival, growth and colouration in gold fish reared at optimized salinity levels.
3. To study the stress responses in gold fish reared at different salinity levels.

Chapter -II

REVIEW OF LITERATURE

Review of literature on effect of salinity (natural/ artificial sea water and inland saline water) on different food and freshwater ornamental fishes is presented under the following heads

2.1 Source and composition of inland saline water

2.2 Osmoregulation mechanism in fish

2.3 Salinity tolerance of freshwater fish

2.4 Effect of salinity on growth of fish

2.5 Effect of salinity on osmoregulatory responses in fish

2.6 Effect of salinity on haematological parameters of fish

2.7 Effect of salinity on hormonal and biochemical parameters of fish

2.8 Effect of salinity on colouration of fish

2.9 Effect of salinity on reproductive performance of fish

2.10 Effect of salinity on histological responses in fish

2.1 Source and composition of inland saline water

Salinity of water is termed as total quantity of salts present in water with major contribution of sodium (Na^+) and chloride (Cl^-) along with several other ions (calcium, magnesium, potassium, bicarbonate and sulphate). Concentration of these salts vary in different water sources viz., natural/ artificial sea water, inland saline water etc. and is generally expressed as milligram per litre (parts per million/ppm) in freshwater and as grams per litre (parts per thousand/ppt) in brackish water and sea water (Boyd and Tucker 1998).

Salinity in inland regions is generally due to availability of naturally salt affected soils or underground saline waters and water logging of lands due to intensive irrigation system. In humid areas, these soils do not exist extensively, because salts are continuously leached out of the soil due to excessive rainfall. However, in semi-arid and arid zones (where rainfall is less than evaporation) accumulation of salts increase many folds. In general, except for low potassium (K^+) concentration, ionic composition and concentration of inland saline water (ISW)

is similar to the sea water. Similar, to sea water, Na^+ and Cl^- are the major ions that determine the salinity of ISW. Low K^+ concentration in ISW is due to absorption of K^+ into the clay (Allan *et al* 2001). Although, K^+ concentration only contribute a small part in total ionic make up of ISW, but it plays an important role in functioning of physiological systems of aquatic organisms (Burton 1995 and Shiau and Hsieh 2001). Therefore, alterations of Na^+ and K^+ ratio may disturb entire physiological functions of aquatic organisms. Moreover, ISW composition varies in terms of both salinity and ionic make-up, which affects the fish survival and growth. The use of ISW as a medium for culture of marine fish and prawns can only be successful, if the K^+ deficiency can be eliminated e.g. by fortification of ISW with K^+, so as to match K^+ concentration of sea water.

2.1.1 Status of Inland Saline Water Aquaculture in World

Salt affected soil is spread over 1000 million hectare (mha) of land worldwide (Sharma *et al* 2011 and Sandeep *et al* 2013), of which secondary salinity has affected over 380 mha of land in more than 20 countries throughout (Lambers 2003). The most severely affected regions are Asia, the Pacific and Australia (Martinez-Beltran and Manzur 2005). Overall, 60 % salt affected inland ground water sources are present in Australia, where salinity varies from 5 ppt to 45 ppt. Moreover, the rising water table and continuously increasing salt content result in fertility loss, which is affecting agricultural activities. According to one of the estimate in 1996, about 2.2 mha of productive agricultural land was affected by salinity (Robertson 1996), with the prediction that if land will not be utilized in a judicious way, then the area will increase up to 3.5 mha by 2050 in West Australia.

Among Asian countries, in China, most of the inland saline water area is concentrated in North-Western China, while coastal salt pans or lagoons are distributed in Eastern China (Wen and Zhi-Hui 1999), with salinity ranging from 0.98 to 175.2 ppt. Inland salinity in Thailand is due to the contact of ground water with saline streets, whereas, in Pakistan, excess irrigation with inadequate drainage system has resulted in surface salinization.

Potassium deficiency is one of the common factor prevalent in all the inland saline affected waters worldwide (Saoud *et al* 2007b and Zhu *et al* 2004). Finfishes and shellfishes have been cultured successfully in inland saline water around the world (Saoud *et al* 2003) with satisfactory growth of various euryhaline species (Patridge and Jenkins 2002). The Western king prawn (*Penaeus latisulcatus*) and White legged shrimp or King prawn (*Litopeneaus vannamei*)

are commonly cultured shellfish species in Australia and USA respectively (Potter *et al* 1991 and Samocha *et al* 2001). Further in addition to shellfishes, saline ground water has also been utilized successfully for culture of various algae species along with finfishes such as tilapia, red drum, sea bream, eels and channel catfish (Ingram *et al* 2002 and Samocha *et al* 2001). Among finfishes, Barramundi, *Lates calcarifer* can tolerate salinity from freshwater to around 55 ppt and hence found to be one of the suitable species for inland saline aquaculture in both Australia and India (Patridge and Creeper 2004). Some of the earlier reports mentioned that about 30 % of shrimp culture in Thailand is carried out in inland saline areas.

2.1.2 Status of Inland Saline Water Aquaculture in India

According to earlier reports of IAB 2000, in India, about 9.38 mha of area is occupied by salt affected soils, out of this, 5.5 mha are saline soils (including coastal areas) and 3.88 mha are alkaline soils. However, as per recent reports (Sharma *et al* 2011), about 6.72 million hectare (mha) of area is occupied by salt affected soils, out of this, 2.95 mha (44%) are saline soils (including coastal areas) and 3.78 mha are alkaline/sodic soils. Saline soils are spread in the states of Gujarat, Andhra Pradesh, West Bengal, Orissa, Bihar, Haryana, Kerala, Karnataka, Maharashtra, Rajasthan, Tamil Nadu and Uttar Pradesh along with UT (Andaman and Nicobar Islands) and 3.77 m ha area is occupied by sodic soils (56%) in Indo-Gangetic Plain, arid and semi-arid region in Western, Central and Peninsular region in Southern India. In Indo-Gangetic Plains, 1.2 mha of inland salt affected soils in non-coastal states have hampered the agricultural output in the region including, Haryana, Rajasthan, Bihar, Uttar Pradesh, Madhya Pradesh, Jammu Kashmir and Punjab. Problem of underground salinity has further worsened due to water logging as a result of non-extraction of underground water and irrigation with canal water (developed to serve the affected areas for crop production). Abundant availability of saline groundwater in county has resulted in development of inland saline aquaculture at commercial level (Singh *et al* 2017).

Dwivedi and Lingaraju (1986) were the first to take up fish and shellfish (prawn) culture in ground saline water of semi-arid regions of Haryana in India. Studies conducted by these researchers reported good survival of brackishwater species viz, *Chanos chanos*, *Mugil cephalus*, *Etroplus suratensis* and *Penaeus monodon* at 8-10 ppt. Likewise, Sharma (2002) also reported salinity tolerance of 0.2-48, 0.5-36 and 0.2-39 ppt in *C. chanos*, *M. cephalus* and *L.*

calcarifer, respectively in surface saline waters of Lunkarnsar lake of Rajasthan. Rahman *et al* (2005) too indicated the possibility of culture of *P. monodon* in inland saline water. He mentioned that ionic composition of inland saline water differs from sea water and if fortification with potassium, magnesium and calcium salts carried out correctly, the survival of *P. monodon* can be improved. Raizada *et al* (2015) reported heavy mortality of *P. monodon* in potassium deficient inland saline waters, while in potassium supplemented inland saline water, the shellfish survived well at 5, 10 and 15 ppt. Reddy and Harikrishna (2014) developed technology for commercial production of *P. monodon* and *L. vannamei* in salt affected soils using inland ground saline water.

Freshwater fish species including Indian major carps were successfully acclimatized with satisfactory growth and survival in saline water of 8-10 ppt (Tewari *et al* 1994, Kumar 2002 and Pillai *et al* 2003). In addition to fish/shellfish culture, inland saline affected waters can be utilized successfully for potential microalgae species like *Spirulina* and *Dunaliella*. Sandeep *et al* (2013) reported suitability of *S. platensis* for mass cultivation in de-calcified inland saline water, with an added advantage of reuse of the spent medium for agriculture and aquaculture purpose. Further, Inland saline water areas of Rohtak (Haryana) with salinity ranging from >0.5 to 165 can also be utilized for producing *Artemia* cyst on commercial level (Singh *et al* 2017).

2.1.3 Status of Inland Saline Water Aquaculture in Punjab

Out of the total 5.0 mha geographical area of Punjab, ground water quality varies from good (59%) to marginal (22%) and poor (19%). The poor water have been further found to be saline (22%), sodic (54%) and saline-sodic (24%) (Sharma *et al* 2011). Punjab state is characterized with two distinct topographical and hydro-geological settings, high yielding fresh groundwater regions in northern and central districts and the saline underground water region in south western districts including Sri Muktsar Sahib, Ferozepur, Fazilka, Faridkot, Bathinda and Mansa. Underground water salinity of this region is neither fit for agriculture nor for industrial use or human consumption. Hence, due to non-extraction of underground saline water for agricultural, industrial or human use, canal irrigation and inadequate drainage, the water table in these districts is rising at an alarming rate of 15-20 cm/annum (Shakya and Singh 2010), which has further led to problem of water logging in the region.

In Punjab, 1.51 lakh ha (3.4%) in the south-western districts (Bathinda, Faridkot, Fazilka, Ferozepur and Sri Muktsar Sahib) is affected by salinity/sodicity and water logging, out of which. Among these districts, Fazilka is found to be severely affected by water logging. Salinity of underground water in these districts varied between 10-15 ppt, whereas salinity of water logged areas varied from 1-47 ppt. Further, water quality parameters (pH, conductivity, total alkalinity and total hardness) of water samples collected from different areas varied within wide range even at same salinities. Calcium hardness found to be many folds higher than even sea water. High salinities with high Ca^{2+} and Mg^{2+} resulted in very high total water hardness (Jana *et al* 2004) and have destroyed the productive agricultural land. Further, surface water salinity in water logged area is highest (1-165 ppt) as compared to village pond water (0-29 ppt) and underground water (0-15 ppt) (Dhawan *et al* 2009).

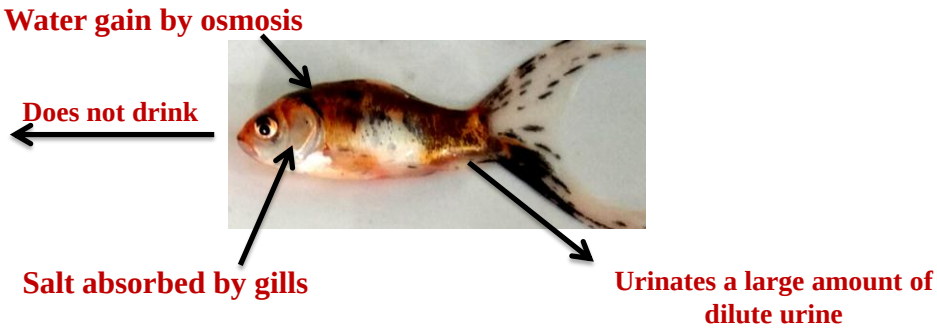
In the recent past, aquaculture technologies have been developed with special reference to rearing freshwater carps in low saline water. Freshwater carps (catls, rohu, mrigal, common carp and grass carp) were reared successfully in inland saline water under semi-intensive polyculture system, with an average productivity 2.48 t $ha^{-1}yr^{-1}$ at stocking density of 10,000 fingerlings ha^{-1}, which was latter enhanced to 3.40 and 4.75 t $ha^{-1}yr^{-1}$ through stocking density enhancement (15,000 and 20,000 fingerlings ha^{-1}, respectively) and species combination selection (Dhawan *et al* 2009, 2010, 2016 and Ansal *et al* 2013, 2016).

2.2 Osmoregulation mechanism in fish

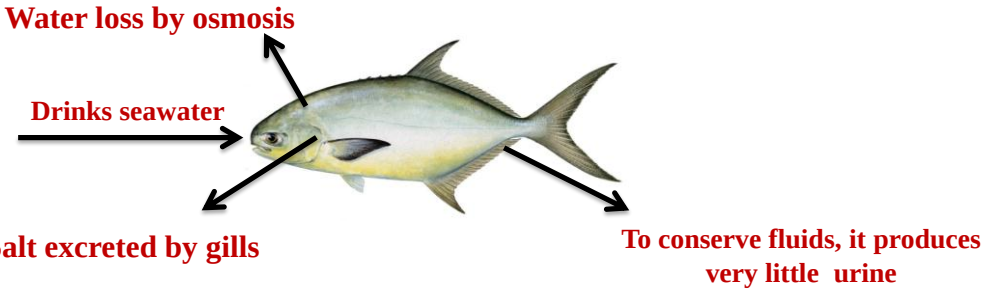
Osmoregulation is the active regulation of the osmotic pressure of an organism's body fluids, detected by osmoreceptors, to maintain the homeostasis of the organism's water content thus, it maintains the fluid balance and the concentration of electrolytes (salts in solution) to keep the body fluids from becoming too diluted or concentrated. Organisms in aquatic and terrestrial environments must maintain the right concentration of solutes and amount of water in their body fluids which involves excretion (getting rid of metabolic nitrogen wastes and other substances such as hormones that would be toxic, if allowed to accumulate in the blood), through organs such as the skin and the kidneys.

The salinity/osmolarity of aquatic habitats can be quite variable. Fish have evolved mechanisms for maintaining fluid and electrolyte homeostasis across a wide range of salinities. Marine teleosts, freshwater teleosts, and marine elasmobranches utilize different physiological

strategies for osmoregulation. Kidneys do play a role in osmoregulation, but overall, extrarenal mechanisms are more important sites for maintaining osmotic homeostasis. Extrarenal sites



Freshwater Fish: Salinity of freshwater much less than body fluids, so its body gains water from the surrounding environment



Marine Fish: Salinity of seawater much greater than body fluids, so its body fluids shed water to the surrounding environment

include the gill tissue, the alimentary tract, the rectal gland (elasmobranchs), and the urinary bladder. Through an understanding of osmoregulatory physiology in these three groups of fishes, the effects of any injury and disease on fluid and electrolyte balance can be explained (Greenwell *et al* 2003). Whether in freshwater or seawater, teleost fish maintain their plasma osmotic concentration at about one-third that of seawater. In freshwater fishes, this requires counteracting the passive gain of water and loss of ions, and is accomplished through the production of large volumes of dilute urine and active uptake of ions across the gills. In seawater, teleosts must counteract the passive gain of ions and loss of water. This is accomplished by drinking seawater, absorbing water and salts across the gut and excreting monovalent ions across the gills and divalent ions through the kidney. It has been estimated that 95% of teleost species are stenohaline, living wholly in either freshwater or seawater (McCormick 2001). The remaining

5% are euryhaline, having the capacity to tolerate a wide range of salinities. This trait is widespread among teleost lineages and has apparently evolved many times, and may be this is one of the reasons that teleosts can be found in almost all aquatic habitats.

The gills are the primary site of Na^+ and Cl^- transport, actively taking up salts in freshwater and excreting them in seawater. However, the site and mechanisms involved in ion uptake through gill in freshwater are less certain. Most of the recent work on the endocrine control of ion transport in fish has focused on the gills. It has been known for some time that the mitochondrion-rich cell (also known as chloride cell) is the site of salt secretion (Foskett *et al* 1983). Both chloride cells and pavement cells may be involved in Na^+ and Cl^- uptake. Cl^- is exchanged for bicarbonate (HCO_3^-) at the apical surface and leaves at the basolateral membrane moving 'downhill' on an electrical gradient. Na^+ may enter the gill epithelia in exchange with H^+ or through an apical Na^+ channel coupled to an apical H^+-ATPase, and then leave at the basolateral surface through Na^+, K^+ ATPase (Hiroi *et al* 1998 and Wilson *et al* 2000).

2.3 Salinity tolerance of freshwater fish

Studies on salinity acclimation, tolerance, metabolism and iono-osmoregulation in fishes have been made long back in various studies (Armitage and Olund 1962, Nelson 1968, Prosser *et al* 1970, Mackay 1974 and Kasim 1983) in a simple or complex manner with the role of temperature leading to multiple osmo-regulatory mechanisms. The highest salinity tolerance is obtained at temperature of 26-30 ^oC for most of the freshwater species (Kasim 1979). Among freshwater carps, common carp (*Cyprinus carpio*) is tolerant to salinity level of 8 ppt at 30^o C for longer time (Kasim 1983). Further, it is evident from the previous studies, that at low temperature, the salinity becomes more lethal even at low level (<12 ppt) and vice versa including factors like location, species and size etc. According to Ansal *et al* (2013), variations in water chemistry in different locations lead to varied salinity tolerance in carps w.r.t. species and size. In their study, common carp appeared most tolerant to inland salinity (up to 12 ppt) followed by rohu (10 ppt), catla (8 ppt), grass carp (8 ppt) and mrigal (2 ppt). Further, higher salinity tolerance was recorded in fry > 5 cm in size depicting the size based tolerance ability.

Mangat and Hundal (2014) also reported varied salinity tolerance of common carp (*C. carpio*) and rohu (*Labeo rohita*). The survival of common carp fingerlings was 100% up to 6 ppt during the 60 days rearing period, whereas 100% mortality was recorded in 12 ppt during

summer and 50% fish survived in the winter seasons. These observations indicated that the fingerlings of *C. carpio* were able to regulate their body physiology in this salinity-temperature regime. Islam *et al* (2014) also reported 100% survival of rohu at 0 to 6 ppt. Mortality was observed from day one at 12 ppt and 100% mortality in 3-4 days. Rani and Gulia (2015) observed the effect of different salinity levels (0, 2 and 4 ppt) on the survival of mrigal, *Cirrihinus mrigala* fingerlings. The results showed 100% fish survival even at highest salinity of 4 ppt along with normal responses up to 2 ppt indicating adaptability of mrigal up to 4 ppt. Further high appetite displayed by fish at 4 ppt is indicative of the fact that the fish is able to maintain normal body metabolism.

Schofield *et al* (2006 a,b) in their study indicated the adaptability of the goldfish *Carassius auratus* L. in low salinity environments (≤10 ppt) for long periods of time and for shorter periods of time at higher salinities. When shifted from fresh to saline water conditions (5-15 ppt), the goldfish survived for at least 72 hours (hrs). However, acute transfer to higher salinities of 20 -25 ppt resulted in 100 % mortality in first 8 hrs of experimental period. Similarly, Tarkhani and Imanpoor (2012) evaluated the effect of salt exposure on stress response in gold fish, *C. auratus*. Fish were kept in salt solution (10 ppt), for a period of 0.5 hr. Salt exposure resulted in rapid increase in cortisol with the peak at 3 hrs, which stayed elevated until 24 hrs and also caused rapid stress response, which get eliminated after 24 hrs in freshwater. According to Küçük (2013) the crucian carp (*C. carassius*) and gold fish (*C. auratus*) can be reared successfully at 8 ppt with 100 % survival along with tolerance up to 20 ppt.

Chervinski (1984) conducted experiments to study salinity tolerance of guppy (*Poecilia reticulata*) to various salt concentrations. The fish were subjected to abrupt and gradual changes from fresh water to various salinities. The results indicated that fish were able to tolerate the abrupt change from 100 or 150% saline water to fresh water. Salinity tolerance of newborn ornamental guppies indicated high tolerance ability even up to 35 ppt seawater (Shikano and Fujio 1997). Seawater tolerance significantly decreased within 5 days after birth and then became stable at a level comparable to adults. These results indicated possibility of culturing guppies in seawater and hence seawater-tolerant strain was established. Further, salinity tolerance was examined in 14 strains of the guppy. Positive correlation was not observed between the strain differences at birth and at the adult stage, but was observed between 10 days after birth and at the adult stage, suggesting genetic control for salinity tolerance at birth differs

from that of specimens over 10 days after birth. The results indicated maternal effect on salinity tolerance of newborn guppies at birth.

Martinez-Palacios *et al* (1990) too extensively investigated the physiology of the Mayan cichlid and reported successful acclimation up to salinities of 35 ppt by gradual increase of salinity to 5 ppt every 48 hours. They found the cichlids to be capable of withstanding direct transfer from freshwater to brackish water of 15 ppt at 28° C. However, half of the experimental fish died within six days, after being transferred directly to 24 ppt salinity. They were found to grow best at 10 and 20 ppt salinities. Despite the fact that Mayan cichlid tolerated salinity shift, other cichlid species may not be having similar flexibility due to stenohaline nature. Salinity tolerance of redhead cichlid (*Vieja synspilum*) was also studied by Martinez-Palacios *et al* (1995), in which fish was directly transferred to different salinity levels at 28° C. Fish showed 90% survival up to 10 ppt which reduced to 50% at 14.5 ppt, hence indicating less salt tolerant capacity as compared to Mayans.

In addition to freshwater carps, effect of salinity stress was also observed in freshwater catfish amd murrels. Kumar *et al* (2017) studied growth performance and survival of *Pangasianodon hypophthalmus* (Sauvage, 1878) in inland saline water with salinities 5, 10, 15, 20 and 25 ppt along with control (0 ppt). The results indicated that *P. hypophthalmus* can tolerate salinities up to 15 ppt in inland saline water, however 100% mortality was observed in 20 ppt and 25 ppt treatments after exposure of 48 hours and 18 hours, respectively. Osmotic study using serum osmolality, water osmolality and osmoregulatory capacity indicated that *P. hypophthalmus* cannot survive at higher salinities. Though *P. hypophthalmus* survived up to 15 ppt, but the optimum salinity for culture is 10 ppt in inland saline water. According to Dubey *et al* (2016), *C. punctata* is also stenohaline freshwater fish, exhibiting tolerances to low salinities, of only $\leq$ 10 ppt.

Euryhaline species respond to salinity stress in a different manner as compared to steno-haline freshwater species. In one of the study by Zaaim *et al* (2018) w.r.to additive effect of stocking density and salinity tolerance on the growth and survival of golden *Anabas* fry. Golden *Anabas* fry was cultured in water with salinities of 0, 5, 10 and 15 ppt, at stocking of 3 fry/ L, in 8 L aquaria. Survival percentage was not significantly different (p>0.05) among the treatments indicating salinity tolerance of golden *Anabas* fry up to 15 ppt salinity. Partridge and Jenkins (2002) studied growth and survival of juvenile black bream (*Acanthopagrus butcheri*) at 0 to 60 ppt (in 12-ppt

increments) and 0 to 12 ppt (in 4-ppt increments). The Juvenile black bream survived and grow well in freshwater to 48 ppt salinity. Osmotic stress was indicated at 60 ppt, however, survival was not significantly affected. Saoud *et al* (2007a) studied the effects of salinity on survival, growth, plasma osmolality and gill Na^+ - K^+ - ATPase (NKA) activity in the rabbitfish *Siganus rivulatus*. In their study they conducted three experiments, in the first experiment fish were kept in nine salinities ranges between 10 and 50 ppt for 3 weeks and the survival and plasma osmolality evaluated. In the second experiment, fish were kept in salinities of 25, 30, 35 and 40 ppt for 6 weeks to study survival, growth, blood osmolality and gill NKA. In the third experiment, fish were kept in salinities of 10, 15, 20 and 25 ppt for 8 weeks and again survival, growth, blood osmolality and gill NKA were evaluated. The results from the first experiment indicated that *S. rivulatus* survived at salinities from 10 to 50 ppt for 3 weeks and they maintained a relatively stable blood osmolality. In the second experiment, no differences in survival and growth of *S. rivulatus* in all treatments. However, gill NKA was lowest at 35 ppt and increased at salinities above and below 35 ppt. In the third experiment, the growth decreased at 10 ppt and survival was similar at all salinity treatments with increase in gill NKA activity with decrease of salinity.

Garcia *et al* (2003) studied the salinity tolerance of seahorse *Hippocampus kuda* juveniles without prior acclimatization to different salinities ranges. *H. kuda* survived in dilute seawater (15 ppt) for at least 18 days, without any change in growth, survival, and total body water. Fish abruptly transferred to freshwater succumbed within 4–24 hrs, while survival of 5 ppt reared fish decreased to 65% in 18 days and the 50 ppt was found to the upper limit of salinity tolerance. Tandler *et al* (1995) studied the effects of salinity (15-40 ppt salinity, 450-1200 mOsm kg^{-1}) on growth, survival and swim bladder (SB) inflation in gilthead seabream (*Sparus aurata*). Within the range tested, 1 day old larvae maintained an osmotic pressure of 224 mOsm kg^{-1}, significantly lower than 410 mOsm kg^{-1} in 13 and 24 day old larvae. A negative relationship was found between salinity and survival as the rearing salinity decreased from 40 to 25 ppt, rate of survival increased from 5.3 to 18.6%. Moreover, reduced salinity induced a significant increase in the rate of SB inflation from 65 to 92.5% in 40 and 25 ppt acclimated larvae, respectively, depicting adaptation mechanism to low salinity

2.4 Effect of salinity on growth of fish

Development and growth are controlled by internal factors including central nervous system (CNS), endocrinological and neuro-endocrinological systems along with environmental conditions. Among the ecological factors, salinity is specific to aquatic environment, as it control and synchronize physiological activities or functions, including growth capacity (Boeuf and Payan 2001). Salinity is one of the most important abiotic factors affecting growth of aquatic organisms and has complex and wide ranging biological effects (Gaumet *et al* 1995 and Boeuf and Payan 2001). It is important to determine the optimum salinity level for every fish species in intensive system, so that the salinity can be altered to fit the optimum range for particular species. Salinity influence growing capacities in larger fish, juveniles or adults and it appear that marine fish showed improved growth rates at lower salinity and fresh water fish at higher salinity as compared to their native environment.

Number of research trials has already been conducted to study on the effects of environmental factors including temperature, photoperiod, salinity etc. on fish growth (Clarke *et al* 1981, Wang *et al* 1997, Swanson 1998, Altinok and Grizzle 2001, Imsland *et al* 2001, Moustakas *et al* 2004, Engstrom- Ost *et al* 2005, Resley *et al* 2006, Arjona *et al* 2008, Imsland *et al* 2008, Kang'ombe and Brown 2008, Kearney *et al* 2008, Luz *et al* 2008, Overton *et al* 2008 and Wang *et al* 2008). Results of these studies clearly indicated that salinity affects the fish growth to a greater extent. There is an accepted hypothesis of how salinity affects energy budget in fish. If salinity is too high or too low in the external environment (depending on fresh water or marine fish), then much of it is being used for active ion transport *i.e.* fish spends more energy to regulate osmotic balance. Therefore, energy available will be less for growth in these environments, because of the use of too much energy for active ion transport (Boeuf and Payan 2001). Further, according to Bishai (1961), the salinity affects the survival and growth of fish directly due to the differences in the ionic composition in internal and external environment or indirectly by affecting the amount of natural food in the form of plankton, that constitute the main food for the early life stages.

Most of the studies have been conducted to know the effect of salinity on growth of food fish (Wang *et al* 1997, Imsland *et al* 2001, Rubio *et al* 2005, Resley *et al* 2006, Arjona *et al* 2008, Imsland *et al* 2008, Kang'ombe and Brown 2008, Overton *et al* 2008 and Arjona *et al*

2009). A few studies have been carried out to know the role of salinity on growth and metabolism in ornamental/aquarium fishes (Altınok and Grizzle 2001, Vasagam *et al* 2005, Schofield *et al* 2006 a,b, Luz *et al* 2008 and Küçük 2013). Altinok and Grizzle (2001) indicated that ≥9 ppt of salinity negatively affected goldfish growth. In this study, growth of goldfish was higher at 8 and 12 ppt (0.53 and 0.34% day^{-1}), when compared with fish reared at 20 ppt (0.06% day^{-1}). Although, growth of crucian carp was not high enough (0.22% day^{-1}, 0.30 % day^{-1}) in the SW (8 ppt) and 50% SW (12 ppt), it was too low in the 100% SW (16 ppt) (0.08% day^{-1}) and became negative (-0.06% day^{-1}) in the 150% SW (20 ppt).

Luz *et al* (2008) studied the adaptability of goldfish *C. auratus* at different salinities (0, 2, 4, 6, 8 and 10 ppt) on food consumption, growth, metabolic resources, and several stress indicators. Additionally, possible changes in feeding regulators, brain neuropeptide Y, circulating ghreline, and the hypothalamic monoaminergic transmission were also examined. Salinities up to and including 6 ppt did not affect weight gain, standard growth and feed conversion rates. The goldfish showed good adaptation to these salinities in terms of metabolic resources (lipids and glycogen content in liver and muscle) after 21 days of salinity exposure. Salinity tolerance of juveniles of comet goldfish, *C. auratus* was exhibited at levels ranging between 0 and 3 ppt (Lawson and Alake 2011). However, maximum adaptation was noticed at 0 ppt or freshwater. 100% survival recorded between 0 and 3 ppt salinities could be attributed to the ability of fish to develop osmoregulatory adaptations, which enabled the species to regulate osmotic pressure of its body fluid (Nikolsky 1963). The 60 and 40 % survival rates were observed at salinities 4 and 5 ppt indicated that fish were drifting away from their tolerance limit and fish can still regulate their body fluid in order to restore the osmotic pressure to normal within these salinities. High mortality rate (100%) from 6 to 10 ppt salinity was due to extreme stress condition, which occurred due to the inability of the fish to maintain osmotic balance between the salt concentration of its body fluid and that of its environment (i.e. 6-10 ppt). There was decrease in total body length with increasing salinity. The length gain was observed between 1 and 5 ppt with highest total length gain of 47.62% in freshwater. However, there was no length gain between 6 and 10 ppt salinities. Similarly, Jalali *et al* (2013) studied the effects of different salinities (0, 3, 6, 9, 12 and 15 ppt) on growth and survival of Shyrbot (*Barbus grypus*) fingerlings for a period of 60 days. They observed no significant effects on weight gain (WG), percentage body weight gain (% BWG), specific growth rate (SGR), daily growth rate (DGR)

and the condition factor (CF) at 0 to 6 ppt while, negative effects were observed at higher salinity i.e. 9, 12 and 15 ppt on FCR and other growth parameters.

Schofield *et al* (2006 a,b) indicated in their study, regarding ability of the gold fish to withstand low levels of salinity and can invade estuarine areas periodically, especially during periods of high rainfall. Therefore, the species may be capable of using estuarine regions to gain access to new river systems and expand its distribution. Although the goldfish can survive at low (≤10 ppt) salinities for an extended period of time, these conditions may be stressful to the fish. Most of the previous reports indicated that holding goldfish at low salinities resulted in changes in the distribution and density of mitochondria rich chloride cells, evokes increase in blood cortisol, increases oxygen consumption by 54 to 64%, increases urea-N excretion and decreases specific growth rate and feed conversion ratio.

Woo and Kelly (1995) found that growth rates of euryhaline sea bream cultured at 15 ppt were consistently higher than those at other salinities. It seems that when saline water used for culture in case of Thai strain koi fish (*Anabas testudineus)*, higher production can be achieved and growth is high in 8 ppt than in 10 ppt and higher than control (Chowdhury *et al* 2014). The survival rate of koi fish during the period of experiment was 91.67 %, 100 % and 95.83 % in control, 8 ppt and 10 ppt. Further, the fish survival in changing salinities depends upon the ability of body fluid to tolerate changes of osmolality and ion concentrations (Stickney 1986). Riley *et al* (2002) reported that keeping Mozambique tilapia, (*Oreochromis mossambicus*) in salt water significantly activates the growth hormone resulting in higher growth rate of fish. The nile tilapia, *O. niloticus* exhibits a moderate tolerance to salinity with 60 day old fish surviving direct transfer upto 25 ppt (Watanbe *et al* 1985). Fish appeared to grow better at moderate salinities than in freshwater (Kamel and Mair 2005, Suresh and Lin 1992). Further, differences in growth for tilapia observed between environments with varying salinity (Massou *et al* 2004).

Rani and Gulia (2015) studied on the effect of different salinity levels (0, 2 and 4 ppt) on the growth of fingerlings of *C. mrigala* fingerlings. Maximum increase in body weight was recorded in fish placed at 0 ppt and minimum in fish kept at 4 ppt salinity. Live weight gain remained high (0.61±0.01) in 0 ppt as compared to 4 ppt (0.03±0.05). Further, specific growth

rate (SGR) decreased as the salinity level increased. The highest SGR was recorded in 0 ppt (3.92±0.01), whereas it was lowest in 4 ppt (0.2±0.04).

2.5 Effect of salinity on Osmoregulatory responses in fish

Salinity affects various physiological processes in aquatic animal such as metabolism and osmoregulation, which is particularly significant for euryhaline migratory fishes that experiences both freshwater and sea water during their life spans. Kim *et al* (1998) and Evans *et al* (1999) mentioned osmoregulation in the maintenance of consistent blood and intracellular volume in the face of changing environmental osmolarity. Osmoregulation involves transportation of Na^+ and Cl^-, because these are the dominant cation and anion in the extracellular fluids and the aqueous environments of fishes (Evans *et al* 1999). There is difference between freshwater and marine fish in prolonging the metabolism of their homeostasis in water (Boyd 1990 and Boyd and Tucker 1998).

It is generally recognized that Na^+, K^+, Ca^{2+} and Cl^- are primarily responsible for osmoregulation in fishes, either by excreting ions i.e. Na^+ and Cl^- or by affecting uptake and by excreting K^+ and Ca^{2+}. Osmoregulation is energy consuming process in fish and it is carried out mainly via branchial chloride cells. A reduction in fish growth when reared in non-optimal salinity waters could be due to an increase in Na^+ and K^+ activity, accompanying energy expenditure (Bouef and Payan 2001). Understanding the physiological characteristics of this transition will help in better understanding of each regulatory system and their most essential components for water and ion transport (Genz *et al* 2008). The various metabolic events in tissue and organs system of fish are regulated by their electrolytes (Prosser 1973). Hilmy *et al* (1982) described that electrolytes of body fluids have various functions, the most of which are to contribute a majority of the osmotically active particles to provide buffer system and mechanism for the regulation of pH (acid-base) balance (Burton 1973), so as to provide ionic balance for normal neuromuscular irritability and tissue functions.

Na^+ is the chief regulator of osmotic pressure of the body fluid, as it initiates and maintains the heart and involuntary muscles and excites the nerves. In freshwater fish, Cl^- exchange across the gill epithelium for bicarbonate ions (or other anion equivalents) and this exchange help to regulate acid-base balance (Williams and Eddy 1988). Further, Cl^- ions along with Na^+ and K^+ play an important role in neuromuscular excitability, acid base balance and

osmotic pressure of the body. A significant decrease in tissue/plasma K^+ concentrations was observed in rainbow trout with in various temperature ranges (Gonzalez and McDonald 2000); in juvenile Siberian sturgeon (*A. baerii*) with various osmolarities (Rodriguez *et al* 2002); in tropical fish, *Prochilodus scrofa* (Cerqueria and Fernandes 2002) in transition from freshwater to seawater.

Salati *et al* (2011) studied the influence of environmental salinity on osmoregulatory mechanisms in the common carp (*C. carpio*). Seventy-five healthy adults of *C. carpio* were randomly divided into five groups kept in salinities of at least 3, 6, 9 and 12 g l^{-1} NaCl and a control (fresh water). The salinity was gradually increased by 3 g l^{-1} daily, until the desired concentration for each group was reached. Then the urinary papilla was catheterized to allow urine collection. Urine was collected once daily for 14 days, blood and gill tissue were sampled and plasma electrolytes and gill NKA activity were assayed. Plasma sodium and chloride significantly increased in response to increased environmental salinity ($P \leq 0.05$). Gill NKA activity significantly increased in response to increased environmental salinity ($P \leq 0.05$). The results indicated that common carp can tolerate environmental salinities up to at least 12 g l^{-1}, but survival at salinities of at least 6 g l^{-1} and higher requires profound changes in function of gill and kidney as principal osmoregulatory organs.

In a comparative study by Nordlie *et al* (1992) on salinity tolerances and osmotic regulatory capabilities in sailfin mollies, *P. latipinna,* inhabiting both fresh and brackish waters. Following extensive acclimation, individuals from freshwater populations tolerated a range of salinities from freshwater through 70 ppt, whereas brackish waters individuals tolerated salinities from freshwater through 80 ppt. Moreover, acclimatization in freshwater and brackish water not altered the physiological capabilities of fish to greater extent with respect to ambient salinity.

Nakano *et al* (1997) studied the metabolic aspects of osmoregulation in tilapia; *O. niloticus*, which was reared in fresh water (FW), and 50% seawater (SW), while *O. mossambicus* was kept in FW, 100% SW and 160% SW for 4 weeks. There were no significant differences in plasma concentrations of growth hormone and Na^+ and CI^- among the fish acclimation to different salinities in both the species, indicating successful adaptation of fish to each salinity. In their results, the activities of phosphofructokinase, glycogen phosphorylase, and glucose-6-phosphate dehydrogenase in *O. niloticus* were higher in 50% SW than in FW, while there were

no difference for *O. mossambicus* among different salinities. In *O. niloticus*, the level of plasma glucose was also higher in hyperosmotic environment than in FW and also the liver metabolic activity showed an increase. Hence the study dictates different efficiency in energy utilization for hypo-osmoregulation between the two species of tilapia.

Sui *et al* (2016) studied the physiological parameters of blood parrot cichlid (*Cichlasoma synspilum*) by adding salt in the water. The effects of salinity on serum osmolality, immune-related enzyme activities, NKA activities in the gill, skin carotenoid content and oxygen consumption were analyzed. Results indicated no significant differences in serum acid phosphatase and alkaline phosphatase activities, skin carotenoid content and oxygen consumption rate among the different groups.

Foskett *et al* (1983) too described that the teleost fish osmoregulation is mainly regulated by integrated transport activities of the gill, gut and renal system. The basic 'epithelial fabric' in each of these tissues is adapted to provide the appropriate transport mechanisms depending upon whether the fish is in fresh water or sea water. Cortisol stimulates chloride cell proliferation and differentiation and appears to interact with NaCl to initiate salt secretion. Prolactin appears to cause chloride cell differentiation by reducing both the active-transport and leak pathways proportionately. Prolactin and cortisol also affect epithelial cell proliferation and differentiation in the other osmoregulatory tissues in fish, suggesting that these hormones are primary agents in the integration of transport activities to achieve overall osmoregulation.

2.6 Effect of salinity on haematological parameters of fish

Haematology is considered as indicator for different stressful condition such as handling, pollutants, metals hypoxia, anesthetics, acclimation to varied conditions of temperature, salinity etc. (Blaxhall 1972, Duthie and Tort 1985, Ogbulie and Okpokwasili 1999 and Alwan *et al* 2009). Fish are known to be in close relationship with the aqueous environment. Hence, blood will reveal conditions within the body of fish long before there is any visible manifestation of disease (Musa and Omoregie 1999 and Okechukwu *et al* 2007). Haematological indices are therefore widely used by fish biologists and researchers, the world over to know the effect of different stresses. Stress due to salinity changes has been reported to alter the standard hematological characteristics of teleosts, elevating plasma corticosteroids (Barton and Iwama 1991 and Yada and Nakansishi 2002), reducing levels of some of the blood parameters and also

increasing the values the some of the blood components. These changes can affect oxygen transports in the blood and across the gills. According to Akinrotimi *et al* (2007), these alterations depend on the species, age, sex, environment and the nature of the stressor.

Red blood cells, is composed mainly of haemoglobin surrounded by flexible protein membrane and outer lipid layer, and the energy required for maintaince of red cell shape, flexibility and osmotic pressure is provided by adenosine triphosphate (ATP), generated by anaerobic glycolysis (Cheesbrough 2005). The depletion of ATP as a result of imposed stress due to acclimation, results in inability of red cells to transport excess sodium out of cell membrane and subsequent haemolysis of the red cells (Emelike *et al* 2008). Thus, the red cell life span becomes so short, that cells are destroyed much faster than they can be formed. The erythrocyte sedimentation rate (ESR) is the speed at which red cells settled out of their plasma (Seiverd 1983). ESR is non-specific haematological parameter that may indicate presence and intensity of diseased state (Gabriel *et al* 2004). The values are usually raised with increased tissue destruction as in acute infections and heavy metal poisoning among others (Blaxhall and Daisley 1973). The increased ESR value may be due to fragility of erythrocytes as a result of stress induced disruption in the formation and release of erythrocyte from hematopoetic tissue (Akinrotimi *et al* 2007). Along with increased ESR, changes in white blood cell (WBC) and the differential counts, neutrophils, lymphocytes and monocytes are also indicator of stressed conditions.

In one of the study, Al-Hilali and Al-Khshali (2016) investigated the effect of high salinity on the blood parameters of common carp (*C. carpio*), which were exposed to different salinities (5, 10 and 5 ppt). As per observations, the level of haemoglobin (Hb), packed cell volume (PCV), red blood cells (RBC) and white blood cells (WBC) increased with increase in salinity. However, according to Akinrotimi *et al* (2012) blood parameters of *Tilapia guineensis* exposed to different salinities i.e. 5, 10 and 15 ppt for a period of 7 days showed different trend. Results of the study indicated that with increased salinity exposure, the Hb, RBC, PCV, mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and thrombocytes decreased, whereas, the WBC, ESR neutrophils, lymphocytes and monocytes increased significantly. According to Imanpoor *et al* (2012), the adaptability of goldfish (*C. auratus*) at different salinities (0, 6 and 12 ppt) had not effected the food consumption, growth, blood biochemistry and the haematocrit level. Moreover,

growth of fish improved in water having maximum salinity (12 ppt) and temperature (31^{o}C). Further, Salinity, temperature and the interaction between these two variables had no effect on haematocrit, glucose and triglyceride of goldfish larvae. In a similar study by Denson *et al* (2003) at various salinities of 5, 15 and 30 ppt on Cobia, *Rachycentron canadum* juveniles for 10 week, the growth and survival of fish was significantly affected with increase in the salinity level. Further, cobia was found to tolerate exposure to low salinity environments for short periods of time with no mortality.

Elarabany *et al* (2017) studied haematological parameters in Nile tilapia *O. niloticuus* exposed to different salinity concentration. Fishes were kept in artificially prepared salt solution by using NaCl @ 4, 8 and 12 ppt in 4 equal groups. Results indicated that RBC, HCT and Hb concentration, were significantly higher in 4ppt, while other parameters such as WBC, haematometric indices (MCV, MCH, MCHC), malondialdehyde (MDA) level, carbonyl protein (CP), glutathione reductase (GR) activity, as well as serum sodium, glucose, cortisol and IgM, did not show any significant differences in the different salinity concentrations.

2.7 Effect of salinity on hormonal and biochemical parameters of fish

Four hormones viz., gonadotropin, cortisol, insulin-like growth factor 1 and thyroid hormones play important role in osmotic regulation (McCormick 2001). Salinity variation is one of the stress causing factors for both freshwater and marine fish, as it affects fish in three dimensions. Under conditions of stress, the body of the fish emits immediate responses recognized as primary and secondary responses. The primary response is the perception of an altered state by the central nervous system (CNS) and the release of the stress hormones, cortisol and catecholamines (adrenaline and epinephrine) into the bloodstream by the endocrine system (Randall and Perry 1992). Secondary responses occur as a consequence of the released stress hormones (Barton and Iwama 1991), causing changes in the blood and tissue chemistry, e.g. an increase of plasma glucose (Barton 1997 and Begg and Pankhurst 2004). This entire metabolic pathway produces a burst of energy to prepare the fish for an emergency situation (Rottmann *et al* 1992).

Some plasma chemicals may be useful tools to evaluate the health and/or stress condition of the fishes (Sadler *et al* 2000 and Wagner and Congleton 2004). Because stress has been reported to elevate plasma cortisol (Wendelaar-Bonga 1997, Pottinger *et al* 2003 and Haukenes

et al 2008**)** and glucose levels (Silbergeld 1974 and David *et al* 2005), many researchers consider as a "rule of thumb" that fish undergoing stressful situations exhibit plasmatic increases of cortisol and glucose (Hattingh 1977, Balm *et al* 1989 and Barcellos *et al* 1999). In spite of the extensive use of cortisol and glucose levels as stress indicators, there are some inconsistencies in the results of various experiments that in some cases would be attributed to unknown situations.

Cortisol has been considered as a primary stress response indicator, which increases rapidly after stress, therefore, circulating levels of this hormone are commonly used as an indicator of the degree of stress experienced by fish (Wendelaar-Bonga 1997 and Barton 2002). Hyperglycemia is secondary stress, which is stimulated by primary stress response (release of catecholamines and corticosteroids) to supply demanded energy to cope stress (Smith and Piper 1972), which could cause respiratory distress. Cortisol not only activates glycogenolysis and gluconeogenesis processes in fish; but also causes chromaffin cells to release of catecholamines, which further increase glycogenolysis and modulate cardiovascular and respiratory function (Reid *et al* 1992, 1998). This whole process increases the substrate levels (glucose) to produce enough energy according to the demand. Glucose is a carbohydrate that has a major role in the bioenergetics of animals, being transformed to chemical energy in the form of ATP, which in turn can be expressed as mechanical energy. In sub-optimum or stressful conditions (internal or external), the chromaffin cells release catecholamine hormones i.e. adrenaline and noradrenaline toward blood circulation (Reid *et al* 1998). These stress hormones in conjunction with cortisol mobilize and elevate glucose production in fish through glucogenesis and glycogenolysis pathways (Iwama *et al* 1999) to cope up with the energy demand produced by the stressor. This glucose production is mostly mediated by the action of cortisol which stimulates liver gluconeogenesis and also halts peripheral sugar uptake. Glucose is then released from liver and muscle toward blood circulation and enters into cells through the insulin action (Nelson and Cox 2005).

Regardless of the wide use of glucose as an indicator of stress, some authors (Mommsen *et al* 1999 and Flodmark *et al* 2001) emphasized that care has to be taken when using plasma glucose as the only indicator. It has been reported that glucose content is a less precise indicator of stress than cortisol (Pottinger 1998). Some authors reported a little rise in glucose level (Davis and McEntire 2009), others found no change (Rotllant and Tort 1997 and Jentoft *et al* 2005) and

even a decrease (Wood *et al* 1990). Sometimes no significant changes in plasma glucose may be observed, because under stress, the fish is rapidly consuming the generated energetic substrates (glucose), since the main function of the central nervous system (CNS) is to maintain homeostasis. Mommsen *et al* (1999) were sceptical about the use of glucose as a stress indicator, whereas, Simontacchi *et al* (2008) stated that glucose and cortisol "cannot be considered itself as reliable stress indicators".

In addition to cortisol and glucose, changes in concentration of serum total protein and its components may be used as clinical indicator in assessment of the fish health, stress status and body conditions of particular species and it is an important non-specific immune parameter (Magnnadottir 2006). Kucuk *et al* (2013) and Kavya *et al* (2015) observed increase in total serum protein with increasing salinity in *O. aureus* and *Notopterus notopterus* exposed to 12 ppt and 1.6 ppt respectively. Whereas, Shahkar *et al* (2015) reported decline in plasma protein during increase of salinity in juvenile ship sturgeon (*Acipenser nudiventris)*. Huang *et al* (2006) were of the view that, as environmental salinity increases, energy consumption increases, which is provided through glucose and lipid through metabolism. Thus, when the sufficient energy is not available in the form of glucose, proteins would be utilized as energy source

2.8 Effect of salinity on colouration of fish

Pigments present in the fish skin and muscle are known as carotenoid, which are responsible for colour expression. Through nervous system and hormonal control, the pigment granules of the chromatophore cells in fish body can be transported leading to intense colouration and can also aggregate resulting in light colouration. Depending upon inheritance, different individuals in the same population can have different deposition abilities for carotenoids. Additionally, fish age, size, physical conditions, internal neuroendocrine systems and androgenic hormones also influences pigment deposition. Further, number of environmental factors including temperature, illumination, pollutants, low dissolved oxygen, high ammonia and salinity changes also influences carotenoid deposition resulting in deviation from normal colouration (Qiufen *et al* 2012).

Pigment patterns of fish vary w.r.t physiology, developmental biology, behavioral ecology, evolutionary biology, etc. (Price *et al* 2008). Physiological color change involves a

displacement of pigment within the chromatophore and is usually seen as an adaptation to background or as a response to acute environmental change (temperature, pollutants, salinity etc.). Morphological color change results from a change in the number of chromatophores and in the quantity of pigment in the skin. The physiological alterations affecting morphological color changes are found in both poikilotherms and homeotherms (Abbott 1973). Colouration/carotenoid content of the skin in ornamental fish is one of the major criteria, which decides its economic value. In a recent study, Eslamloo *et al* (2015) indicated that background colour could affect goldfish skin pigmentation. The carotenoid concentration in the skin significantly decreases in white background in comparison with the other groups. They also revealed that red and blue backgrounds are chronically stressful and immunosuppressive for goldfish, therefore white backgrounds should be used for culture of gold fish.

Weber and Holmes (2010) suggested salinity variation pattern resulting in changing colouration pattern of the common starfish (*Asterias rubens*). The colouration pattern of this species is very broad ranging from bright purple to orange, pale brown and beige. It is suggested that salinity is involved in shaping colouration in *A. rubens*. In saline water of 30- 34 PSU (practical salinity unit), the bright colours which are found in water with lower salinity (15-30 PSU) disappear. The results confirm the hypothesis that coloration patterns are genetically independent (Harley *et al* 2006).

Moreover, during transportation of ornamental fish, salt is usually added to water as sedative and prophylaxis to maintain freshwater fish in a good state of health (active and brightly coloured). However, the physiological effects of increased salinity on freshwater ornamental fish are unclear. In one of study by Lawson and Alake (2011) in gold fish exposed to different salinities, normal gold colour in fish was maintained up to four days and thereafter a change to bleached yellow colour was observed. According to Sui *et al* (2016), normal body colouration and carotenoid content of ornamental blood parrot fish did not show variation from normal colour, when exposed to from freshwater (0 ppt) to 7.5 ppt for 7 days.

2.9 Effect of salinity on reproductive performance of fish

Among various water quality parameters, salinity is one of the major factors influencing reproductive physiology in fish as observed by Vasagam *et al* (2005) in terms of enhanced fry yield at 25 ppt and improved growth at 10 ppt in sailfin molly (*P. latippina*) along with better

colouration as compared to freshwater reared counterparts. Salinity has direct effect on fertilization, embryogenesis, larval and egg survival and growth (Boeuf and Payan 2001). Several investigators have demonstrated the wide salinity tolerance of ovoviviparous poecilid fishes such as *P. reticulate* (Aria *et al* 1998) and *Limia melanonotata* (Haney and Walsh 2003). Further, the salinity may also have affected the endocrine system of the fish, as evidenced by continous chasing of female fish by the male fish. Similar results were made by Milton and Arthington (1983) in molly. The gradual increase in fry production with each spawning may be attributed to the positive correlation between fish body weight and fry production (Tamaru *et al* 2001). Haddy and Pankhurst (2000) investigated the effects of varied salinities of 5, 20 or 35 ppt on seasonal reproductive development, plasma steroid levels, the efficacy of luteinizing hormone releasing hormone ethylamide (LHRHa) to stimulate ovulation, sperm motility, egg fertility and hatching. Gonadal maturation was not affected by salinity in both the sexes. In females, the plasma steroid females were unaffected by salinity, while in males the level of plasma steroid were higher. Dhawan *et al* (2010) reported successful acclimation of freshwater fish common carp in saline water (inland) along with satisfactory growth at optimum salinity level of 8 ppt. Morover, the fish was able to attain maturity and bred successfully with > 90% and 95-98% fertilization and hatching rate respectively and satisfactory growth up to fry (3-5 cm) stage within 30 days. The logical reason for successful breeding can be the potential of common carp to produce progeny having high salinity tolerance, more than that of their parents.

Low concentration of salt is found to have positive impact on egg hatching in African catfish, *Heterobranchus longifillis* (Fashina – Bombata and Busari 2003). Salinity is of particular importance in salty or slightly salty water fish, for example in gilthead sea bream *Sparus aurata* L. and flat fish (Gavlik and Specker 2004, Wang *et al* 2007, Kearney *et al* 2008 and Appelbaum and Jesuarockiaraj 2009). Similar relationships have been observed in poecilids (molly) indicating successful rearing and breeding in water having salinity ranging from 6.7 to 20 ppt. Male and female of euryhaline species like nile tilapia (*O. nitoticus*) responded differently to increasing salinity (Schofield *et al* 2011). Mean GSI for male did not differ significantly among treatments (0, 10, 20, 30, 40, 50, 60, 70 and 80 ppt), but ovary development delayed with increasing salinity along with significant decline in number of eggs produced significantly at salinity of 40 ppt and above. Further, it was also observed that nile tilapia withstood acute transfer (direct transfer from freshwater to saline water) up to 20 ppt and survived chronic

gradual transfer up to 40 ppt. In general, fresh water teleosts embryos are able to tolerate low salinity, as demonstrated by Bart *et al* (2013) in nile tilapia. Embryo hatchability was higher at 4 ppt as compared to fresh water or 12 ppt.

It was studied that the mother's environmental salinity enhances the salt water tolerance of the offsprings, indicating a successful mechanism of reproduction of an ovo-viviparous fish in saline water. The influence of parent's environmental salinity on their offspring was also observed in oviparous fish. Lee *et al* (1981) reported that tolerance to low salinity was higher in fertilized eggs from parents acclimatized to low salinity than in those without parental acclimation in *Sillago sikama.*

2.10 Effect of salinity on histological responses in fish

Histopathological changes in animals tissues are powerful indicators of prior exposure to environment stressors and are net result of adverse biochemical and physiological changes in an organism. The fish gills play an important role in maintaining whole animal ionic homeostasis in both freshwater and marine environment. Fish gills are the organs, where gas exchange, ionic regulation, acid base adjustment, as well as the discharge of nitrogenous wastes occurs. An increase in mucous cell density and secretions in fish has been shown to be correlated with an increase in salinity (Roberts and Powel 2003), elevated pH and ammonia concentrations (Lease *et al* 2003), along with microbial and parasitic infection (Dezfuli *et al* 2010). Because fish gills are always exposed to the external environment and are quite sensitive to the chemical and physical changes of water (Pandey *et al* 2003). Histopathological changes in the fish gills are used as an important tool to determine the negative effects of many pollutants (Rašković *et al* 2013). Injury to gill epithelium is a common response observed in fish exposed to a variety of contaminants. The severity of damage to the gills depends on the concentration of the toxicants and the period of exposure. Along with gill, liver is also one of the target organ due to its large blood supply that causes noticeable toxicant exposure and accumulation and also its clearance function and its pronounced metabolic capacity (Reddy and Kusum 2013).

Al-Amoudi and Aguis (1991) reviewed the mechanism of ion exchange across the gills. Depending on the salinity of the external medium, the gill epithelium has to pump salt from or to the body against a varying osmotic gradient. Light and electron microscopic examination of the gill epithelia with their chloride cells is described for both fresh and saline water adapted *O.*

mossambicus and *O. spilurus.* Vesicles of electron dense material were concentrated in the apical region. In saline water adapted fish, the chloride cells were more abundant and bigger in size, cytoplasm was darker and the tubular system more compact than those in their fresh water equivalents. No evident relationship between the vesicles and the tubular system was observed.

Ahmadmoradi *et al* (2012) investigated the histopathological lesions of kidney, liver and intestine in goldfish (*C. auratus*) and angelfish (*Pterophyllum sp.*) due to environmental contamination. Histological alterations was observed in kidney which include glomerulonephritis, cell swelling of epithelial cells, tubular epithelium necrosis, hyaline droplets and hyaline cast in renal tubules and tubular dilation. It was observed that unfavourable environmental contamination including salinity can induce several histopathological alterations in the tissues of goldfish and angel fish. Further, Oğuz (2015) studied gill histology of the endemic Lake Van Fish (*Chalcalburnus tarichi*) in two different water systems during the reproductive migration from saline water to freshwater. The histological examination of gill samples showed that freshwater samples had a thinner primary lamella, while the cells that secrete mucous into the gills showed no volumetric change. However, a significant increase in their number was observed. In fish acclimated to freshwater, a small number of mucous cells were identified in the secondary lamella. Epithelial lifting was observed in both types of aquatic environments.

Salinity tolerance of Nile tilapia, *O. niloticus* was studied by Azevedo *et al* (2015) to evaluate the effect of salinity on the growth performance, haematological parameters and histological characteristics of the gills. There were no significant differences of the salinity levels on daily feed intake and the best results were observed in 0 ppt. Histopathological alterations were observed in chloride cell hypertrophy, epithelial lifting, structure alteration, primary lamellae cells aggregation, fusion and occurrence of aneurisms of different sizes in some secondary lamellae. Hassan *et al* (2013) investigated the adaptability and tolerance of the tilapia fingerlings, *Oreochromis* sp. to different salinities and the histopathological and behavioral changes were observed in fish exposed to salinities of 0, 5, 20 and 35 ppt. The tilapia fingerlings with 10-14 cm total length acclimated successfully to freshwater before introduced to hyper-saline environment. The results showed 100 % fish survived in 0 ppt and 5 ppt, while 75% and 100% mortality in 20 ppt and 35 ppt respectively. The mortality rate increased with increased salinity. Fish exposed to different salinities exhibited clinical signs such as respiratory distress,

abnormal nervous behavior and resulting in mortality. Degeneration, necrosis, hemorrhage and hyperplasia of kidney and gills were observed as major histopathological changes. The gills sections in the control group presented a normal appearance and did not reveal any histopathological lesions in the tissues, because the histological lesions were severe with increased of the salinity. Histopathological lesions observed in the gills of the fish exposed to the salinity showed different lesions, which include hyperplasia of the epithelium, fusion of secondary lamellae and necrosis.

According to Liu *et al* (2013), the impact of salinity on the liver tissue of juvenile chum salmon was high; exposure to low salinity caused some liver cells to breakdown leading to serious vacuolization of the tissue. Long-term exposure to freshwater caused varying degrees of injury to the liver and other organs. Histological observations confirmed ruptured liver cells. In the freshwater and low-salinity treatment groups (0 and 5 ppt), a large number of vacuoles appeared at the same time in liver tissue. This suggested that at low salinity, it is beyond the ability of juvenile chum salmon to make physiological adjustments, which results in pathological changes to the structure and function of liver tissue.

Chapter -III

MATERIALS AND METHODS

3.1 Site of the Experiment

The experimental study was carried out at the PG research unit of College of Fisheries, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. Water quality analysis, growth, survival, carotenoid content and physiological (haematological, biochemical, antioxidant) parameters were analyzed in the Water Quality Lab, Aquaculture Nutrition Lab and Central Equipment Lab of College of Fisheries, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. Digital colour analysis of fish skin was carried out in Department of Livestock Products Technology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana.

3.1.1 Collection of inland saline water and preparation of experimental salinities

Inland saline water (stock) was collected from salt affected/ water logged areas of village Shajrana, district Fazilka (Punjab) to carry out the experiments (Plate-I). Stock inland saline water having salinity of 15 ppt and 12 ppt for experiment I and II respectively was analyzed for its physico-chemical properties w.r.t. temperature, pH, Salinity, Dissolved Oxygen (DO), Electrical Conductivity (EC), Total Alkalinity (TA), Total Hardness (TH), Ammonical Nitrogen (NH_3-N) and Ionic Composition in terms of cations [Calcium (Ca^{2+}), Magnesium (Mg^{2+}), Sodium (Na^{+}) and Potassium (K^{+})] and anions [Chloride (Cl^{-}) and Sulphate (SO_4^{2-})]. From this stock water, different experimental salinities [2 ppt (S2), 4 ppt (S4), 6 ppt (S6), 8 ppt (S8) and 10 ppt (S10)] were prepared after dilution with freshwater having salinity of 0 ppt (S0).

3.1.2 Preparation of the experimental aquaria

The experiments were carried out in glass aquaria (50 litre capacity with water volume of 40 liters). All the aquaria were properly cleaned before the stocking of the fish. The experimental aquaria (in triplicate) were filled with water having different experimental salinities (0-10 ppt). Each aquarium was supplied with continous oxygen supply through aerator (Plate-II).

3.1.2.1 Procurement, acclimatization and stocking of the experimental fish

Fingerling of freshwater ornamental goldfish *Carassius auratus* (Linnaeus) var. shubunkin were procured from local market. Fish were conditioned for one month in indoor

cemented pools. After proper conditioning, fish were acclimatized by gradual increase in salinity @ 1 ppt at 1-hr interval (Plate II) and randomly distributed @10 fish/ treatment into six (one control and five experimental salinity levels) treatments (0, 2, 4, 6, 8, 10 ppt) in triplicate in the month of June 2017 for experiment I [Salinity tolerance test- Effect of salinity on survival of freshwater ornamental goldfish, *Carassius auratus* (Linn.) var. Shubunkin] to be carried out for 10 days and in the month of August 2017 for experiment II [To study survival, growth, colouration and stress responses of freshwater gold fish, *Carassius auratus* (Linn.) var. Shubunkin reared at selected salinity (inland saline water)] to be carried out for 120 days (4 months) from August 2017 to November 2017. At the time of stocking (for both the experiments I and II), the mean initial total length and weight of fish varied from 6.5-8.5 cm and 4.5-8.5 g respectively.

3.1.2.2 Feeding of fish

During experiment I, fish were fed once a day with commercial feed [(OPTIMUM with nutritional composition: crude protein – 28%, crude fiber – 4%, crude fat – 3% and moisture – 10 %)] @ 0.5-1.0 % fish body weight. During experiment II, fish were fed twice a day (9:00 am and 4:00 pm) with same commercial feed up to satiation throughout the experimental period. The amount of feed was adjusted at every fortnight sampling according to increase in fish weight during experiment – II.

3.1.2.3 Observations recorded

EXPERIMENT – I	**EXPERIMENT – II**
Daily Observations I. Physico-chemical parameters • Temperature, pH, EC, TH, TA, NH_3- N and ionic composition – Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^- and SO_4^{2-} II. Fish Survival III. Fish Behavior **At completion of the salinity tolerance test** I. Colouration studies - Digital colour analysis	**Fortnightly Observations** I. Physico-chemical parameters • Temperature, pH, EC, TH, TA, NH_3- N and ionic composition – Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^- and SO_4^{2-} II. Fish Behavior III. Fish Growth **At completion of the experiment** • Fish Survival and Growth Parameters • Haematological, Biochemical, Anti-oxidant and Histopathological Parameters • Colouartion studies - Carotenoid and Digital colour analysis

3.1.3 Physico-chemical parameters of water

Water samples were collected daily and at fortnightly intervals for experiment I and II respectively in the morning hours for the analysis of various physico-chemical parameters mentioned below

3.1.3.1 Temperature

Water temperature (°C) was recorded by using an ordinary mercury thermometer (0 to 50 °C).

3.1.3.2 pH

pH of water was recorded by using digital pH meter (Metler Toledo- FE 20-1).

3.1.3.3 Dissolved oxygen (DO)

Dissolved oxygen of water was estimated (only during experiment II) by modified Winkler's method (APHA 2012).

Reagents:

- **Sodium thiosulphate titrant (0.025 N):** 6.205 g of sodium thiosulphate dissolved in freshly boiled and cooled distilled water and diluted to 1 litre.
- **Manganous sulphate solution:** Dissolve 182 g of $MnSO_4$ in distilled water, filter it and dilute in 500 ml of distilled water.
- **Alkali iodide-azide solution:** Dissolve 350 g KOH separately and 75 g of KI in distilled water. Mixed it and make the total volume of 500 ml by adding distilled water. Dissolve separately 5 g of sodium azide (NaN_3) in 20 ml distilled water. Add this azide solution to the alkaline- iodide reagent.
- **Concentrated sulphuric acid**
- **Starch indicator**

Procedure

i. Water sample collected in 250 ml BOD bottle without any air bubble and stoppered.

ii. 1 ml each of manganous sulphate and alkaline iodide- azide solutions added one after the other at the bottom of the bottle.

iii. The contents were then mixed by inverting the bottle 2-3 times and allow the brown precipitate to settle at the bottom.

iv. Precipitates were dissolved by adding 2 ml of concentrated sulphuric acid.

v. After that 50 ml of sample was taken in a conical flask and titrated against standard sodium thiosulphate solution till the colour changes to pale straw.

vi. To this, add 2 drops of starch indicator and again titrated till the blue colour disappears.

vii. Total amount of titrant used was recorded and DO calculated as per following formula

Calculations

$$DO \text{ in } mgl^{-1} = T \times N \times 8 / 50 \times 1000$$

Where,

T = Volume of titrant ($Na_2S_2O_3$) used in ml

N = Normality of $Na_2S_2O_3$ solution (0.025 N)

3.1.3.4 Electrical Conductivity (EC)

Electrical Conductivity (mS cm^{-1}) of water was recorded by using conductivity meter (EC- 3, COM – 80, HM Digital)

3.1.3.5 Salinity

Salinity (ppt) of water was recorded by using digital salinity meter (ATAGO ATC- S/Mill-E).

3.1.3.6 Total Alkalinity (Phenolphthalein and Methyl Orange Alkalinity)

Total alkalinity (TA) of water was determined by volumetric method (APHA 2012).

Principle: Alkalinity is determined by titrating the sample with standard solution of the sulphuric acid (H_2SO_4). Alkalinity due to hydroxyl (OH^-) and carbonate (CO_3^{2-}) ions is determined to the first end point (pH 8.3) using phenolphthalein indicator and bicarbonates (HCO_3^{2-}) alkalinity is determined to the second end point (pH 4.5) using methyl orange indicator.

Reagents:

i. Sulphuric acid (0.02 N)

ii. Phenolphthalein indicator

iii. Methyl orange indicator

Procedure:

i. Take 50 ml sample in Erlenmeyer's flask and add few drops of phenolphthalein indicator.

ii. If a slight pink colour appears, titrate with acid titrant (0.02 N H_2SO_4) to colorless end point and the amount of volume of acid titrant used was recorded (A) to calculate phenolphthalein alkalinity (PA).

iii. To the colourless solution, add few drops of methyl orange in the same flask and continue to titrate further till the color changes from yellow to orange.

iv. The amount of volume of H_2SO_4 used was recorded as (B) to calculate methyl orange alkalinity (MOA).

v. The total alkalinity (TA) was calculated as the total sum of phenolphthalein alkalinity (PA) and methyl orange alkalinity (MOA).

Calculations:

$$PA\ (CaCO_3 mgl^{-1}) = \text{ml of titrant used / Volume of sample x } 1000$$

$$MOA\ (CaCO_3 mgl^{-1}) = \text{ml of titrant used / Volume of sample x } 1000$$

$$\text{Total alkalinity}\ (CaCO_3\ mgl^{-1}) = PA + MOA$$

3.1.3.7 Total hardness

The total hardness of water was determined by volumetric method (APHA 2012)

Principle: Eriochrome black T forms wine red complex compound with metal ions (Ca^{2+} and Mg^{2+}). The disodium- metallation complexes as colourless chelate complex leaving a blue coloured aqueous solution of the dye.

Reagents:

i. **Standard EDTA titrant:** Dissolve 3.723 g of disodium salt of EDTA in distilled water to prepare 1 L of titrant.

ii. **Ammonia buffer solution:** Add 114 ml of concentrated NH_4OH to 13.5 mg of NH_4Cl and make volume upto 200 ml

iii. **Eriochrome black T- indicator:** Dissolve 0.5 g of dye in 100 ml of 80% ethyl alcohol

Procedure:

i. 50 ml of sample was taken in Erlenmeyer's flask

ii. 1 ml of ammonia buffer solution was added to the sample followed by 5 drops of Eriochrome black T- indicator resulting in wine red colouration.

iii. The above solution is titrated with EDTA solution, till a clear blue colour appears

iv. The amount of titrant used is noted and the total hardness is calculated.

Calculations:

Hardness ($CaCO_3 mgl^{-1}$) = ml of titrant used/ Volume of sample x 1000

3.1.3.7.1 Calcium hardness

Principle: Murexide indicator forms pink coloured complex with Ca^{2+} ions with the addition of di-sodium salt of EDTA; the Ca^{2+} form a colorless chelate complex leaving behind a purple solution of dye.

Reagents

i. Standard EDTA solution (0.01 M): 3.723 g EDTA sodium salt was dissolved and diluted to 1000 ml with distilled water.

ii. Murexide indicator solution: 200 mg of murexide and 100 g of NaCl were mixed and grounded.

iii. Sodium hydroxide solution (8%): 80 g of NaOH was dissolved and diluted to 1000 ml with distilled water.

Procedure:

i. 50 ml of well mixed sample was taken in a flask.

ii. To this, 1 ml of 8% sodium hydroxide solution was added followed by a pinch of murexide indicator mixture.

iii. The colour of sample changed to salmon pink.

iv. The sample was immediately titrated with standard EDTA solution, until pink colour changed to purple.

Calculations:

Calcium hardness (Ca^{2+} ions as $CaCO_3$ mgl^{-1}) = ml of titrant used / Volume of sample x 400.5 x 1.05

3.1.3.7.2 Magnesium hardness

Magnesium hardness was calculated as

Magnesium ($CaCO_3 mgl^{-1}$) = (Total hardness – Calcium hardness) x 0.243

3.1.3.8 Sodium (Na^+) and Potassium (K^+)

Sodium and potassium of water was estimated by flame emission photometric method (APHA 2012)

3.1.3.8.1 Sodium (Na^+)

Principle: A characteristics light is produced by excitation of electrons when the sample with sodium is sprayed into flame. The intensity of this radiation is directly proportional to concentration of sodium and can be read at 589 nm by using a suitable filter device.

Reagents

i. Deionised distilled water

ii. **Stock sodium solution:** 2.542 g of NaCl dried at 140°C was dissolved and diluted to 1000 ml with distilled water.

iii. **Intermediate sodium solution:** 10 ml of stock sodium solution was diluted with distilled water to 100 ml.

iv. **Standard sodium solution:** 10 ml of intermediate sodium solution was diluted with distilled water to 100 ml.

Procedure:

Direct- intensity measurement:

i. A blank was prepared and sodium calibration standards were prepared in stepped amounts in the following applicable ranges: 0 to1.0, 0 to 10 or 0 to 100 mgl^{-1}

ii. The operation was repeated with both calibration standards and samples for enough times to secure a reliable average reading for each solution**.** A calibration curve was constructed from the sodium standards.

iii. The sodium concentrations of sample was determined from the calibration curve.

Calculations

For direct reference to the calibration curve (mg Na l^{-1}) = (mg Na l^{-1} in portion) x D

Where, D = Dilution ratio (ml sample + ml water/ ml sample)

3.1.3.8.2 Potassium (K^+)

Principle: The characteristics emission linearly corresponds to the concentration of potassium being spread in the flame, which can be read at 769 nm on scale by using a suitable filter.

Reagents

i. Deionised distilled water

ii. **Stock potassium solution**: 1.907 g of KCl dried at 110°C was dissolved and diluted to 1000 ml with distilled water.

iii. **Intermediate potassium solution**: 10 ml of stock potassium solution was diluted with distilled water to 100 ml.

iv. **Standard potassium solution:** 10 ml of intermediate potassium solution was diluted with distilled water to 100 ml.

Procedure:

Direct- intensity measurement:

i. A blank and a potassium calibration standard were prepared in stepped amounts in the following applicable ranges: 0 to1.0, 0 to 10 or 0 to 100 mgl^{-1}

ii. The emission intensity was determined at 769 nm. The calibration standards and samples were aspirated for enough time to secure a reliable average reading for each.

iii. A calibration curve was constructed from the potassium standards.

iv. The potassium concentrations of sample was determined from the calibration curve.

Calculations

For direct reference to the calibration curve (mg K l^{-1}) = (mg K l^{-1} in portion) x D

Where, D = Dilution ratio (ml sample + ml water/ ml sample)

3.1.3.8 Chloride

Chloride of water was estimated by following Argentometeric method (APHA 2012).

Principle: Chloride is titrated with soluble silver salt (in weak acid solution) in presence of chromate; yielding relatively insoluble silver chloride. The brick red silver chromate is formed as the end point.

Reagents

i. **Silver nitrate titrant (0.0141 N):** 2.395 g of silver nitrate was dissolved in distilled water, diluted to 1 litre and stored in dark bottle.

ii. **Potassium chromate indicator:** 10 g of potassium chromate was dissolved in little quantity of distilled water and silver nitrate solution was added to prepare red precipitate. It was left overnight, filtered and diluted to 200 ml with distilled water.

Procedure:

i. 50 ml of sample was taken in Erlenmeyer's flask

ii. 5 drops of potassium chromate indicator were added to the flask till yellow colored appeared.

iii. The above solution is titrated with silver nitrate solution, till the appearance of brick red end point.

iv. The volume of titrant was recorded.

Calculations:

Chloride (mgl^{-1}) = ml of titrant used x N x 35.45/ Volume of sample x 1000

Where, N = Normality of titrant (0.0141 N)

3.1.3.9 Sulphate (SO_4^{2-})

Sulphate of water was determined by turbid metric method (APHA 2012)

Principle: A known volume of water sample is treated with barium chloride in the presence of gum acacia solution and the turbidity produced by the precipitation of sulphate as barium sulphate is measured with a colorimeter, using blue filter, at a wave length of 420 nm. Gum acacia, in this determination, helps to preventrapid settling of the barium sulphate precipitate.

Reagents

i. **Sodium acetate acetic acid buffer:** Dissolve 100 g of sodium acetate in 500 ml distilled water followed by 30 ml of 99.5% acetic acidand make volume up to 1 litre.

ii. **Gum acacia solution (0.25%):** Dissolve 0.25 g of gum acacia and dilute to 100 ml with distilled water.

iii. **Barium chloride crystals:** Grind crystals of barium chloride in pestle and mortar to pass through 30 mesh sieve.

iv. **Standard sulphate solution (100 ppm sulphur):** Dissolve 0.5434 g of reagent potassium sulphate and dilute in 1 litre with distilled water.

Procedure

i. Take 5 ml of sample and dilute to 100 ml into a 250 ml Erlenmeyer flask.

ii. 20 ml of sodium acetate acetic acid buffer was added and mixed well.

iii. Flasks were constantly stirred with the help of stirrer and 0.25 g of $BaCl_2$ crystals were added while stirring. Mixture was continuously stirred for one minute after addition of $BaCl_2$.

iv. Suspension was poured into an absorption cell of photometer and turbidity was measured at 5±0.5 min.

v. To correct for sample colour and turbidity, a blank was run simultaneously without adding $BaCl_2$.

Calculations:

SO_4^{2-} (mgl^{-1}) = [mg (SO_4^{-2}) x 1000] / [ml of sample]

3.1.3.10 Ammonical Nitrogen (NH_3-N)

Ammonical nitrogen of water was estimated as per standard method given by APHA (2012)

Principle: Ammonia reacts with phenol and alkaline hypochlorite to form indophenole blue. The reaction is catalysed by the nitroprusside or ferrocyanide. The resulting absorbance is proportional to the concentration of ammonia and is measured spectrophotometerically at 635 nm.

Reagents:

i. **Phenol:** Dissolve 10 g of phenol in 100 ml of 95% ethyl alcohol.

ii. **Sodium Nitroprusside reagent:** Dissolve 1 g of sodium nitroprusside in 200 ml distilled water and stored in amber coloured bottle.

iii. **Oxidizing Reagent:**

- **Alkaline hypochlorite:** Dissolve 10 g of NaOH in 300 ml of distilled water. To this add 2.7 ml of 10 % hypochloriteand dilute to 500 ml with distilled water.
- **Tris sodium citrate:** 100 g of tris sodium citrate and 5 g of NaOH and dissolved in distilled water to make final volume to 500 ml.
- Oxidizing reagent is prepared by mixing alkaline hypochlorite and tris sodium citrate in 1:4 ratio

iv. **Standard ammonia chloride solution:** Dissolved 3.82 g of anhydrous ammonium chloride in ammonia free distilled water and make total volume of 1 litre.

Procedure:

i. 50 ml of water sample was taken in amber coloured flask.

ii. To this 2 ml of phenol and 2 ml of sodium nitroprusside reagent were added followed by 5 ml of Oxidizing reagent in 1:4 ratio.

iii. Water sample was mixed well and incubated at 25° C for one hour in the dark and absorbance was read at 640 nm.

3.1.4 Survival, growth and behaviour of fish

3.1.4.1 Fish Survival

Experiment I

Fish survival in each salinity treatment was recorded daily for experimental period of 10 days.

Experiment II

Fish survival in each salinity treatment was determined by comparing the number of live fish recovered at the end of experiment with that of total fish stocked.

3.1.4.2 Fish Growth

Experiment II

At every fortnight, fish sampling was done by taking out the the fish from aquaria for minimum time to record the length and weight of the fish. Total length gain (TLG), Net weight gain (NWG), percent total length gain (% TLG), percent net weight gain (% NWG) and specific growth rate (SGR) for every treatment were calculated according to the following formulae:

TLG = Av. final total body length (cm) - Av. initial total body length (cm)

NWG = Av. final body weight (g) - Av. initial body weight (g)

%TLG = Av. final total body length (cm) - Av. initial body length (cm)/ Av. initial total body length (cm) x 100

% NWG = Av. final body weight (g) - Av. initial body weight (g)/ initial body weight (g) x 100

SGR (% increase in weight/day) = ln Av. final body weight (g) - ln Av. initial body weight/ culture days x 100

Where, ln = Natural Logarithm

3.1.4.3 Fish Behaviour

Experiment I

Fish behaviour was observed daily w.r.to swimming activity and feeding response.

Experiment II

Fish behaviour was observed at fortnightly intervals w.r.to swimming activity, feeding response, colour response, body fragility and mucus secretion.

3.1.5 Haematological parameters

Haematological parameters were studied at the completion of the experiment II. Blood from the experimental fish was collected and pooled for each replicate for haematological analysis.

3.1.5.1 Total Red Blood Cells Count (RBC) (Mukherjee 1988)

Principle: The technique is popularly known as haematocytometry and analyzed by Neubaur grid, on the haemocytometer, which show cell counting areas for the estimation. It consists of an accurate dilution of measured quantity of blood with a fluid, which is isotonic with the blood and prevents coagulation.

Reagents

Hayem's solution: Sodium sulphate (2.5 g), sodium chloride (0.5 g) and mercuric chloride (0.25 g) dissolved in 100 ml of distilled water.

Procedure

i. The blood was drawn into the RBC pipette upto 0.5 mark followed by immediate addition of diluting fluid containing, Hayem's solution was drawn upto 101 mark.

ii. This gives a dilution of 1:200 (Blood: Haeyem's solution).

iii. The solution is added by shaking gently and allowed to settle for 2 to 3 minutes.

iv. The counting chamber and cover glass were properly cleaned and the cover glass was placed over the ruled area.

v. Again the solution was mixed gently and the stemfull of solution was expelled and a drop of fluid was allowed to flow under the cover slip by holding the pipette at an angle of 45°C.

vi. It was allowed to settle for 2 to 3 minutes, the RBC's after settling without air bubble under the coverslip were counted.

vii. Then the ruled counting area was focused under the microsope and the number of RBC's were counted in fine small squares of the counting area under high power lense.

viii. The number of RBC were calculated by using the following formula:

Calculation

Total RBC ($x10^6$ mm^{3-1}) = No. of cells counted x dilution factor (1:200) x
OR ($x10^6/mm^3$) depth factor (0.1 mm) / Total No. of squares (5)

3.1.5.2 Total White Blood Cell Count (WBC) (Mukherjee 1988)

Principle: It consists of an accurate dilution of measured quantity of blood with a fluid which is isotonic with the blood and prevents coagulation.

Reagents

Turk's fluid: Glacial acetic acid (3 ml) and gentian violet (1 ml) dissolved in distilled water to make the volume to 100 ml.

Procedure

i. Blood collection and processing procedure was same as described for RBC except for the dilution factor, which is 1: 20.

ii. As far as the counting (Neubaur counting chamber) procedure of WBC, each of these 4 square millimeter area was sub divided into 16 squares, by using low power objective and a maximum ocular care was taken for ounting cells of the Neubaur chamber.

iii. The following formula was taken for the enumeration of WBC.

Total WBC ($x10^3$ mm^{3-1}) = No. of cells counted x Volume of the square x
OR ($x10^3/mm^3$) dilution factor (20) / No. of small squares (4)

3.1.5.3 Haemoglobin (Hb) (Sahli 1962)

Haemoglobin is a reasonable index of the red cell population and was estimated by acid haematin method.

Principle: When haemoglobin reacts with N/10 HCl, it forms acid haematin that is brown in colour.

Procedure

i. N/10 Hydrochloric acid was taken up to the mark 20 in the graduated tube and a drop (0.1 ml) of blood was added.

ii. It was allowed to stand for 5 minutes until it changes to dark brown colour.

iii. The solution was diluted by adding distilled water drop by drop (each time mixing the solution with a stirring rod), until it matches standard colour.

iv. Reading was taken from the scale on the graduated tube and the Hb concentration was expressed as gram percent (g %).

3.1.5.4 Packed Cell Volume (PCV) or Haematocrit Value (Mukherjee 1988)

PCV was estimated by micro- capillary method.

Principle: It is based on the principle of separation of blood by centrifugation into three distinct the top.

Procedure

i. The blood filled capillaries were sealed and centrifuged at 10,000 r.p.m. for 8 minutes.

ii. Final observations were recorded from micro-capillary scale and expressed as %

3.1.5.5 Mean Corpuscular Volume (MCV) (Mukherjee 1988)

MCV is calculated by using the following formula

$$MCV\ (\mu m^3) = PCV\ (\%) / R.B.C.\ in\ millions\ ml^{-1} \times 10$$

3.1.5.6 Mean Corpuscular Haemoglobin (MCH) (Mukherjee 1988)

Mean Corpuscular Haemoglobin (MCH) is the average haemoglobin content of the RBC. MCH is influenced by the size of the cell and concentration of haemoglobin. It is calculated by the following formula

$$MCH\ (g\ \%) = Hb\ (g\ \%) / R.B.C.\ in\ millions\ ml^{-1} \times 10$$

3.1.5.7 Mean Cell Haemoglobin Concentration (MCHC) (Mukherjee 1988)

The MCHC is an expression of the average Hb concentration per unit volume of packed cells (W/V). Hence, expressed as $g\ dl^{-1}$ or %.

MCHC (g %) = MCH (g %) / MCV (μm³) x 100 **OR** Hb (g %) / PCV (%) x 100

3.1.6 Biochemical parameters

Biochemical parameters were analyzed from blood serum by using Erba Manhelm Kit

3.1.6.1 Total protein

Total protein (TP) in blood serum was analyzed by following the principle of Biuret reaction (Gornall *et al* 1949)

Principle: The peptide bonds of protein react with copper (Cu^{2+}) ions in alkaline solution to form a blue-violet ion complex, (biuret reaction); each copper ion complexing with 5 or 6 peptide bonds. Tartrate is added as a stabilizer, whilst iodide is used to prevent auto-reduction of the alkaline copper complex. The colour formed is proportional to the protein concentration and is measured at 546 nm (520-560 nm).

Reagents

i. Copper II sulphate - 12 mmol l^{-1}
ii. Potassium sodium tartrate - 31.9 mmol l^{-1}
iii. Potassium iodide - 30.1 mmol l^{-1}
iv. Sodium hydroxide - 0.6 mol l^{-1}

Procedure

Reagent blank, standard and test samples were prepared as follows

	Reagent blank	**Standard**	**Sample (Test)**
Reagent (R1)	1000 μl	1000 μl	1000 μl
Distilled water	20 μl	–	–
Standard Reagent (R2)	–	20 μl	–
Sample (Test)	–	–	20 μl

i. Reagent 1, Standard Reagent 2 and Test Sample were mixed and incubated for 10 minutes at 37^{o} C.

ii. Absorbance of the standard and each test sample was read at 546 nm (520 – 560 nm) against reagent blank

Calculations

TP (gdl^{-1}) = Absorbance of Test sample/Absorbance of standard x Concentration of standard

3.1.6.2 Albumin

Principle: Albumin binds with Bromo Cresol Green (BCG) at pH 4.2 causing a shift in absorbance of the yellow BCG dye. The blue-green colour formed is proportional to the concentration of albumin, when measured photometrically between 540–630 nm with maximum absorbance at 625 nm.

Reagent

i. Bromocresol green - 0.21 mmol l^{-1}

ii. Succinate buffer - 100 mmol l^{-1}

iii. Sodium azide - 0.5 g l^{-1}

Procedure

Reagent blank, standard and test samples were prepared as follows

	Reagent blank	**Standard**	**Sample (Test)**
Reagent (R1)	1000 µl	1000 µl	1000 µl
Distilled water	10 µl	–	–
Standard Reagent (R2)	–	10 µl	–
Sample (Test)	–	–	10 L

i. Reagent 1, Standard Reagent 2 and Test Sample were mixed and the absorbance of the standard and each test sample was read at 630 nm (580 – 630 nm) against reagent blank, after one minute of incubation at 37^0C.

Calculation

Albumin (gdl^{-1}) = Absorbance of Test / Absorbance of Standard x Concentration of Standard

3.1.6.3 Globulin

Globulin was calculated as

Globulin (gdl^{-1}) = Total protein (gdl^{-1}) – Albumin (gdl^{-1})

3.1.6.4 Blood glucose (Trinder 1969)

Principle: Glucose in the sample is oxidised to yield gluconic acid and hydrogen peroxide in the presence of Glucose oxidase. The enzyme peroxidase catalyses the oxidative coupling of 4-aminoantipyrine with phenol to yield a coloured quinonemine complex, with absorbance proportional to the concentration of glucose in sample.

$$\beta\text{-D-Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{phenol} + 4\text{AAP} \xrightarrow{\text{Peroxidase}} \text{Red Dye} + 2H_2O$$

Reagent

i. Phosphate buffer - 250 mmol l^{-1}
ii. Glucose oxidase - > 25 Uml^{-1}
iii. Peroxidase - > 2 Uml^{-1}
iv. Phenol - 5 $mmoll^{-1}$
v. 4-aminoantipyrine - 0.5 mmol l^{-1}

Procedure

	Reagent blank	**Standard**	**Sample (Test)**
Reagent (R1)	1000 µl	1000 µl	1000 µl
Distilled water	10 µl	–	–
Standard Reagent (R2)	–	10 µl	–
Sample (Test)	–	–	10 µl

i. Reagent 1, Standard Reagent 2 and Test Sample were mixed and incubated for 10 minutes at 37^o C.

ii. Absorbance of the standard and each test sample was read at 505 nm (500 – 540 nm) against reagent blank or 505 / 670 nm on bichemistry analyzer.

Calculation

Glucose (gdl^{-1}) = Absorbance of Test / Absorbance of standard x Concentration of standard

3.1.7 Stress (Anti-oxidant) parameters

Stress parameters were analysed in blood haemolysate w.r.t. anti-oxidant enzymes i.e. Superoxide Dismutase (SOD), Lipid Peroxidation (LPO) and Glutathione Reductase (GR).

Preparation of haemolysate: Blood haemolysate was prepared before proceeding for different markers to determine oxidative damage in terms of antioxidant parameters. Blood samples were centrifuged at 3000 rpm for 15 minutes and supernatant was separated out. The sedimented cells were washed thrice with chilled 0.85% Nacl solution. Washed erythrocytes were lysed with nine parts of distilled water to prepare 10% haemolysate. Haemolysate was stored at -20°C for determination of different parameters.

3.1.7.1 Superoxide Dismutase (SOD) (Nishikimi *et al* 1972)

Principle: The assay is based on the principle that the nitroblue tetrazolium inhibits superoxide dismutase with reduced nicotinamide adenine dinucleotide (NADH) mediated by phenazonium methosulphate under aerobic conditions.

Reagents

i. **0.017 M sodium phosphate buffer (pH 8.3):**

- Solution 1: 2.052 g of sodium dihydrogen phosphate (NaH_2PO_4) L^{-1} distilled water
- Solution 2: 2.413 g of disodium hydrogen phosphate (Na_2HPO_4) L^{-1} distilled water.
- Solution 1 (7.36 ml) and solution 2 (92.64 ml) were mixed and diluted to 200 ml with distilled water after adjusting the pH to 8.3.

ii. **1.5 mM Nitroblue tetrazolium chloride (NBT):** 132.26 mg of nitroblue tetrazolium /100 ml distilled water.

iii. **2.34 mM Nicotinamide adenine dinucleotide- disodium salt (NADH):** 16.6 mg of NADH/10 ml distilled water.

iv. **0.093 mM Phenazine methosulphate (PMS)**: 2.85 mg of Phenazolium methosulphate/ 100 ml distilled water.

Procedure

i. To 2.6 ml of phosphate buffer at 20^{o} C in the cuvette, 100 µl each of PMS, NBT and haemolysate (1:100 v/v) were added.

ii. The reaction was initiated by adding 100 µl of NADH and increase in absorbance was recorded at 560 nm for 2 minutes at 30 seconds interval on spectrophotometer.

iii. The unit of superoxide dismutase was defined as activity of enzyme concentration required to inhibit chromogen production by 50 % in 1 minute under assay conditions.

Calculations:

SOD activity (U mg^{-1} Hb) = $\Delta T/(\Delta C/2) \times 100 / Y$

Where

ΔT: Change in optical density of test at 30 sec interval

ΔC: Change in optical density of control at 30 sec interval

Y: Haemoglobin concentration in haemolysate (mg)

3.1.7.2 Lipid peroxidation (Placer *et al* 1966)

Principle: The principle is based on the reaction of malondialdehyde (MDA), an end product of lipid peroxidation with thiobarbituric acid to yield a pink coloured trimethine complex exhibiting an absorption maximum at 548 nm wavelength.

Reagents

i. **0.2 M Tris- 0.16 M KCl buffer (pH- 7.4):** 2.422 g Tris and 1.192 g KCl per 100 ml distilled water.

ii. **7 % perchloric acid**

iii. **1 N sodium hydroxide (NaOH):** 4 g NaOH per 100 ml distilled water.

iv. **Thiobarbituric acid (TBA) reagent:**

- **TBA solution:** 0.8 g TBA per 100 ml 1N NaOH

- **TBA reagent:** Two volumes of TBA solution per one volume of 7 % perchloric acid.

v. **Pyridine – n – butanol reagent (3:1: v/v)**

Procedure

i. 0.1 ml haemolysate was taken in a test tube and 1.4 ml tris buffer was added to it.

ii. The contents were incubated for 30 minutes followed by addition of 1.5 ml TBA reagent.

iii. Mixture was heated in boiling water bath for 10 minutes.

iv. 3 ml pyridine- n- butanol reagent and 1 ml of 1 N NaOH was added to test tube after cooling and mixed properly by shaking.

v. Control was neither incubated nor heated.

vi. Absorbance was read at 548 nm on spectrophotometer against distilled water blank.

Calculations: Lipid peroxidation (nmol MDA g Hb^{-1}) = (A_{test}-$A_{control}$) x 46 x 1000/Y

Where Y: Haemoglobin concentration in g 0.1^{-1} ml haemolysate

3.1.7.3 Glutathione reductase (GR)/ whole blood reduced glutathione (Ellman 1959)

Principle: This Spectrophotometric assay involves the oxidation of GSH by the sulfhydryl reagent DTNB to form the yellow derivatives 5- thio-2 nitrobenzoic acid (TNB), which is measured at 412 nm.

Reagents

i. **5 % TCA:** 5 g TCA/100 ml distilled water.

ii. **Phosphate buffer (0.1 M, pH 7.4):** KH_2PO_4 (13.6 g) and $NaHPO_4$ (14.19 g)/1 L distilled water (pH adjusted to 7.4).

iii. **10 mM DTNB solution:** 0.396 g DTNB dissolved in 100 ml distilled water.

Procedure

i. 0.2 ml of whole blood was double diluted with distilled water and incubated for 10 minutes at 37^{o} C for complete haemolysis.

ii. After haemolysis, 3 ml of 5 % TCA was added to precipitate the protein content.

iii. Contents were centrifuged at 2500 rpm for 15 minutes, the supernatant (1 ml) was taken, mixed with 1 ml phosphate buffer (0.1 M, pH 7.4) and 0.5 ml of 10 mM DTNB solution.

iv. The absorbance was recorded at 412 nm using UV/VIS spectrophotometer.

A standard curve was prepared with different known concentrations of GSH solution. GSH concentration was expressed as Mm l^{-1}

3.1.8 Colouration studies

Fish muscle and skin colour analysis was carried out by spectrophotometer and digital methods.

3.1.8.1 Carotenoid (Spectrophotometeric) analysis:

Carotenoid ($\mu g\ g^{-1}$) analysis of fish skin and muscle (at initiation and completion of experiment II) was done by following method of Olson (1979).

i. The entire body tissue (without head and alimentary canal) was taken in a 10 ml screw capped clear glass vials and 2.5 g of anhydrous sodium sulfate was added.

ii. The sample was gently meshed with a glass rod against the side of the vial, to which 5 ml of chloroform was added and left overnight at room temperature under dark conditions.

iii. Chloroform formed a clear 1-2 cm layer above the caked residue, the optical density of which was read at 380, 450, 470 and 500 nm in a spectrophotometer.

iv. A blank was prepared in a similar manner was used for comparison.

v. The wavelength at which maximum absorption observed was used for the calculation.

3.1.8.2 Digital analysis

The digital photographs of experimental fish were taken at completion of experiment I and II and at termination of experiment I and II with chroma meter KONICA MINOLTA CR-400. Colour of the fish was measured from at least three fishes from each replicate from following three areas:

1. Mid dorsal region
2. Mid ventral region
3. Lateral region

The instrument was first calibrated using a white tile provided with the instrument. Three parameters i.e. L (Lighting ranging from 0 for black to 100 for white), a* (balance between red/green) and b* (balance between yellow/blue) was measured. The hue and chroma (saturation) values were calculated using the formula: [tan-1 (b/a)] and $(a^2+b^2)^{½}$, respectively.

3.1.9 Histological studies

The histological studies were aimed to assess any changes at tissue level due to stress caused by rearing of freshwater fish in inland saline water. Histological studies were carried out w.r.t gill, kidney and liver tissues from all salinity treatments and control (Titford 2009).

Reagents

i. Bouins fixative: Glacial acetic acid- 5 ml + Picric acid- 75 ml + Formaldehyde- 25 ml

ii. 70, 80 and 95 % Methanol

iii. 70, 95 and 100 % Ethanol

iv. Chloroform

v. Xylene

vi. Hematoxylin

vii. 1% acid ethanol

viii. 0.2% ammonia water

ix. Eosin

x. DPX mount

Procedure

I. Dissection of the fish

i. Fish were carefully cut opened from the anal fin along the belly up to the operculum using sharp scissors (taking care not to cut into the fish's internal organs).

ii. Gills, kidneys and liver were taken carefully with the help of scalpel and scissor.

II. Preservation of tissues in bouin's solution: After collecting the tissue samples, samples were preserved in bouins solution and kept at room temperature for one month (change the solution after 4-5 days).

III. Processing of tissues:

(a) Dehydration of the tissues

i. Tissues were washed by submerging in 70 % methanol followed by 80 % methanol and 95 % methanol respectively and kept in refrigerator at 4^{o}C for 20 minutes at each steps.

ii. Washing procedure was repeated after 20 minutes by changing, removing and washing with 95 % methanol.

iii. Tissues were washed with 100 % methanol in preserving container and submerged in methanol and kept in refrigerator at 4^{o} C for 20 minutes. Procedure was repeated two more times after every 20 minutes by changing, removing and washing with 95 % methanol.

iv. Tissues were washed with chloroform in the container andsubmerged in chloroform and kept in refrigerator at 4^{o} C for 30 minutes. Procedure was repeated for 2 more times after every 30 minutes by changing and washing with chloroform.

(b) Embedding (Fixing) of the tissue

i. After dehydration, tissues (gills, kidneys and liver) were poured into melted paraffin wax and kept in hot air oven at 55 ± ^{o}C for 30 minutes.

ii. By using warm forceps, the tissues samples were transferred into the paraffin wax. Gently pressed the samples flat. Paraffin wax solidify within 30 minutes, which hold the samples in position.

iii. Fixing procedure was repeated for 2 more times after every 30 minutes by changing, removing and washing with dropper. Carefully add hot liquid paraffin wax in the steel plates inside the hot air oven.

iv. Tissues were kept for one hour in hot air oven (55 ^{o}C).

v. After one hour, steel plates were taken out from hot air oven and labeled (treatment wise).

vi. Tissues were left overnight at room temperature for complete cooling and hardening of paraffin wax in blocks.

(c) Tissue sectioning and slide preparation

Trimmed sample blocks were fixed on rotary microtome (LEICA RM 2125 RTS) tissue holder, and sections were cut from a paraffin block to get thin tissues sections (6-8 μ) in the form of a ribbon, which were spread in hot water bath. The spread wax ribbons along with tissue sections were carefully lifted, placed on the glass slides and processed further for staining.

(d) Staining and Photography

The slides with tissue sections were stained as per following steps

Staining steps

Xylene (3 times)	5 mins
100% ethanol (2 times)	5 mins
95% ethanol	5 mins
70% ethanol	5 mins
Running tap water	1 min
Hematoxylin	1 min
Running tap water	1 min
1% acid ethanol	1-2 seconds
Running tap water	1 min
0.2% ammonia water	1 min
Running tap water	1 min
70% ethanol	1 min
Eosin	1 min
95% ethanol (2 times)	5 mins
100% ethanol (3 times)	5 mins
Xylene (3 times)	5 mins
The stained slides were finally mounted using DPX	

The specific areas in each slide of the tissues were observed under microscope for recording significant structural changes and the marked areas were subsequently photographed by light microscope LEICA DM3000 LED with digital camera at various levels of magnifications.

3.1.10 Statistical analysis

Statistical analysis of data was performed with a statistical package (SPSS 16.0 for Windows, SPSS Inc., Richmond, CA, USA). One way ANOVA and Duncan's multiple range test was applied to work out the effect of different salinities on physico-chemical parameters, survival, growth, colouration, anti-oxidant, haematological, biochemical and histological parameters of experimental fish and to determine differences among the salinity treatments ($P \leq 0.05$).

Chapter IV

RESULTS AND DISCUSSION

The results of the present study are covered under the following heads:

4.1 Experiment-I: Salinity tolerance test: Effect of salinity on survival of freshwater gold fish, *Carassius auratus* (Linn.) var. Shubunkin

4.1.1 Physico-chemical parameters of inland saline water (stock water)

4.1.2 Physico-chemical parameters of water with different salinities

4.1.3 Survival of fish

4.1.4 Fish behavior

4.1.5 Colouration studies – Digital colour analysis

4.2. Experiment-II: To study survival, growth, colouration and stress responses of freshwater gold fish, *Carassius auratus* (Linn.) var. Shubunkin reared at selected salinity (inland saline water)

4.2.1 Physico-chemical parameters of inland saline water (stock water)

4.2.2 Physico-chemical parameters of water with different salinities

4.2.3 Survival and growth of fish

4.2.4 Fish behavior

4.2.5 Haematological parameters

4.2.6 Biochemical parameters

4.2.7 Antioxidant parameters

4.2.8 Histopathological studies

4.2.9 Colouration studies

4.2.9.1 Carotenoid analysis

4.2.9.2 Digital colour analysis

4.1 Experiment-I: Salinity tolerance test: Effect of salinity on survival of freshwater gold fish, *Carassius auratus* (Linn.) var. Shubunkin

Inland saline water was collected from salt affected / water logged areas of village Shajrana, district Fazilka (Punjab) to carry out the salinity tolerance test

4.1.1 Physico-chemical parameters of inland saline water (stock water)

The mean physico-chemical parameters of inland saline water (stock water), including temperature, pH, salinity, electrical conductivity (EC), total alkalinity (TA), total hardness (TH), ammonical nitrogen (NH_3-N), ionic composition in terms of cations i.e. calcium (Ca^{2+}), magnesium (Mg^{2+}) sodium (Na^{+}) potassium (K^{+}) and anions i.e. chloride (Cl^{-}) and sulphate (SO_4^{2-}) are presented in Table 1.

Table 1: Physico-chemical parameters of inland saline water (stock water) collected from salt affected / water logged areas of village Shajrana, district Fazilka, Punjab used for experiment I.

PARAMETERS	MEAN± SE
Salinity (ppt)	**15 ± 0.05**
Temperature (^{0}C)	28.0 ± 0.28
pH	7.28 ± 0.13
EC ($mScm^{-1}$)	19.78 ± 0.33
TA ($CaCO_3$ mgl^{-1})	340.0 ± 0.00
TH ($CaCO_3$ mgl^{-1})	3320.0 ±60.09
NH_3-N (mgl^{-1})	0.36 ±0.01
Ca^{2+}($CaCO_3$ mgl^{-1})	497.4 ±62.06
Mg^{2+} ($CaCO_3$ mgl^{-1})	482.8 ±3.60
Na^{+} (mgl^{-1})	1121.0 ±1.24
K^{+}(mgl^{-1})	85.11 ±2.33
Cl^{-} (mgl^{-1})	5199.3 ±2.08
SO_4^{2-} (mgl^{-1})	50.5 ±7.08

Values are Mean ± SE ($p \leq 0.05$)

Mean temperature and salinity of inland saline water (stock) at the time of analysis was 28.0 ^{o}C and 15 ppt, respectively. Ionic profile of stock water revealed Na^{+} and Cl^{-} as dominant cation and anion, respectively.

4.1.2 Physico-chemical parameters of water with different salinities

From the inland saline water (stock water), different experimental salinities [2 ppt (S2), 4 ppt (S4), 6 ppt (S6), 8 ppt (S8) and 10 ppt (S10)] were prepared after dilution with freshwater having salinity of 0 ppt (S0). Mean physico-chemical parameters of water in different treatments during the experimental period of 10 days (June 2017) are presented in Table 2.

4.1.2.1 Temperature

The water temperature (^{o}C) varied from 29.00 to 29.90 in different treatments during the experimental period. Among different treatments, mean water temperature was 29.68, 29.59, 29.68, 29.78, 29.66 and 29.58 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were insignificant ($p \leq 0.05$).

4.1.2.2 pH

The water pH varied from 6.51 to 8.26 in different treatments during the experimental period. Among different treatments, mean water pH was 6.69, 7.24, 7.55, 7.29, 8.13 and 8.12 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant ($p \leq 0.05$), which revealed that pH of water increased with increase in salinity.

4.1.2.3 Electrical conductivity (EC)

The EC of water (mS cm^{-1}) varied from 0.54 to 13.41 in different treatments during the experimental period. Among different treatments, mean EC of water was 0.60, 2.34, 6.21, 7.24, 11.53 and 13.23 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant ($p \leq 0.05$). The EC of water in different treatments increased with increase in salinity.

4.1.2.4 Total alkalinity (TA)

The TA ($CaCO_3$ mg l^{-1}) of water varied from 255 to 418 in different treatments during the experimental period. Among different treatments, mean TA of water was 278.8, 310.3, 348.6,

375.0, 400.3 and 405.3 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant ($p \leq 0.05$). TA values of water in different treatments increased significantly with increase in salinity, showing positive co-relation with salinity.

4.1.2.5 Total Hardness (TH)

The TH ($CaCO_3$ mg l^{-1}) of water varied from 285 to 1295 in different treatments during the experimental period. Among different treatments, mean TH of water was 289.0, 505.0, 625.3, 945.3, 1030.0 and 1260.0 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant ($p \leq 0.05$). TH values increased significantly with increase in salinity of water in different treatments, showing positive co-relation with salinity.

4.1.2.6 Ammonical nitrogen (NH_3- N)

The NH_3-N (mgl^{-1}) varied from 0.125 to 0.285 in different treatments during the experimental period. Among different treatments, mean NH_3-N of water was 0.16, 0.17, 0.15, 0.16, 0.18 and 0.24 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant ($p \leq 0.05$), which revealed that the NH_3-N of water in different treatments increased with increase in salinity.

4.1.2.7 Calcium (Ca^{2+})

The Ca^{2+} ($CaCO_3$ mgl^{-1}) ions varied from 45.31 to 230.80 in different treatments during the experimental period. Among different treatments, mean Ca^{2+} of water was 51.33, 95.87, 126.20, 160.70, 206.10 and 224.70 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant ($p \leq 0.05$). The Ca^{2+} of water in different treatments increased with increase in salinity.

4.1.2.8 Magnesium (Mg^{2+})

The Mg^{2+} ($CaCO_3$ mgl^{-1}) ions varied from 59.50 to 291.25 in different treatments during the experimental period. Among different treatments, mean Mg^{2+} of water was 60.31, 109.30, 135.40, 208.20, 227.40 and 282.80 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant ($p \leq 0.05$). The Mg^{2+} of water in different treatments increased with increase in salinity.

4.1.2.9 Sodium (Na^+)

The Na^+ (mgl^{-1}) ions varied from 54.60 to 765.31 in different treatments during the experimental period. Among different treatments, mean Na^+ of water was 57.93, 162.10, 199.50, 320.20, 472.10 and 759.70 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant ($p \leq 0.05$). The Na^+ ions in different treatments increased with increase in salinity of water.

4.1.2.10 Potassium (K^+)

The K^+ (mgl^{-1}) ions varied from 6.90 to 44.50 in different treatments during the experimental period. Among different treatments, mean K^+ of water was 7.56, 9.41, 10.18, 19.18, 36.56 and 42.30 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant ($p \leq 0.05$). The K^+ ions in different treatments increased with increase in salinity of water.

4.1.2.11 Chloride (Cl^-)

The Cl^- (mgl^{-1}) ions varied from 22.05 to 1280 in different treatments during the experimental period. Among different treatments, mean Cl^- of water was 67.1, 268.8, 666.6, 957.0, 1048.0 and 1230.0 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant ($p \leq 0.05$). The Cl^- ions in different treatments increased with increase in salinity of water.

4.1.2.12 Sulphate (SO_4^{2-})

The SO_4^{2-} (mgl^{-1}) ions varied from 8.20 to 146.50 in different treatments during the experimental period. Among different treatments, mean SO_4^{2-} of water was 9.24, 64.46, 79.37, 93.96, 117.2 and 140.8 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant ($p \leq 0.05$). The SO_4^{2-} ions in different treatments increased with increase in salinity of water.

The overall result, with respect to physico-chemical parameters of different salinity treatments, revealed that at temperature range of 29.00-29.90 °C, increasing salinity of water (0-10 ppt) in different treatments resulted in significant increase in all the water quality parameters including pH (6.51-8.26), EC (0.54-13.41), TA (255-418), TH (285-1295) and NH_3-N (0.125-

Table 2. Mean physico-chemical parameters of water in different salinity treatments during salinity tolerance test

PARAMETERS	TREATMENTS*					
	S0	S2	S4	S6	S8	S10
Temperature (^{O}C)	29.68^{a}±0.14 (29.00-29.91)	29.59^{a}±0.12 (29.05-29.81)	29.68^{a}±0.08 (29.3-29.82)	29.78^{a}±0.04 (29.6-29.90)	29.66^{a}±0.12 (29.07-29.81)	29.58^{a}±0.12 (29.12-29.80)
pH	6.69^{d}±0.06 (6.51-6.98)	7.24^{c}±0.03 (7.13-7.35)	7.55^{b}±0.10 (7.21-7.99)	7.29^{b}±0.03 (7.21-7.39)	8.13^{a}±0.04 (7.91-8.21)	8.12^{a}±0.04 (8.01-8.26)
EC (mS cm^{-1})	0.60^{f}±0.02 (0.54-0.69)	2.34^{e}±0.01 (2.31-2.39)	6.21^{d}±0.03 (6.05-6.29)	7.24^{c}±0.01 (7.21-7.27)	11.53^{b}±0.26 (10.5-12.11)	13.23^{a}±0.49 (13.11-13.41)
TA ($CaCO_3$ mgl^{-1})	278.8^{e}±8.53 (255-301)	310.3^{d}±4.50 (295-320)	348.6^{c}±3.46 (332-355)	375.0^{b}±1.96 (370-382)	400.3^{a}±1.92 (393-406)	405.3^{a}±5.61 (385-418)
TH ($CaCO_3$ mgl^{-1})	289.0^{f}±1.63 (285-295)	505.0^{e}±5.68 (490-518)	625.3^{d}±3.61 (612-635)	945.3^{c}±4.28 (925-955)	1030.0^{b}±8.62 (1012-1064)	1260.0^{a}±13.06 (1216-1295)
NH_3-N (mgl^{-1})	0.16^{b}±0.013 (0.125-0.189)	0.17^{b}±0.011 (0.128-0.193)	0.15^{b}±0.019 (0.128-0.182)	0.16^{b}±0.011 (0.127-0.185)	0.18^{b}±0.003 (0.165-0.186)	0.24^{a}±0.014 (0.210-0.285)
Ca^{2+} ($CaCO_3$ mgl^{-1})	51.33^{f}±1.76 (45.31-56.85)	95.87^{e}±4.04 (85.12-110.23)	126.20^{d}±2.34 (115.60-131.10)	160.70^{c}±4.09 (145.60-175.20)	206.10^{b}±3.30 (190.90-213.80)	224.70^{a}±1.91 (220.30-230.80)
Mg^{2+} ($CaCO_3$ mgl^{-1})	60.31^{f}±0.36 (59.50-60.96)	109.30^{e}±1.36 (104.44-112.09)	135.40^{d}±0.80 (132.40-137.70)	208.20^{c}±1.02 (203.70-211.70)	227.40^{b}±2.04 (223.50-234.61)	282.85^{a}±2.95 (273.05-291.25)
Na^{+} (mgl^{-1})	57.93^{f}±0.88 (54.60-60.31)	162.10^{e}±2.65 (150.91-169.20)	199.50^{d}±2.19 (190.70-205.31)	320.20^{c}±1.83 (315.20-325.61)	472.10^{b}±4.35 (455.20-485.20)	759.70^{a}±1.83 (752.01-765.31)
K^{+} (mgl^{-1})	7.56^{e}±0.25 (6.90-8.40)	9.41^{d}±0.11 (9.10-9.80)	10.18^{d}±0.22 (9.70-11.20)	19.18^{c}±0.78 (15.60-20.90)	36.56^{b}±0.67 (34.10-38.30)	42.30^{a}±0.62 (40.10-44.50)
Cl^{-} (mgl^{-1})	67.1^{f}±0.94 (64.10-70.21)	268.8^{e}±15.32 (22.05-310.50)	666.6^{d}±8.87 (630.51-685.10)	957.0^{c}±6.43 (940.80-980.60)	1048.0^{b}±7.82 (1021-1074)	1230.0^{a}±13.58 (1190-1280)
SO_4^{2-} (mgl^{-1})	9.24^{f}±0.28 (8.20-10.10)	64.46^{e}±1.23 (59.30-68.10)	79.37^{d}±2.27 (71.65-88.50)	93.96^{c}±3.19 (82.60-102.60)	117.2^{b}±2.82 (105.50-125.90)	140.80^{a}±1.29 (138.10-146.50)

*** S0 = 0 ppt, S2= 2ppt, S4 = 4 ppt, S6 = 6ppt, S8= 8 ppt, S10= 10ppt**
Values are Mean ± S.E. ($p \leq 0.05$)
Values with same superscripts (a,b,c…..f) in a row do not differ significantly ($p \leq 0.05$)

0.285), which can be attributed to increasing concentration of salts with increasing salinity. The relative abundance of different ions was in accordance to that of stock inland saline (15 ppt) water from which different salinity water for different treatments was prepared (Na^{+}> Mg^{2+}> Ca^{2+}> K^{+} and Cl^{-}> SO_4^{2-}). At particular temperature range, increase in salinity has direct effect on pH, TA and NH_3-N. In the present study, temperature and pH remained in favourable range (Boyd 1990, Delince 1992, Wurts and Durborrow 1992 and Bhatnagar *et al* 2004) for freshwater fish culture. Further, significantly higher NH_3-N in S10 (10 ppt) may be due to high level of salinity and pH, because both these parameters has positive correlation with ammonia (Wurts, 2000). For commercial production, un-ionized ammonia and total ammonical nitrogen (TAN) should be below 0.05 mgl^{-1} and 1.0 mgl^{-1} respectively for long-term exposure. In the present study, although NH_3-N increased with increasing salinity, but it remained in tolerance range for freshwater fish (DWAMD 1994). It is also pertinent to mention here that the warm-water fish are more tolerant to ammonia toxicity than cold-water fish, and freshwater fish are more tolerant than saltwater fish. As the salinity is the total concentration of dissolved ions in water, with major contribution of calcium, sodium, potassium, bicarbonate, chloride and sulphate, therefore, most of the parameters (TH, EC and ionic composition) during 10 days of salinity tolerance test, showed linear increase, indicating direct co-relation with the salinity.

4.1.3 Survival of fish

For experiment I (salinity tolerance test), fish were acclimatized by gradual increase in salinity @ 1 ppt at 1-hr interval before distribution into six (one control and five experimental) treatments (0, 2, 4, 6, 8, 10 ppt) in triplicate. At completion of salinity tolerance test (10 days), % survival did not vary significantly, however, in control (S0) and S2 (2 ppt), it was 100%, which decreased to 96.66% in S4, S6 and S8 and 93.33 % in S10 (Table 3).

Every species has their optimum salinity tolerance range depending upon its physiological conditions, which is the complex interaction of nervous system along with metabolism. Salinity acclimation and tolerance leading to iono-osmoregulation in fishes occurs in a simple or complex manner with role of temperature leading to multiple osmo-regulatory mechanisms (Armitage and Olund 1962, Nelson 1968, Prosser *et al* 1970, Mackay 1974, Kasim 1983). 100 % survival of gold fish up to 2ppt (S2) could be attributed to the ability of the fish to regulate osmotic pressure of its body fluid (Nikolsky 1963). Gold fish is one of the examples of

cold water freshwater versatile fish species w.r.t. its physiology and metabolism, as it survives even up to 10 ppt during salt tolerance test. Küçük (2013) observed 100 % survival of gold fish (*Carassius auratus)* and crucian carp (*C. carassius*) up to salinity of 16 ppt. At 20 ppt, survival of both the species however decreased by 81.25 % and 83.33 % respectively. Lawson and Alake (2011) too reported 100 % survival of comet gold fish only up to 3 ppt, which decreased to significantly low value of 60 and 40 % at 4 and 5 ppt. Further, at 6-10 ppt, 100% fish mortality was observed, which indicated inability of the fish to maintain osmotic balance between the salt concentration of its body fluid and that of its environment. In the present study, shubunkin gold fish showed adaptability for short term exposure (10 days) to salinity up to even 10 ppt.

Table 3. Survival (%) of shubunkin gold fish, *C. auratus* (L.) in different salinity treatments during the salinity tolerance test in different salinity treatments

Day	TREATMENTS *					
	S0	**S2**	**S4**	**S6**	**S8**	**S10**
1	100±0.00[a]	100±0.00 [a]	100±0.00 [a]	100±0.00 [a]	100±0.00 [a]	100±0.00 [a]
2	100±0.00 [a]	100±0.00 [a]	100±0.00 [a]	100±0.00 [a]	100±0.00 [a]	100±0.00 [a]
3	100±0.00 [a]	100±0.00 [a]	100±0.00 [a]	100±0.00 [a]	100±0.00 [a]	100±0.00 [a]
4	100±0.00 [a]	100±0.00 [a]	100±0.00 [a]	100±0.00 [a]	100±0.00 [a]	100±0.00 [a]
5	100±0.00 [a]	100±0.00 [a]	100±0.00 [a]	96.66±3.33 [a]	96.66±3.33 [a]	96.66±3.33 [a]
6	100±0.00 [a]	100±0.00 [a]	96.66±3.33 [a]	96.66±3.33 [a]	96.66±3.33 [a]	96.66±3.33 [a]
7	100±0.00 [a]	100±0.00 [a]	96.66±3.33 [a]	96.66±3.33 [a]	96.66±3.33 [a]	96.66±3.33 [a]
8	100±0.00 [a]	100±0.00 [a]	96.66±3.33 [a]	96.66±3.33 [a]	96.66±3.33 [a]	93.33±3.33 [a]
9	100±0.00 [a]	100±0.00 [a]	96.66±3.33 [a]	96.66±3.33 [a]	96.66±3.33 [a]	93.33±3.33 [a]
10	100±0.00 [a]	100±0.00 [a]	96.66±3.33 [a]	96.66±3.33 [a]	96.66±3.33 [a]	93.33±3.33 [a]

*** S0 = 0 ppt, S2= 2ppt, S4 = 4 ppt, S6 = 6ppt, S8= 8 ppt, S10= 10ppt**
Values are Mean ± S.E. ($p \leq 0.05$)

4.1.4 Fish behavior

The behavior response towards salinity stress was observed w.r.to swimming activity and feeding response (Table 4). During salinity tolerance test, normal swimming activity of the fish

Table 4: Swimming activity and feeding response of shubunkin gold fish, *Carassius autaus* (L.) in different salinity treatments during the salinity tolerance test

Behaviour	Day	TREATMENTS*					
		S0	S2	S4	S6	S8	S10
Swimming activity	1	A	A	A	A	A	A
	2	A	A	A	A	A	A
	3	A	A	A	LA	LA	LA
	4	A	A	LA	LA	LA	LA
	5	A	A	LA	LA	LA	LA
	6	A	A	LA	LA	S	S
	7	A	A	LA	LA	S	S
	8	A	A	LA	LA	S	S
	9	A	A	LA	LA	S	S
	10	A	A	LA	LA	S	S
Feeding response	1	HAp	HAp	HAp	HAp	HAp	HAp
	2	HAp	HAp	HAp	HAp	HAp	HAp
	3	HAp	HAp	HAp	HAp	HAp	HAp
	4	HAp	HAp	HAp	HAp	HAp	HAp
	5	HAp	HAp	LAp	HAp	HAp	Hap
	6	HAp	HAp	LAp	LAp	Lap	Lap
	7	HAp	HAp	LAp	LAp	Lap	Lap
	8	HAp	HAp	LAp	LAp	Lap	Lap
	9	HAp	HAp	LAp	LAp	Lap	Lap
	10	HAp	HAp	LAp	LAp	Lap	Lap

*** S0 = 0 ppt, S2= 2ppt, S4 = 4 ppt, S6 = 6ppt, S8= 8 ppt, S10= 10ppt**
Swimming Activity - A = Active, LA = Less Active, S = Sluggish
Feeding Response – HAp – High Appetite, LAp = Low Appetite

was observed up to highest salinity i.e. 10 ppt for first 2 days, as the fish was gradually acclimatized to the salinity stress. However, from 4th day onward, fish showed low swimming activity in S4 and from 3rd day onward in S6-S10. Fish was found to be sluggish in S8 and S10 from 6th day onward. In terms of feeding behaviour, response of fish diverted from high appetite to low appetite in S4-S10 from 5th day onwards. The behaviorial changes in terms of swimming activity and feeding response clearly indicated adaptive strategy towards changing environmental conditions w.r.t. salinity. Fish was able to adapt easily up to 6 ppt, whereas at 8 and 10 ppt (S8

and S10), low activity to sluggishness along with low appetite indicated gradual drifting away from normal physiological responses. According to Lawson and Alake (2011), gold fish was very active from 0-3 ppt throughout the study period of 14 days, 4-6 ppt for for 3 days and in 7 ppt for 2 days. Fish showed erretic swimming behavior from day 5 to 8, in 8-10 ppt salinity. Likewise, feeding behavior was normal from 0-3 ppt in terms of very high appetite up to 3 ppt, thereafter behavior declined to moderate and low appetite and finally no appetite from 4-10 ppt in a gradual pattern. Behavioural changes during salinity tolerance test in the present study indicted adaptive capability of gold fish up to 4 ppt for shorter exposure period of 10 days. Mangat and Hundal (2014) too reported high to moderate appetite of freshwater common carp, when exposed to 6 ppt salinity for 60 days during summer, autumn and winter seasons. At higher salinity (12 ppt), appetite was low during winter season, but 100 % fish mortality was observed during summer and autumn.

4.1.5 Colouration studies – Digital colour analysis

At the completion of the salinity tolerance test, digital parameters of skin (dorsal and ventral region) was measured in terms of L (luminosity), a* (balance between red/green) and b* (balance between yellow/blue) values. From L, a* and b* values, colour indices i.e. chroma and hue were calculated (Table 5, Plate-III).

4.1.5.1 L a* b* colouration

Among different treatments, mean L, a* b* values in dorsal region were 41.43, 44.95, 54.40, 54.18, 44.02 and 44.14; 1.37, 3.19, 1.83, 5.20, 5.53 and 0.87; 2.63, 5.73, 6.25, 20.56, 16.33 and 5.15 in S0, S2, S4, S6, S8 and S10 respectively. The differences for L, a* and b* values were insignificant ($p \leq 0.05$) among different salinity treatments.

Among different treatments, mean L, a* and b* values in ventral region were 52.88, 47.45, 46.07, 49.57, 43.14 and 47.04; 1.75, 0.98, 0.36, 3.86, 7.68 and 0.52; 14.27, 4.62, 7.70, 16.85, 19.25 and 2.36 in S0, S2, S4, S6, S8 and S10 respectively. The differences for L and b* values were insignificant ($p \leq 0.05$) among different salinity treatments, whereas, values for a* (S8 > S6 > S0=S2=S10=S4) were significantly ($p \leq 0.05$) different.

4.5.1.2 Chroma and hue

Among different treatments, mean chroma and hue values in dorsal region were 41.47, 45.06, 54.46, 54.55, 44.44 and 44.18; 2376, 2582, 3120, 3125, 2546 and 2531 in S0, S2, S4, S6, S8 and S10 respectively. The difference for chroma values were insignificant ($p \leq 0.05$) among different treatments, while values for hue were significantly ($p \leq 0.05$) higher in all salinity treatments in comparison to S0 (0ppt).

Among different treatments mean chroma and hue values in ventral region were 52.92, 47.49, 46.08, 49.76, 43.83 and 47.05; 3032, 2721, 2640, 2851, 2511 and 2696 in S0, S2, S4, S6, S8 and S10 respectively. The difference for chroma values were insignificant ($p \leq 0.05$) among different treatments, while for hue (S0=S6=S2=S10≥S4=S8) values differences were significant ($p \leq 0.05$) among different salinity treatments.

Table 5: Comparative L a*b* colouration and colour indices of skin in dorsal and ventral regions of shubunkin gold fish, *C. auratus* (L.) in different salinity treatments at the completion of the salinity tolerance test

La*b* value/colour indices	TREATMENTS*					
	S0	**S2**	**S4**	**S6**	**S8**	**S10**
Dorsal region						
L	$41.43^{a}\pm4.69$	$44.95^{a}\pm4.24$	$54.40^{a}\pm2.88$	$54.18^{a}\pm8.83$	$44.02^{a}\pm3.44$	$44.14^{a}\pm7.37$
a*	$1.37^{a}\pm0.63$	$3.19^{a}\pm0.39$	$1.83^{a}\pm1.43$	$5.20^{a}\pm2.45$	$5.53^{a}\pm2.15$	$0.87^{a}\pm1.38$
b*	$2.63^{a}\pm2.01$	$5.73^{a}\pm2.02$	$6.25^{a}\pm3.84$	$20.56^{a}\pm11.83$	$16.33^{a}\pm7.35$	$5.15^{a}\pm7.35$
Chroma	$41.47^{a}\pm4.65$	$45.06^{a}\pm4.24$	$54.46^{a}\pm2.88$	$54.55^{a}\pm8.81$	$44.44^{a}\pm3.64$	$44.18^{a}\pm7.38$
Hue	$2376^{b}\pm266.9$	$2582^{a}\pm243.3$	$3120^{a}\pm165.2$	$3125^{a}\pm205.2$	$2546^{a}\pm208.7$	$2531^{a}\pm422.3$
Ventral Region						
L	$52.88^{a}\pm2.93$	$47.45^{a}\pm2.24$	$46.07^{a}\pm3.05$	$49.57^{a}\pm6.43$	$43.14^{a}\pm2.90$	$47.04^{a}\pm0.73$
a*	$1.75^{c}\pm0.71$	$0.98^{c}\pm1.27$	$0.36^{c}\pm0.81$	$3.86^{b}\pm1.18$	$7.68^{a}\pm0.50$	$0.52^{c}\pm0.70$
b*	$14.27^{a}\pm4.26$	$4.62^{a}\pm2.38$	$7.70^{a}\pm6.33$	$16.85^{a}\pm9.12$	$19.25^{a}\pm4.95$	$2.36^{a}\pm0.65$
Chroma	$52.92^{a}\pm2.94$	$47.49^{a}\pm2.24$	$46.08^{a}\pm3.05$	$49.76^{a}\pm6.38$	$43.83^{a}\pm2.86$	$47.05^{a}\pm0.73$
Hue	$3032^{a}\pm168.2$	$2721^{a}\pm128.8$	$2640^{ab}\pm175.2$	$2851^{a}\pm365.1$	$2511^{ab}\pm163.9$	$2696^{a}\pm320.3$

*** S0 = 0 ppt, S2= 2ppt, S4 = 4 ppt, S6 = 6ppt, S8= 8 ppt, S10= 10ppt**
Values are Mean ± S.E. ($p \leq 0.05$); Values with same superscripts (a,b,c…..f) in a row do not differ significantly ($p \leq 0.05$)

During the salinity tolerance study of 10 days, the digital observation of skin colouration in terms of L a* and b* values have not shown any remarkable departure from normal body pigmentation with increasing salinity. Maintenance of normal colour with slight variation in terms of hue (colour appearance) up to highest salinity, indicated the fish were able to maintain their normal body metabolism during short term salinity exposure. Lawson and Alake (2011) too observed similar results in gold fish exposed to different salinities. Normal gold colour was maintained up to 5 ppt up to four days and thereafter a change to bleached yellow colour was observed. There was restoration to normal gold colour between days 9 to 14.

4.2. Experiment II: To study survival, growth, colouration and stress responses of freshwater gold fish, *Carassius auratus* (Linn.) var. Shubunkin reared at selected salinity (inland saline water)

Experiment II was conducted for 120 days (4 months) with all the salinity treatments [2 ppt (S2), 4 ppt (S4), 6 ppt (S6), 8 ppt (S8), 10 ppt (S10)] tested during experiment 1 by using inland saline water (stock water) of 12 ppt salinity (Table 6).

4.2.1 Physico-chemical parameters of inland saline water (stock water)

The mean physico-chemical parameters of inland saline water (stock water), including temperature, pH, salinity, EC, TA, TH, NH_3-N, ionic composition in terms of cations i.e. Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} and anions i.e. Cl^{-} and SO_4^{2-} are presented in Table 6. Mean temperature and salinity of inland water (stock) at the time of analysis was 29.33^0C and 12 ppt respectively. Ionic profile of stock water revealed Na^{+} and Cl^{-} as dominant cations and anions respectively.

4.2.2 Physico-chemical parameters of water with different salinities

The water from different salinity treatments after completion of experiment I was pooled and used for experiment II after filteration. From the inland saline water (stock water), different experimental salinities [2 ppt (S2), 4 ppt (S4), 6 ppt (S6), 8 ppt (S8), 10 ppt (S10)] were prepared after dilution with freshwater having salinity of 0 ppt (S0). Mean physico- chemical parameters of water in different treatments during the experimental period of 120 days (August – November 2017) are presented in Table 7, Figures 1-11.

Table 6: Physico-chemical parameters of inland saline water (stock water) collected from salt affected / water logged areas of village Shajrana, district Fazilka, Punjab

PARAMETERS	MEAN± SE
Salinity (ppt)	**12 ± 0.08**
Temperature (^{0}C)	29.33 ± 0.12
pH	8.87 ± 0.01
EC (mScm^{-1})	17.92 ± 0.36
TA ($CaCO_3$ mgl^{-1})	254.7 ± 6.76
TH ($CaCO_3$ mgl^{-1})	2316.7 ± 15.27
NH_3-N (mgl^{-1})	0.21 ± 0.02
Ca^{2+}($CaCO_3$ mgl^{-1})	360.2 ± 1.40
Mg^{2+} ($CaCO_3$ mgl^{-1})	587.3 ± 1.48
Na^{2+}(mgl^{-1})	765.9 ± 9.91
K^{+}(mgl^{-1})	91.1 ± 0.26
Cl^{-} (mgl^{-1})	3539.7 ± 1.45
SO_4^{2-} (mgl^{-1})	87.1 ± 2.41

Values are Mean ± SE ($p \leq 0.05$)

4.2.2.1 Temperature

The water temperature (^{0}C) varied from 14.2 to 30.5 in different treatments during the experimental period. Among different treatments, mean water temperature was 24.06, 24.14, 24.01, 24.00, 24.10 and 24.07 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were insignificant ($p \leq 0.05$).

4.2.2.2 pH

The pH of water varied from 7.14 to 8.99 in different treatments during the experimental period. Among different treatments, mean pH of water was 7.41, 7.99, 8.28, 8.30, 8.46 and 8.48 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant ($p \leq 0.05$), which revealed significant increase in pH of water with increasing salinity.

4.2.2.3 Dissolved Oxygen (DO)

The DO of water (mg l^{-1}) varied from 5.50 to 9.52 in different treatments during the experimental period. Among different treatments, mean DO of water was 9.13, 8.47, 8.19, 7.99, 7.19 and 6.49 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments

were significant ($p \leq 0.05$). The DO of water in different treatments decreased with increase in salinity.

4.2.2.4 Electrical conductivity (EC)

The EC of water (mS cm^{-1}) varied from 0.64 to 18.91 in different treatments during the experimental period. Among different treatments, mean EC of water was 0.84, 5.94, 8.82, 10.78, 13.45 and 17.22 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant ($p \leq 0.05$). The EC of water in different treatments increased with increase in salinity.

4.2.2.5 Total alkalinity (TA)

The TA ($CaCO_3$ mg l^{-1}) of water varied from 232 to 336 in different treatments during the experimental period. Among different treatments, mean TA of water was 260, 279, 295, 300, 305 and 317 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant ($p \leq 0.05$). TA values increased significantly with increasing salinity of water in different treatments, showing positive co-relation with salinity.

4.2.2.6 Total Hardness (TH)

The TH ($CaCO_3$ mg l^{-1}) of water varied from 305 to 2930 in different treatments during the experimental period. Among different treatments, mean TH of water was 364.60, 1045.60, 1550.50, 2168.80, 2250.80 and 2699.90 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant ($p \leq 0.05$). TH values increased significantly with increasing salinity of water in different treatments, showing positive co-relation with salinity.

4.2.2.7 Ammonical nitrogen (NH_3- N)

The NH_3-N (mgl^{-1}) ions varied from 0.010 to 0.298 in different treatments during the experimental period. Among different treatments, mean NH_3-N of water was 0.06, 0.15, 0.14, 0.14 0.14 and 0.18 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant ($p \leq 0.05$). The NH_3-N content of water in different treatments increased with increasing salinity.

4.2.2.8 Calcium (Ca^{2+})

The Ca^{2+} ($CaCO_3$ mgl^{-1}) ions varied from 82.40 to 302.70 in different treatments during the experimental period. Among different treatments, mean Ca^{2+} of water was 91.20, 115.00, 141.30, 195.70, 233.70 and 281.10 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant ($p \leq 0.05$). The Ca^{2+} of water in different treatments increased with increase in salinity.

4.2.2.9 Magnesium (Mg^{2+})

The Mg^{2+} ($CaCO_3$ mgl^{-1}) ions varied from 63.78 to 914.50 in different treatments during the experimental period. Among different treatments, mean Mg^{2+} of water was 83.29, 231.50, 333.20, 466.20, 558.10 and 586.60 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant ($p \leq 0.05$). The Mg^{2+} of water in different treatments increased with increase in salinity.

4.2.2.10 Sodium (Na^{+})

The Na^{2+} (mgl^{-1}) ions varied from 14.50 to 685.80 in different treatments during the experimental period. Among different treatments, mean Na^{+} of water was 19.80, 192.90, 264.20, 364.30, 460.40 and 592.60 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant ($p \leq 0.05$). The Na^{+} of water in different treatments increased with increase in salinity.

4.2.2.11 Potassium (K^{+})

The K^{+} (mgl^{-1}) ions varied from 0.50 to 91.20 in different treatments during the experimental period. Among different treatments, mean K^{+} of water was 0.84, 16.21, 24.91, 39.73, 58.35 and 79.56 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant ($p \leq 0.05$). The K^{+} of water in different treatments increased with increase in salinity.

Table 7. Mean physico- chemical parameters of water in different salinity treatments during the experimental period

PARAMETERS	TREATMENTS*					
	S0	**S2**	**S4**	**S6**	**S8**	**S10**
Temperature (^{O}C)	24.06^{a}±1.13 (14.2-29.6)	24.14^{a}±1.14 (14.3-29.8)	24.01^{a}±1.13 (14.1-29.6)	24.0^{a}±1.12 (14.5-29.3)	24.10^{a}±1.15 (14.2-29.8)	24.07^{a}± 1.13 (14.2-30.5)
pH	7.41^{d}±0.03 (7.14-7.59)	7.99^{c}±0.03 (7.75-8.30)	8.28^{b}±0.02 (8.07-8.73)	8.30^{b} ±0.05 (8.06-8.92)	8.46^{a}±0.05 (8.13-8.99)	8.48^{a}±0.41 (8.12-8.89)
DO (mgl^{-1})	9.13^{a}±0.07 (8.10 - 9.52)	8.47^{b}±0.04 (8.20- 8.92)	8.19^{c}±0.09 (7.15- 8.54)	7.99^{c}±0.10 (7.01- 8.65)	7.19^{d}±0.11 (6.20- 7.98)	6.49^{e}±0.11 (5.50- 7.65)
EC ($mScm^{-1}$)	0.84^{f}±0.02 (0.64-0.99)	5.94^{e}±0.31 (3.08-8.22)	8.82^{d}±0.20 (7.1-10.56)	10.78^{c}±0.13 (10.12-11.98)	13.45^{b}±0.17 (11.46-14.82)	17.22^{a}±0.25 (14.65-18.91)
TA ($CaCO_3$ mgl^{-1})	260^{e}±3.12 (232-280)	279^{d}±3.24 (244-304)	295^{c}±1.88 (280-316)	300^{bc}±2.57 (276-326)	305^{b} ±2.42 (289-320)	317^{a}±2.46 (303-336)
TH ($CaCO_3$ mgl^{-1})	364.60^{f} ± 7.11 (305-410)	1045.60^{e} ± 14.93 (948-1210)	1550.50^{d}±25.21 (1302-1800)	2168.80^{c}±21.85 (2010-2320)	2250.80^{b}±12.00 (2164-2420)	2699.90^{a}±25.96 (2514-2930)
NH_3-N (mgl^{-1})	0.06^{c} ± 0.011 (0.010-0.177)	0.15^{b} ± 0.006 (0.101-0.199)	0.14^{b}±0.005 (0.101-0.184)	0.14^{b}± 0.005 (0.102-0.193)	0.14^{b} ± 0.006 (0.104-0.204)	0.18^{a} ± 0.011 (0.115-0.298)
Ca^{2+} ($CaCO_3$ mgl^{-1})	91.23^{f} ± 0.99 (82.41-98.62)	115.00^{e}±0.93 (105.10-121.50)	141.30^{d}±1.97 (124.20-158.61)	195.70^{c} ± 3.37 (160.50-216.71)	233.70^{b}±4.24 (190.20-272.31)	281.10^{a}±2.83 (250.40-302.70)
Mg^{2+} ($CaCO_3$ mgl^{-1})	83.29^{f} ± 2.38 (63.78-107.40)	231.50^{e}±6.60 (199.11-325.51)	333.20^{d}±10.61 (250.60-410.31)	466.20^{c} ± 8.19 (421.70-532.21)	558.10^{b}±16.74 (497.70-716.50)	586.60^{a}±19.65 (587.91-914.50)
Na^{2+} (mgl^{-1})	19.80^{f} ± 0.58 (14.50-23.21)	192.90^{e} ± 6.26 (159.60-252.21)	264.20^{d} ± 16.1 (189.11-390.80)	364.30^{c} ± 8.23 (310.31-450.80)	460.40^{b} ±6.25 (425.31-520.51)	592.60^{a} ± 7.41 (514.30-685.80)
K^{+} (mgl^{-1})	0.84^{f} ± 0.02 (0.50-0.94)	16.21^{e}±0.27 (13.51-17.6)	24.91^{d}±0.73 (21.31-33.40)	39.73^{c}± 1.29 (30.40-49.51)	58.35^{b}±1.27 (50.20-69.01)	79.56^{a} ± 1.12 (70.21-91.20)
Cl^{-} (mgl^{-1})	72.50^{f} ± 3.20 (39.90-109.90)	621.20^{e}±1.21 (399.80-651.21)	950.70^{d}±2.19 (850.61-1269.60)	1571.70^{c} ± 4.21 (1350.50-2160.71)	2194.50^{b}±2.27 (2014.5-2439.2)	2854.90^{a} ± 2.40 (2621.30-3010.51)
SO_4^{2-} (mgl^{-1})	5.80^{d} ± 0.45 (2.32-8.24)	38.56^{c}±1.79 (30.59-52.03)	43.58^{c}±1.73 (37.26-59.08)	60.21^{b}± 5.62 (40.25-121.68)	68.50^{b}±6.33 (43.65-125.69)	81.79^{a} ± 5.55 (62.16-121.69)

*** S0 = 0 ppt, S2= 2ppt, S4 = 4 ppt, S6 = 6ppt, S8= 8 ppt, S10= 10ppt**

Values are Mean ± S.E. ($p \leq 0.05$)

Values with same superscripts (a,b,c…..f) in a row do not differ significantly ($p \leq 0.05$)

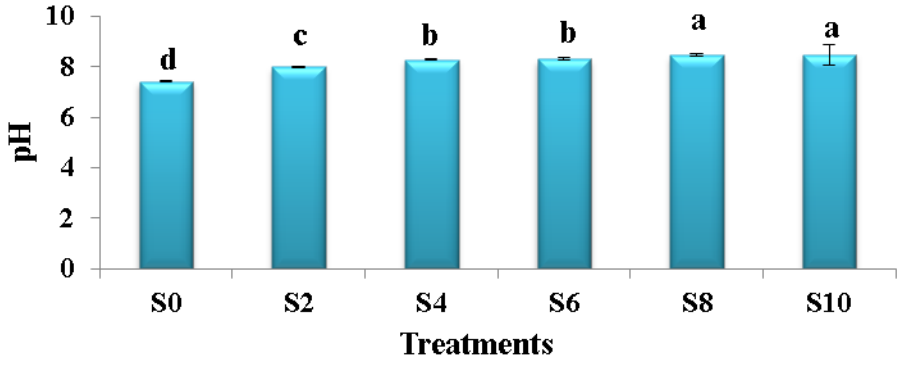


Figure 1: Mean water pH in different salinity levels during the experimental period

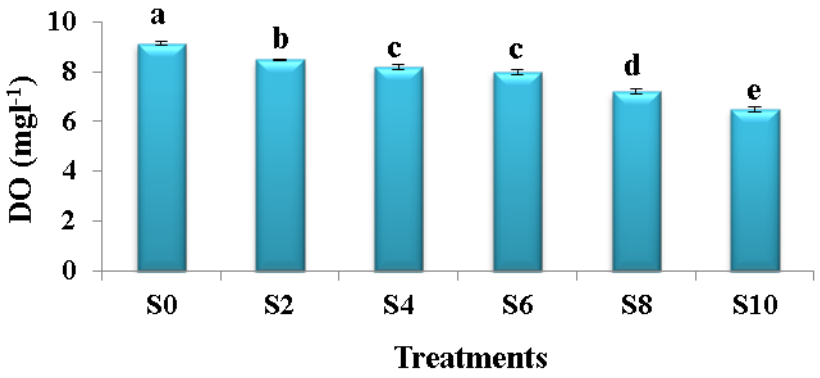


Figure 2: Mean water DO (mgl^{-1}) in different salinity levels during the experimental period

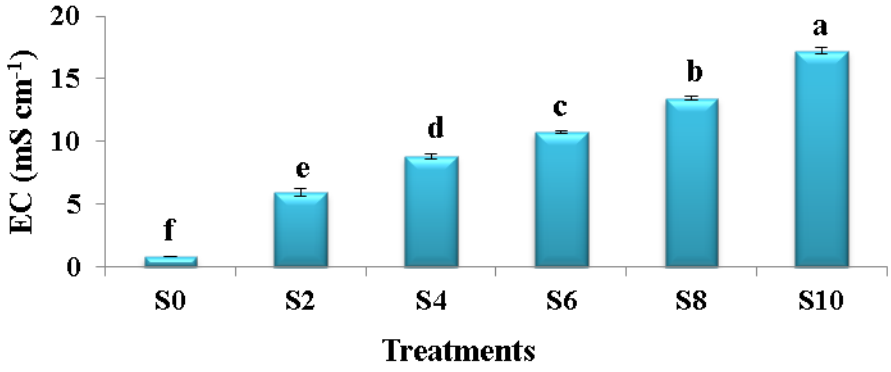


Figure 3: Mean EC ($mS\ cm^{-1}$) of water in different salinity levels during the experimental period

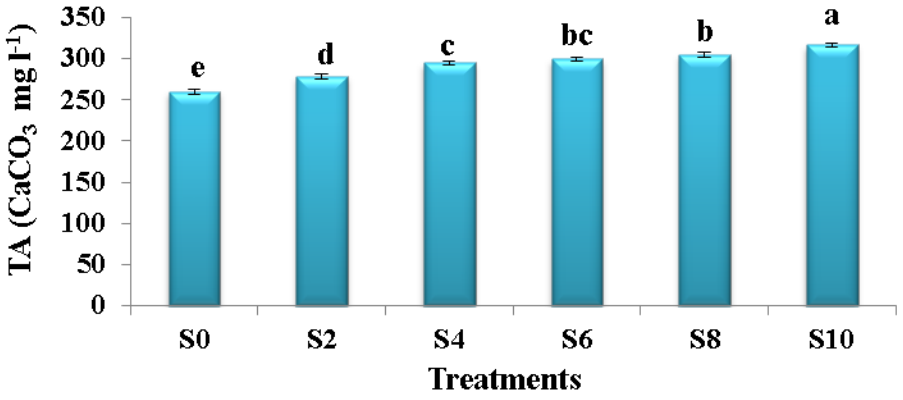


Figure 4: Mean TA ($CaCO_3$ mg l^{-1}) of water in different salinity levels during the experimental period

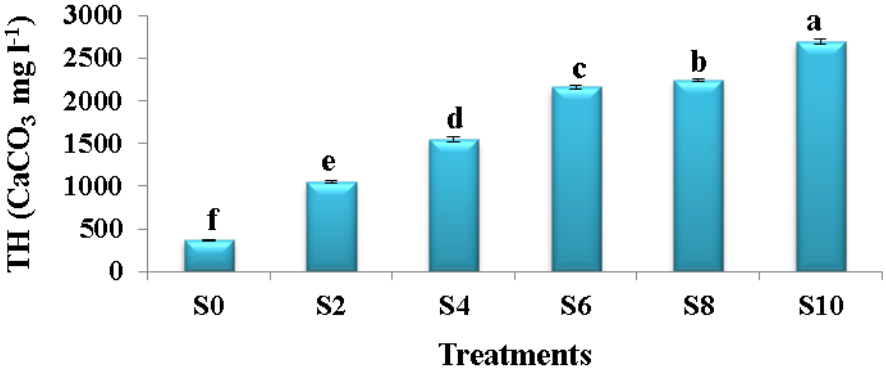


Figure 5: Mean TH ($CaCO_3$ mg l^{-1}) of water in different salinity levels during the experimental period

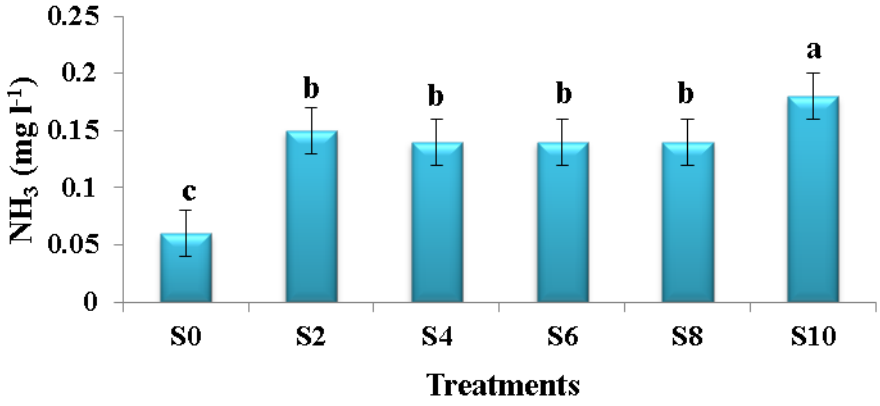


Figure 6: Mean NH_3-N (mg l^{-1}) of water in different salinity levels during the experimental period

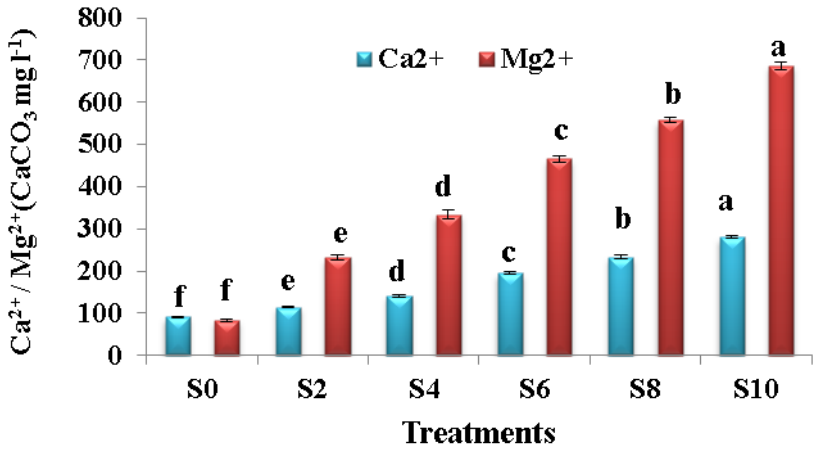


Figure 7: Mean Ca^{2+} ($CaCO_3$ mg l^{-1}) and Mg^{2+} ($CaCO_3$mg l^{-1}) of water in different salinity levels during the experimental period

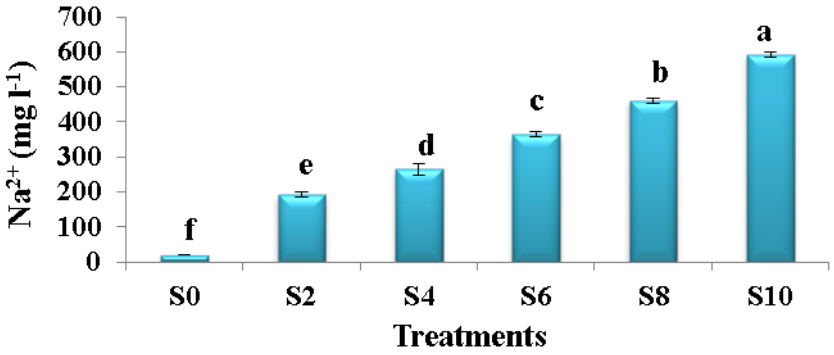


Figure 8: Mean Na^+ (mg l^{-1}) of water in different salinity levels during the experimental period

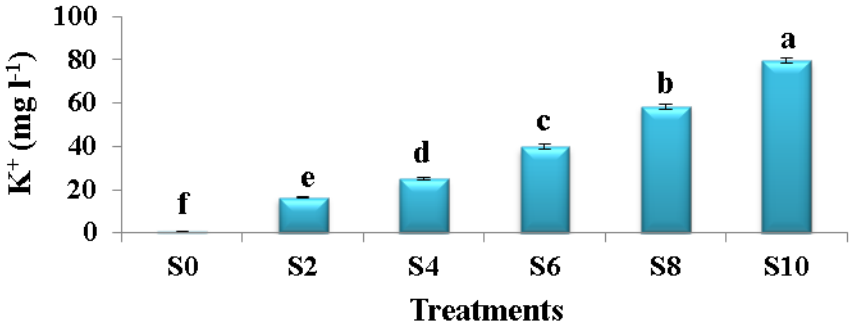


Figure 9: Mean K^+ (mg l^{-1}) of water in different salinity levels during the experimental period

4.2.2.12 Chloride (Cl^-)

The Cl^- (mgl^{-1}) ions varied from 39.90 to 3010.51 in different treatments during the experimental period. Among different treatments, mean Cl^- of water was 72.50, 621.20, 950.70, 1571.70, 2194.50 and 2854.90 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant ($p \leq 0.05$). The Cl^- of water in different treatments increased with increase in salinity.

4.2.2.13 Sulphate (SO_4^{2-})

The SO_4^{2-} (mgl^{-1}) ions varied from 2.32 to 121.69 in different treatments during the experimental period. Among different treatments, mean SO_4^{2-} of water was 5.80, 38.56, 43.58,60.21, 68.50 and 81.79 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant ($p \leq 0.05$). The SO_4^{2-} of water in different treatments increased with increase in salinity.

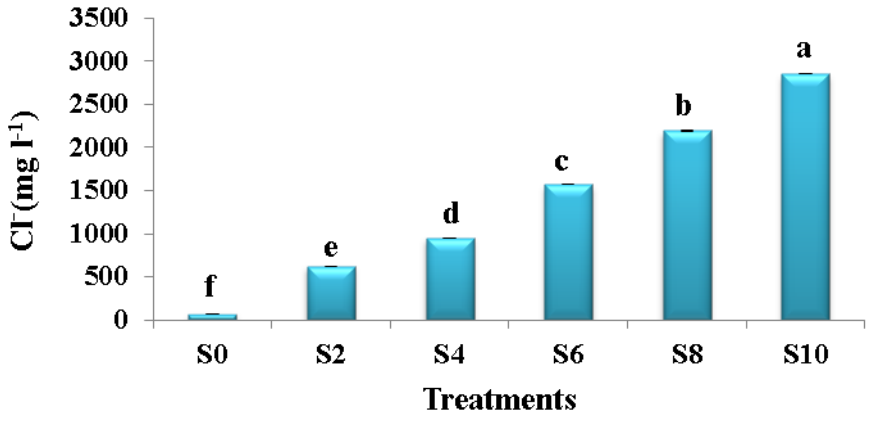


Figure 10: Mean Cl^- ($mg\ l^{-1}$) of water in different salinity levels during the experimental period

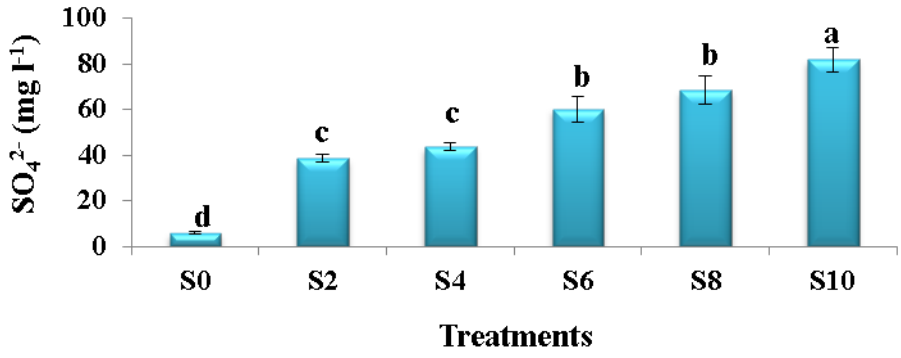


Figure 11: Mean $SO4^{2-}$ (mg l^{-1}) of water in different salinity levels during the experimental period

The overall result, with respect to physico-chemical parameters of different treatments, revealed that at temperature range of 14.2 to 30.5 ^{0}C for 120 days of experimemental period (August 2017 – November 2017), increasing salinity of water (0-10-ppt) in different treatments showed significant effect on pH (7.14-8.99) and NH_3-N (0.010 to 0.298). pH remained in the desirable range (6.5-9.0), but values for NH_3-N were higher (average NH_3-N level in all salinity treatments was >0.1 mgl^{-1}) with significantly higher level (0.298 mgl^{-1}) in S10 (desirable range – 0-0.025), which is one of the stress causing factor for freshwater fish at higher salinity (Delince 1992, Wurts and Durborow 1992 and Bhatnagar *et al* 2004). Further, the increase in salinity resulted in low dissolved oxygen (9.13 in S0 to 6.49 mgl^{-1} in S10), however it remained in desirable range (5 and above) to support good health (Bhatnagar *et al* 2004, Bhatnagar and Singh 2010). Salinity is the total concentration of electrically charged ions (cations - Ca^{2+}, Mg^{2+}, K^+, Na^+ and anions - CO_3^{2-}, HCO_3^-, SO_4^{2-}, Cl^- and other constituents like NO_3^-, NH_4^+ and PO_4^-) having its direct effect on number of parameters. Hence, during the experimental period, the parameters like EC, TA and TH of water showed linear trend with increasing salinity (due to increasing concentration of salts in accordance to salinity levels). The relative abundance of different ions was also similar to that of stock inland saline water (12 ppt), from which different salinity water for different treatments was prepared (Na^+> Mg^{2+}> Ca^{2+}> K^+ and Cl^-> SO_4^{2-}).

4.2.3 Survival and growth of fish

4.2.3.1 Survival

During the experiment period of 120 days (4 months), fish survival was 100 % in S0 and S2 for experimental period of 120 and 90 days respectively, whereas for rest of the salinity treatments, gradual mortality was observed from 45th day onwards (Table 8). At the completion of the experiment, fish survival was 100 % in S0 (0ppt), 93.33% in S2 (2ppt), 86.66% in S4 (4 ppt), 80% in S6 (6 ppt) and S8 (8 ppt); and 60 % in S10 (10 ppt) and the differences were significant (S0=S2≥S4=S6=S8≥S10). These results indicated adaptive ability of gold fish up to 8 ppt (80% survival) at temperature range of 14.2 to 30.5^{0}C.

Table 8. Survival (%) of shubunkin gold fish, *C. auratus* (L.) in different salinity levels during the experimental period

Days	TREATMENTS*					
	S0	**S2**	**S4**	**S6**	**S8**	**S10**
0	100.0^a±0.00	100.0^a±0.00	100.0^a±0.00	100.0^a±0.00	100.0^a±0.00	100.0^a±0.00
15	100.0^a±0.00	100.0^a±0.00	100.0^a±0.00	100.0^a±0.00	100.0^a±0.00	100.0^a±0.00
30	100.0^a±0.00	100.0^a±0.00	100.0^a±0.00	100.0^a±0.00	100.0^a±0.00	100.0^a±0.00
45	100.0^a±0.00	100.0^a±0.00	86.66^a ±6.66	86.66^a ±6.66	86.66^a ±6.66	86.66^a ±6.66
60	100.0^a±0.00	100.0^a±0.00	86.66^a ±6.66	86.66^a±6.66	80.00^b±11.54	60.00^b±11.54
75	100.0^a±0.00	100.0^a±0.00	86.66^{ab}±6.66	80.00^{ab}±11.54	80.00^{ab}±11.54	60.00^b±11.54
90	100.0^a±0.00	100.0^a±0.00	86.66^{ab}±6.66	80.00^{ab}±11.54	80.00^{ab}±11.54	60.00^b±11.54
105	100.0^a±0.00	93.33.0^a±6.66	80.00^{ab}±11.54	80.00^{ab}±11.54	80.00^{ab}±11.54	60.00^b±11.54
120	100.0^a±0.00	93.33.0^a±6.66	86.66^{ab}±6.66	80.00^{ab}±11.54	80.00^{ab}±11.54	60.00^b±11.54

*** S0 = 0 ppt, S2= 2ppt, S4 = 4 ppt, S6 = 6ppt, S8= 8 ppt, S10= 10ppt**
Values are Mean ± S.E. (p≤0.05)
Values with same superscripts (a,b,c…..f) in a row do not differ significantly (p≤0.05)

Survival of fish depends on overall adapting ability to ever-changing environment conditions (Koedjick *et al* 2012). Freshwater fish produce large volume of dilute urine, which is low in salt. Moreover, high concentrations of environmental calcium help reduce salt loss through gills and body surfaces in freshwater environment (Wurts 1998). Most fish are adapted and only survive in the freshwater (stenohaline); however some have great adaptations for changing salinity levels. The results of present study too indicated adaptation of gold fish towards salinity in range of 0-8 ppt (100-80% survival), however, survival gradually reduced after 2 ppt. The results of the present study are in agreement with the studies of Kasim (1983) and Küçük (2013) with freshwater fishes. Further, it has also been documented that regulation of body physiology is in accordance to the salinity-temperature regime (Islam *et al* 2014, Wang *et al* 1997), indicating that at low temperature, osmoregulatory system breaks down at higher salinity over a longer duration of time and vice versa at high temperature. In the present study too, temperature and salinity showed coupled effect on fish survival in salinity tolerance test (experiment I) for 10 days (mortality was observed from 3rd day onwards in S4-S10 and 5th day onwards in S2) at temperature range of 29.00 to 29.90 ^{0}C and prolonged exposure (experiment II) for 120 days (mortality was observed from 45 day onwards in S4-S10 and 90 day onwards in S2) at averegae temperature range of 14.2 to 30.5^{0}C. Results of present study also exhibited that, during prolonged salinity exposure, goldfish was tolerant to low salinity (2 ppt), suggesting it as a stenohaline freshwater fish (Luz *et al* 2008). The results are in accordance with the previous studies on stenohaline freshwater species like *Amblypharyngodon mola* (Dubey *et al* 2014), *Cirrhinus mrigala* and *Labeo fimbriatus* (Kasim 1983), *Labeo rohita* (Pillai *et al* 2003, Ansal *et al* 2013) and *Catla catla* (Ghosh *et al* 1973, Ansal *et al* 2013). Some of the freshwater species viz., common carp, Koi carp and spotted murrel, however revealed salinity tolerance up to higher level of 12 ppt (Wang *et al* 1997, Dubey *et al* 2016 and Sharma *et al* 2017).

Optimum range of salinity tolerance of different fish species could be attributed to number of factor including age of fish, temperature optima, and experimental design with variations of other environmental factors and genetic stock having genetic variations between different populations (Kefford *et al* 2004 and Overton *et al* 2008). Moreover, survival of freshwater fish could be affected due to increasing osmotic maintenance requirement due to sudden influx of saline water beyond isotonic point at higher salinity resulting in altered enzymatic functions (Lutz 1972, Kilambi 1980 and Handerland *et al* 2000).

4.2.3.2 Growth

The fish growth was assessed in terms of total body length (TBL) and body weight (BW) at fortnightly intervals during experimental period of 120 days. At the termination of the experiment, growth parameters i.e. total body length gain (TLG), net weight gain (NWG) and specific growth rate (SGR) of fish for each treatment were calculated, to compare the effect of increasing salinity on fish growth.

Among different treatments, the mean final total body length (cm) and mean body weight (g) of fish was 8.11, 8.19, 8.17, 8.23, 8.01 and 7.76; 7.98, 7.28, 7.38, 7.01, 5.37 and 5.16 in S0, S2, S4, S6, S8 and S10, respectively, which revealed significant ($p \leq 0.05$) effect of increasing salinity from 0-10 ppt on fish growth. The differences for growth parameters in terms of TLG (S2=S0≥S6=S4>S8=S10), %TLG (S2=S0≥S6=S4>S8>S10), NWG (S0≥S2≥S4=S6>S8=S10), %NWG (S0≥S2=S6≥S4>S8=S10) and SGR (S0≥S2≥S4=S6>S8=S10) were significant ($p \leq 0.05$) and are indivative of negative impact of salinity on fish growth (Table 9 and 10; Figure 12). Fish performane in terms of all the growth parameters revealed ≤ 2ppt salinity as comfortable level for long term rearing. Further, there was remarkable decline in growth at higher salinities of 8 and 10 ppt (negative values in S8 and S10). The adverse effects of salinity in fish growth can be explained as metabolic and physiological breakdown at higher salinities of 8 and 10 ppt (Mangat and Hundal 2014).

Previous studies have also indicated that the rising salinity in freshwater had no negative effect on growth rate of fish up to certain level, thereafter it declines, which indicates tolerance level of particular fish species. Although fish can survive at higher salinities, but after certain level, growth gets compromised. Similar to present study, Luz *et al* (2008) too observed high growth rate in gold fish adapted to 0 to 2 ppt (1.2 % day^{-1}), but as the salinity increased to 8 and 10 ppt, growth rate declined (0.4 % day^{-1} and 0.2 % day^{-1} respectively). However, Altinok and Gizzle (2001) reported that growth of freshwater ornamental gold fish affected at ≥ 9 ppt. In their study, growth of gold fish was maximum in 8 ppt (0.53 % day^{-1}) and 12 ppt (0.34 % day^{-1}), as compared to fish reared at 20 ppt (0.06 % day^{-1}). Similar results were observed by Küçük (2013) in their study on gold fish, *C. auratus* and crucian carp, *C. carassius.* Both fish species can tolerate salinity up to 20 ppt with 100 % survival, but growth gets compromised. Maximum growth was recorded up to 12 ppt (0.30% day^{-1}), get too low in 16 ppt (0.08 % day^{-1}) and

Table 9: Changes in total body length (cm) of shubunkin goldfish, *C. auratus* (L.) in different salinity levels during the experimental period

Month	Days	TREATMENTS*					
		S0	**S2**	**S4**	**S6**	**S8**	**S10**
August	**0**	8.04^{a}±0.14	8.10^{a}±0.14	8.15^{a}±0.14	8.19^{a}±0.14	8.26^{a}±0.15	8.25^{a}±0.15
	15	8.08^{a}±0.14	8.12^{a}±0.14	8.16^{a}±0.14	8.20^{a}±0.14	8.23^{a}±0.07	8.13^{a}±0.12
	30	8.09^{a}±0.14	8.13^{a}±0.14	8.18^{a}±0.14	8.21^{a}±0.14	8.26^{a}±0.15	8.13^{a}±0.14
September	**45**	8.10^{a}±0.14	8.06^{a}±0.14	8.18^{a}±0.14	8.21^{a}±0.14	8.26^{a}±0.14	8.07^{a}±0.14
	60	8.15^{a}±0.13	8.17^{a}±0.14	8.18^{a}±0.14	8.22^{a}±0.14	8.16^{a}±0.14	7.98^{a}±0.11
October	**75**	8.07^{a}±0.12	8.17^{a}±0.14	8.19^{a}±0.14	8.21^{a}±0.14	8.15^{a}±0.14	7.95^{a}±0.12
	90	8.08^{a}±0.12	8.16^{a}±0.14	8.20^{a}±0.14	8.22^{a}±0.14	8.14^{a}±0.14	7.88^{a}±0.11
November	**105**	8.10^{a}±0.12	8.18^{a}±0.14	8.16^{a}±0.17	8.23^{a}±0.14	8.03^{a}±0.17	7.80^{a}±0.12
	120	8.11^{ab}±0.12	8.19^{ab}±0.15	8.17^{ab}±0.17	8.23^{a}±0.15	8.01^{ab}±0.17	7.76^{b}±0.11
TLG		0.07^{a}±0.09	0.09^{a}±0.18	0.02^{ab}±0.18	0.04^{ab}±0.12	-0.25^{c}±0.20	-0.48^{c}±0.06
% TLG		0.87^{a}±0.20	1.12^{a}±0.07	0.25^{ab}±0.41	0.50^{ab}±0.27	-2.82^{c}±0.54	-5.66^{d}±0.99

*** S0 = 0 ppt, S2= 2ppt, S4 = 4 ppt, S6 = 6ppt, S8= 8 ppt, S10= 10ppt**

TLG =Total Length Gain

Values are Mean ± S.E. ($p \leq 0.05$)

Values with same superscripts (a,b,c…..f) in a row do not differ significantly ($p \leq 0.05$)

Table 10: Growth parameters of shubunkin goldfish, *C. auratus* (L.) in different salinity levels during and at the completion of the experiment

Month	Days	TREATMENTS*					
		S0	**S2**	**S4**	**S6**	**S8**	**S10**
August	**0**	$6.40^{a}\pm0.27$	$6.40^{a}\pm0.22$	$6.80^{a}\pm0.29$	$6.60^{a}\pm0.42$	$6.50^{a}\pm0.27$	$6.30^{a}\pm0.10$
	15	$7.0^{a}\pm0.25$	$6.90^{a}\pm0.17$	$6.80^{a}\pm0.29$	$6.90^{a}\pm0.27$	$5.90^{b}\pm0.23$	$6.00^{b}\pm0.36$
	30	$7.32^{a}\pm0.22$	$6.71^{b}\pm0.25$	$6.55^{b}\pm0.21$	$6.67^{b}\pm0.06$	$5.84^{c}\pm0.15$	$5.80^{d}\pm0.03$
September	**45**	$7.69^{a}\pm0.18$	$6.90^{b}\pm0.25$	$6.63^{b}\pm0.20$	$6.50^{b}\pm0.07$	$5.75^{c}\pm0.15$	$5.77^{c}\pm0.06$
	60	$7.79^{a}\pm0.19$	$6.99^{b}\pm0.26$	$6.73^{b}\pm0.20$	$6.05^{c}\pm0.21$	$5.67^{c}\pm0.16$	$5.76^{d}\pm0.04$
October	**75**	$7.85^{a}\pm0.18$	$7.10^{b}\pm0.25$	$6.83^{b}\pm0.20$	$6.18^{c}\pm0.23$	$5.60^{d}\pm0.16$	$5.73^{e}\pm0.05$
	90	$7.90^{a}\pm0.18$	$7.22^{b}\pm0.25$	$6.93^{b}\pm0.20$	$6.37^{c}\pm0.20$	$5.55^{d}\pm0.17$	$5.44^{d}\pm0.05$
November	**105**	$7.93^{a}\pm0.18$	$7.26^{b}\pm0.26$	$7.08^{b}\pm0.21$	$6.95^{b}\pm0.27$	$5.47^{c}\pm0.16$	$5.35^{d}\pm0.06$
	120	$7.98^{a}\pm0.17$	$7.28^{b}\pm0.27$	$7.38^{b}\pm0.15$	$7.01^{b}\pm0.27$	$5.37^{c}\pm0.17$	$5.16^{d}\pm0.10$
NWG		$1.58^{a}\pm0.36$	$0.89^{ab}\pm0.31$	$0.58^{b}\pm0.27$	$0.41^{b}\pm0.50$	$-1.14^{c}\pm0.20$	$-1.12^{c}\pm0.25$
% NWG		$26.67^{a}\pm5.80$	$14.67^{ab}\pm4.88$	$9.90^{b}\pm3.99$	$11.05^{ab}\pm9.35$	$-17.11^{c}\pm2.54$	$-17.03^{c}\pm3.15$
SGR		$1.32^{a}\pm0.29$	$0.73^{ab}\pm0.26$	$0.48^{b}\pm0.22$	$0.34^{b}\pm0.42$	$-0.95^{c}\pm0.17$	$-0.93^{c}\pm0.21$

*** S0 = 0 ppt, S2= 2ppt, S4 = 4 ppt, S6 = 6ppt, S8= 8 ppt, S10= 10ppt**

NWG = Net Weight Gain, SGR = Specific Growth Rate

Values are Mean ± S.E. ($p\leq0.05$)

Values with same superscripts (a,b,c…..f) in a row do not differ significantly ($p\leq0.05$).

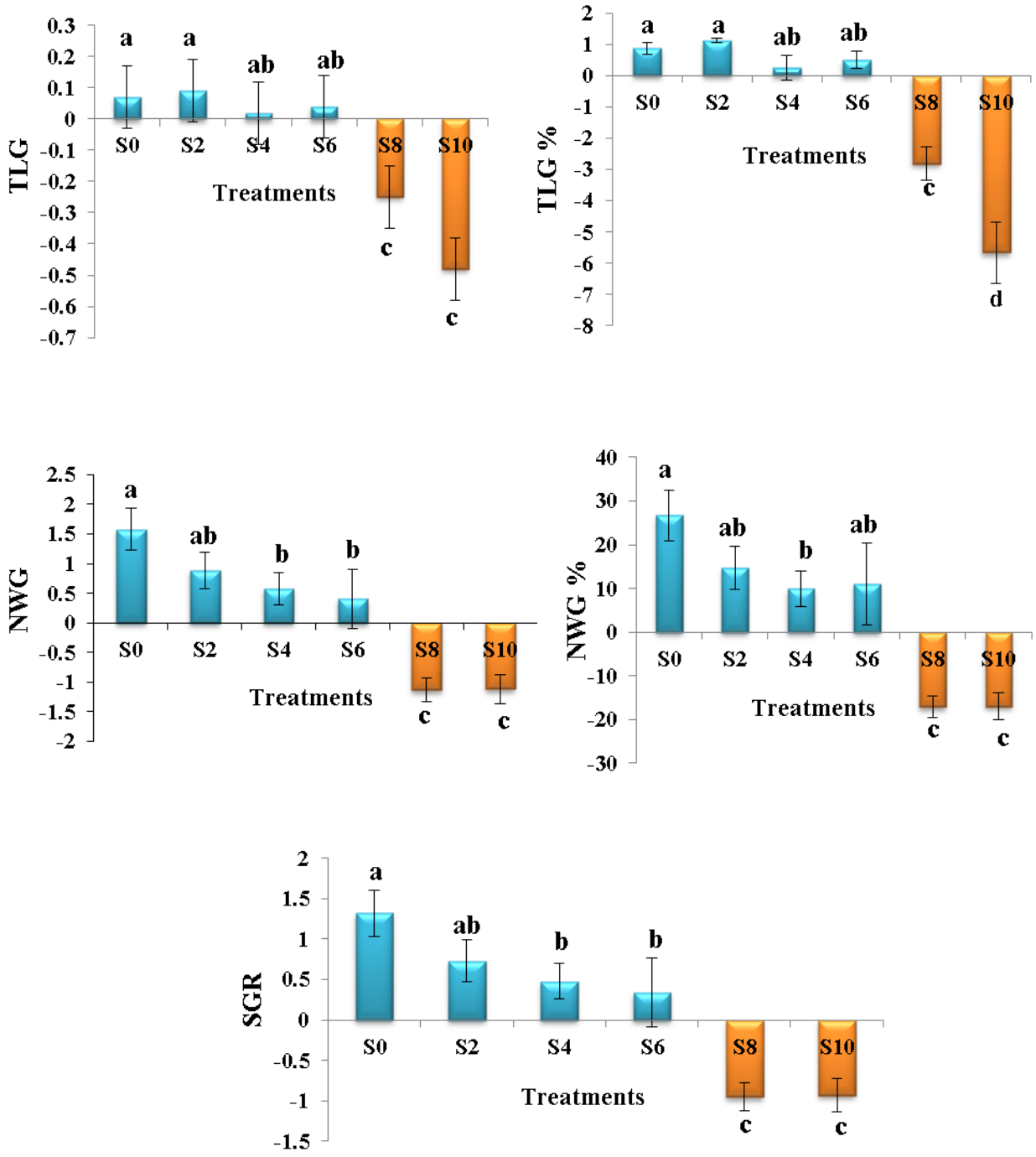


Figure 12: Comparative TLG, % TLG, NWG, % NWG and SGR of shubunkin goldfish, *C. auratus* (L.) in different salinity levels at the completion of the experimental period

become negative at 20 ppt (-0.06 % day^{-1}). Similarly growth rates of *Ctenopharyngodon idella* and *Anabas testudineus* reared at different salinities were not significantly hampered up to 5 ppt and 10 ppt (Kilambi 1980 and Dubey *et al* 2015) respectively.

Many researchers found that freshwater ornamental fishes grew better in brackish water than freshwater or sea water (Vonck *et al* 1998, Imsland *et al* 2001, Luz *et al* 2008, and Küçük *et al* 2013 and Sharma *et al* 2017). There is an accepted hypothesis that iso-osmotic waterdecreases the use of energy for osmoregulation compared to fresh or salt water and the saved energy is used for growth. Boeuf and Payan (2011) reported that fish in an isotonic medium has the lowest standard metabolic rates, whereas osmoregulation in sea water appears to be energetically more expensive than in freshwater. According to Wurts (1998), vertebrate animals (fish, bird, mammal, amphibians and reptiles) have a unique and common characteristic. The salt content of their blood is virtually identical having salinity of 9 g l^{-1} (0.9 percent salt solution) and almost 77 percent of salts in blood are sodium and chloride. The remainder is made up of bicarbonate, calcium and potassium. Among these, sodium, potassium and calcium are critical for the normal functioning of heart, nerve and muscle tissue. Further, high calcium concentration in terms of high alkalinity, hardness, bicarbonates, and electrical conductivity of surrounding water makes the fish to hold salt in their body in freshwater. Fish may have high salt in their blood and do not spend more energy to regulate osmotic balance and thus, grow rapidly to appropriate salinity level. Salinity is one of the important abiotic factors having significant effect on physiological well being of the cultured organism, and physiological status determine the growth of particular organism coupled with number of factors (Boeuf and Payan 2001). There was significant decrease in growth parameters from S4 to S10 in the present study showing negative impact of increased salinity along with higher pH, ammonical nitrogen and less dissolved oxygen. Lawson and Alake (2011) too observed appreciable decrease in the weight gain with increasing salinity in *C. auratus*, similar to the present study, proving that 0 ppt is the most favourable level but adaptability can be up to 4-6ppt.

Comparable growth of freshwater fish at higher salinities (> 0ppt) in the previous studies by different researchers and in present study suggested that the fish were able to regulate osmotic pressure of the body fluids. Further, the more the osmo-regulatory adaptations, lesser the difference between the compositions and pressures of the internal fluid of the organism and its

external environment (Nikolsky 1963). Islam *et al* (2014) too reported that rohu (*L. rohita*) showed similar growth at 0 and 4 ppt. It is also pertinent to mention here that, fish can survive for long and perform well at low salinity, whereas for shorter period at higher salinities. Schofield *et al* (2006 a,b) reported that gold fish can survive at < 10 ppt for longer period of time, as compared to higher salinity. Higher salinities result in significant muscle dehydration, which leads to adverse effect on growth, food intake and hence food conversion ratio (Luz *et al* 2008). Kumar *et al* (2017) too reported that though freshwater sutchi pangas catfish, *Pangasianodon hypophthalmus* survived up to 15 ppt, but 10 ppt was found to be optimum salinity for culture in inland saline waters. Reduction in growth in particular freshwater species at higher salinity may be attributed to lower food consumption due to reduced appetite resulting in decreased food conversion efficiency (Overton *et al* 2008).

In the present study, observations w.r.to feeding behaviour during salinity tolerance test and during growth experiment clearly depicted effect of increasing salinity on appetite, which showed gradual change from high to low appetite (Table 11). Further, De Boeck *et al* (2000) reported that exposure to salt reduced food intake by 70 % in freshwater fishes. Stress response due to toxicity of pollutants like chlorine for the freshwater ornamental fishes like koi carp and sword tail resulted in significant decrease in body weight and weight gain along with altered haematology (El-Bouhy *et al* 2006).

4.2.4 Fish behavior

Fish behaviour in terms of swimming activity, feeding responses and morphological characters like colouration, body fragility and presence of mucus on skin were recorded at fortnightly intervals, are depicted in Table 11. The results indicated stressful condition of fish reared in higher salinity levels of 8 ppt (S8) and 10 ppt (S10). However, deviations from normal responses (low activity, sluggishness, low appetite, skin discolouration, high fragility and excessive mucus) were also seen at 6 ppt (S6) after experimental period of 45 days. These results are in accordance to the decreased fish survival and growth in S8 and S10 (Table 8-10). Altered appetite due to increasing salinity have been reported in several species (Arunachalam and Reddy 1979, Kilambi 1980, MacEina and Shireman 1980, McKay and Gjerde 1985, Wang *et al* 1997, De Boeck *et al* 2000, Altinok and Grizzle 2001, Imsland *et al* 2001, Lawson and Alake 2011 and Dubey *et al* 2016).

Table 11: Behavioural and morphological responses in shubunkin gold fish, *C. auratus* (L.) in different salinity levels during the experimental period

Behaviour	Days	TREATMENTS*					
		S0	**S2**	**S4**	**S6**	**S8**	**S10**
Swimming activity	**0**	VA	VA	VA	VA	VA	VA
	15	VA	VA	VA	VA	VA	LA
	30	VA	VA	VA	VA	LA	LA
	45	VA	VA	LA	LA	LA	S
	60	VA	VA	LA	LA	LA	S
	75	VA	VA	LA	LA	LA	S
	90	VA	VA	LA	LA	S	S
	105	VA	VA	LA	LA	S	S
	120	VA	VA	LA	LA	S	S
Feeding response	**0**	Hap	Hap	HAp	Hap	HAp	HAp
	15	Hap	Hap	HAp	Hap	HAp	HAp
	30	Hap	Hap	HAp	Hap	HAp	HAp
	45	Hap	Hap	HAp	Hap	HAp	HAp
	60	Hap	Hap	HAp	Hap	HAp	LAp
	75	Hap	Hap	HAp	Hap	HAp	LAp
	90	Hap	Hap	HAp	Hap	LAp	LAp
	105	Hap	Hap	HAp	Hap	LAp	LAp
	120	Hap	Hap	Lap	Lap	LAp	LAp
Colouration	**0**	NC	NC	NC	NC	NC	NC
	15	NC	NC	NC	NC	NC	NC
	30	NC	NC	NC	NC	NC	NC
	45	NC	NC	NC	NC	DC	DC
	60	NC	NC	NC	DC	DC	DC
	75	NC	NC	NC	DC	DC	DC
	90	NC	NC	NC	DC	DC	DC
	105	NC	NC	NC	DC	DC	DC
	120	NC	NC	NC	DC	DC	DC
Body fragility	**0**	NF	NF	NF	NF	NF	NF
	15	NF	NF	NF	NF	NF	NF
	30	NF	NF	NF	NF	NF	NF
	45	NF	NF	NF	HF	HF	HF
	60	NF	NF	NF	HF	HF	HF
	75	NF	NF	HF	HF	HF	HF
	90	NF	NF	HF	HF	HF	HF
	105	NF	NF	HF	HF	HF	HF
	120	NF	NF	HF	HF	HF	HF
Mucus	**0**	NM	NM	NM	NM	NM	NM
	15	NM	NM	NM	NM	NM	NM
	30	NM	NM	NM	NM	NM	NM
	45	NM	NM	NM	EM	EM	EM
	60	NM	NM	NM	EM	EM	EM
	75	NM	NM	NM	EM	EM	EM
	90	NM	NM	NM	EM	EM	EM
	105	NM	NM	NM	EM	EM	EM

	120	NM	NM	NM	EM	EM	EM

*** S0 = 0 ppt, S2= 2ppt, S4 = 4 ppt, S6 = 6ppt, S8= 8 ppt, S10= 10ppt**

Decreased sensitivity of the olfactory nerves to amino acids, reducing the stimulus to eat can be the most probable reason for reduced appetite (Shoji *et al* 1996). Further, shorter time of feed retention in the gut along with increased drinking rates for osmoregulation get coupled to have negative affect on fish growth (Ferraris *et al* 1986). The normal swimming activity and high appetite displayed by fish towards food indicates regulation and maintenance of body metabolism at appropriate salinities (0-6 ppt in present study), while low appetite with sluggishness revealed total body metabolic breakdown (Mangat and Hundal 2014). Lawson and Alake (2011) too observed erratic swimming behaviour of gold fish at 10 ppt salinity along with different levels of response to feeding in 4-9 ppt salinities. All these responses were normal at lower salinity level of up to 3 ppt. Morphological change w.r.to dull skin colouration, high fragility with excessive mucus secretion also indicated stress condition of fish at higher salinity levels. Islam *et al* (2014) too reported high appetitive behaviour between 0 to 4 ppt salinities, and with increasing salinity from 6-12 ppt, moderate appetite, low appetite and death were displayed. Adverse effects of higher salinities were prominent in gold fish in terms of food intake with altered feeding response by Luz *et al* (2008) and Lawson and Alake (2011), along with deviation from normal body colour. Further, stress resulting in abnormal swimming behaviour, decreased respiratory rate with sign of anoxia, excessive mucus secretion on body surface, loss of reflexes and fade colouration are some of the changes easily depicted in koi carp and sword tail (El-Bouhy *et al* 2006).

4.2.5 Haematological parameters

The mean haematological parameters of fish including Haemoglobin (Hb), Red Blood Cell (RBC), White Blood Cells (WBC), Packed Cell Volume (PCV), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Content (MCHC) analyzed/ calculated at the completion of the experiment, are presented in Table 12.

4.2.5.1 Haemoglobin (Hb)

Among different treatments, mean Hb (g %) of fish was 5.83, 4.50, 3.50, 3.56, 3.53 and 3.60 in S0, S2, S4, S6, S8 and S10 respectively and the values for control (S0) was significantly

higher as compared to all the treatments (S0>S2=S10=S6=S8=S4), however, diffrences for all the salinity treatments were insignificant. The Hb in fish decreased significantly ($p \leq 0.05$) from S0 (0ppt) to S2 (2ppt) and afterwards it remained constant.

4.2.5.2 Red blood cells (RBC)

Among different treatments, mean RBC ($\times 10^6/mm^{3-1}$) of fish was 1.31, 2.10, 2.90, 3.56, 3.86 and 4.46 in S0, S2, S4, S6, S8 and S10 respectively and the differences among treatments were significant (S10≥S8≥S6>S4>S2>S0). The RBC in fish increased significantly ($p \leq 0.05$) up to highest salinity level i.e. 10 ppt (S10).

4.2.5.3 White blood cells (WBC)

Among different treatments, mean WBC ($\times 10^3/mm^{3-1}$) of fish was 3.32, 3.40, 4.04, 4.83, 6.19 and 6.28 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S10=S8>S6≥S4≥S2=S0). The WBC in fish increased significantly ($p \leq 0.05$) as the salinity increased to highest level i.e. 10 ppt (S10).

4.2.5.4 Packed cell volume (PCV)

Among different treatments, mean PCV (%) of fish was 5.96, 7.23, 7.90, 8.26, 8.33 and 9.03 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S10≥S8=S6=S4>S2>S0). The PCV in fish was significantly ($p \leq 0.05$) higher in all the salinity treatments up to 10 ppt (S10), however, the PCV remained stable from S4-S8 (4-8ppt).

4.2.5.5 Mean corpuscular volume (MCV)

Among different treatments, mean MCV (μm^3) of fish was 45.85, 36.50, 27.28, 23.33, 21.61 and 20.36 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S0≥S2≥S4≥S6=S8=S10). The level of MCV in fish decreased significantly ($p \leq 0.05$) as the salinity increased to 6 ppt (S6), thereafter the values remained stable.

4.2.5.6 Mean corpuscular haemoglobin (MCH)

Among different treatments, mean MCH (g %) of fish was 44.85, 23.17, 12.09, 10.01, 9.11 and 8.03 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S0>S2>S4=S6=S8=S10). The level of MCH in fish decreased significantly ($p \leq 0.05$) as the salinity increased to 4 ppt (S4) and the differences were insignificant for rest of the salinity treatments (S4-S10).

4.2.5.7 Mean corpuscular haemoglobin content (MCHC)

Among different treatments, mean MCHC (g %) of fish was 97.72, 62.09, 44.24, 43.07, 42.37 and 39.88 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S0>S2>S4=S6=S8=S10). The level of MCHC in fish decreased significantly. ($p \leq 0.05$) as the salinity increased to 4 ppt (S4) and like MCV and MCH, the differences for MCHC were insignificant for rest of the salinity treatments (S4-S10).

Haematological parameters in terms of RBC, WBC and PCV showed increasing trend with increasing salinity (S0 to S10) and the differences among treatments were significant. (Table 12; Figures 13-19). However, Hb and haematological indices i.e. MCV, MCH and WBC showed significant decrease up to S10. The increase and decrease of different parameters was significant but gradual, which can be due to low temperature coupled with increasing salinity conditions for longer duration (120 days), resulting in stress, which must have regulated the physiological responses gradually, so as to reach internal homeostasis.

Fish lives in very close contact with their environment and are therefore always at risk to chemical and physical changes, which may be indicated in their blood components (Wilson and Taylor 1993). Salinity is one of the most significant abiotic factors for aquaculture and its most favourable levels are species specific for survival, growth and efficient production. Furthermore, osmotic stress has been reported to draw physiological responses corresponding to haematological alterations. Numerous phases have been described in fish following salinity challenges. Houston (1959) was the first to describe these phases. He described crisis phase as a period of rapid and significant changes leading to the stabilization phase, in which homeostatic mechanisms regulate internal conditions to a stable value within the tolerance range. Increase in

Table 12: Haematological parameters of shubunkin goldfish, *C. auratus* (L.) in different salinity levels at the completion of the experiment

PARAMETERS	TREATMENTS*					
	S0	**S2**	**S4**	**S6**	**S8**	**S5**
Hb (g %)	$5.83^{a}\pm0.21$	$4.50^{b}\pm0.35$	$3.50^{b}\pm0.17$	$3.56^{b}\pm0.29$	$3.53^{b}\pm0.33$	$3.60^{b}\pm0.30$
RBC ($x10^6$ mm^{3-1}) OR $x10^6/mm^3$	$1.31^{e}\pm0.10$	$2.10^{d}\pm.0.32$	$2.90^{c}\pm0.05$	$3.56^{b}\pm0.20$	$3.86^{ab}\pm0.18$	$4.46^{a}\pm0.26$
WBC ($x10^3$ mm^{3-1}) OR $x10^3/mm^3$	$3.32^{c}\pm3.49$	$3.40^{c}\pm2.76$	$4.04^{bc}\pm2.96$	$4.83^{b}\pm1.54$	$6.19^{a}\pm3.34$	$6.28^{a}\pm1.54$
PCV (%)	$5.96^{d}\pm0.033$	$7.23^{c}\pm0.145$	$7.90^{b}\pm0.173$	$8.26^{b}\pm0.176$	$8.33^{b}\pm0.176$	$9.03^{a}\pm0.26$
MCV (μm^3)	$45.85^{a}\pm3.52$	$36.50^{ab}\pm6.85$	$27.28^{bc}\pm1.14$	$23.33^{c}\pm1.49$	$21.61^{c}\pm0.68$	$20.36^{c}\pm1.33$
MCH (g %)	$44.85^{a}\pm4.11$	$23.17^{b}\pm5.82$	$12.09^{c}\pm0.84$	$10.01^{c}\pm0.71$	$9.11^{c}\pm0.58$	$8.03^{c}\pm0.22$
MCHC (g %)	$97.72^{a}\pm3.14$	$62.09^{b}\pm3.72$	$44.24^{c}\pm1.22$	$43.07^{c}\pm2.86$	$42.37^{c}\pm3.81$	$39.88^{c}\pm3.41$

*** S0 = 0 ppt, S2= 2ppt, S4 = 4 ppt, S6 = 6ppt, S8= 8 ppt, S10= 10ppt**

Values are Mean±S.E. ($p\leq0.05$)

Values with same subscripts (a,b...e) in a row do not differ significantly ($p\leq0.05$)

RBC - red blood cells, WBC - white blood cells, Hb - haemoglobin, PCV - packed cell volume, MCV- mean corpuscular volume, MCH-mean corpuscular haemoglobin, MCHC- mean corpuscular haemoglobin content

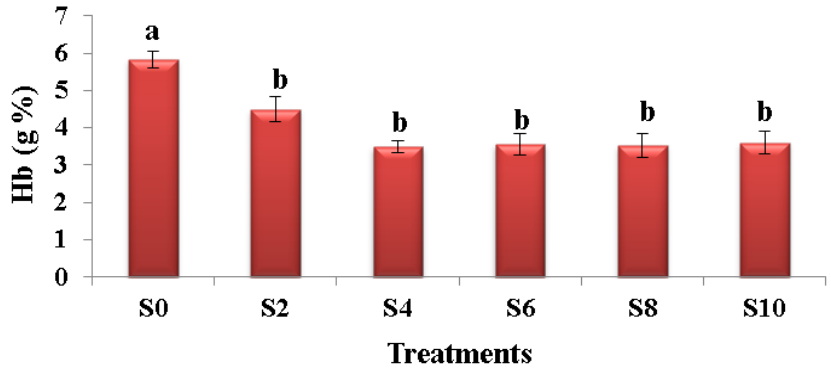


Figure 13: Comparative Hb (g %) content in blood of shubunkin goldfish *C. auratus* (L.) in different salinity levels at the completion of the experiment

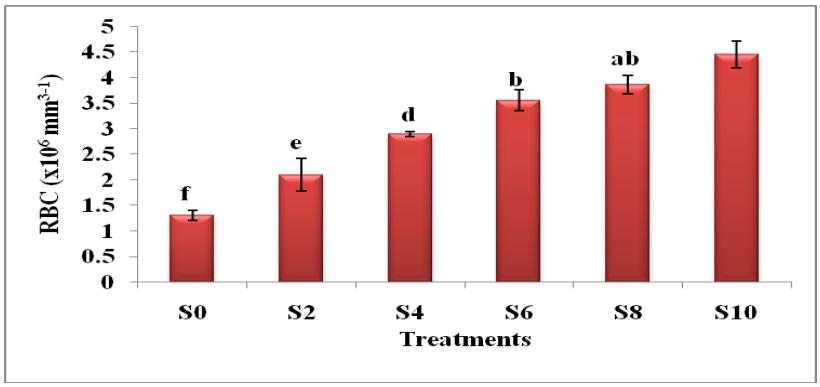


Figure 14: Comparative RBC (x10^6 mm^{3-1}) in blood of shubunkin goldfish *C. auratus* (L.) in different salinity levels at the completion of the experiment

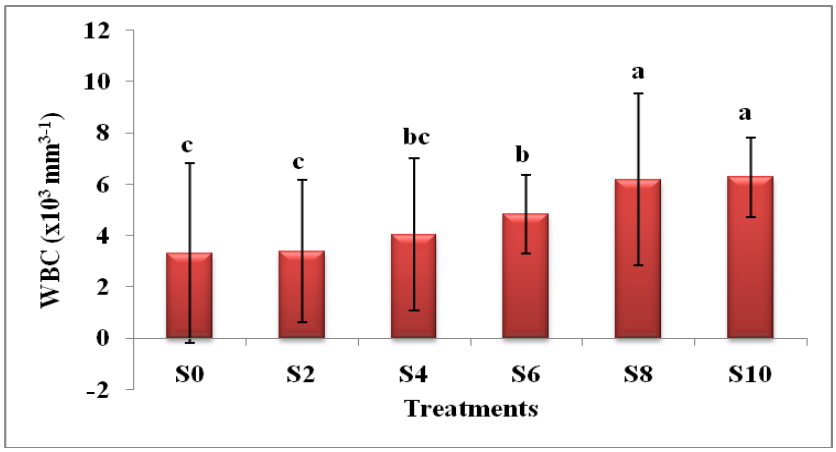


Figure 15: Comparative WBC (x10^3 mm^{3-1}) in blood of shubunkin goldfish *C. auratus* (L.) in different salinity levels at the completion of the experiment

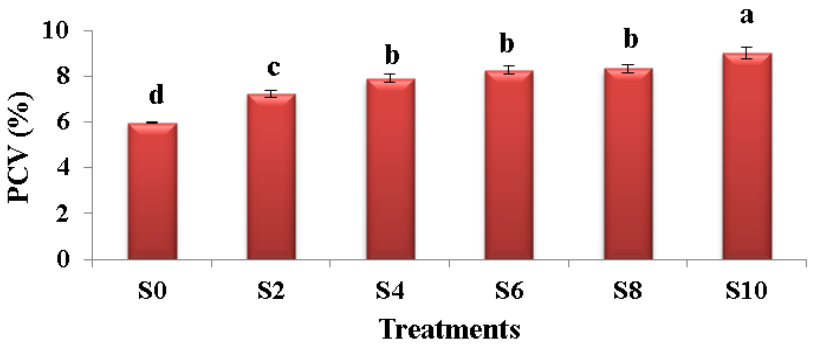


Figure 16: Comparative PCV (%) content in blood of shubunkin goldfish *C. auratus* (L.) in different salinity levels at the completion of the experiment

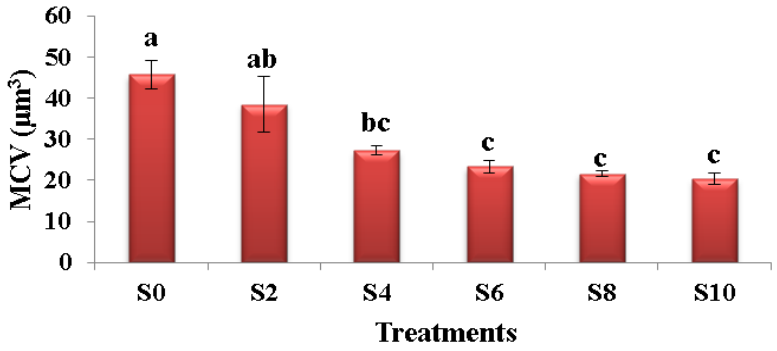


Figure 17: Comparative MCV (µm^3) content in blood of shubunkin goldfish *C. auratus* (L.) in different salinity levels at the completion of the experiment

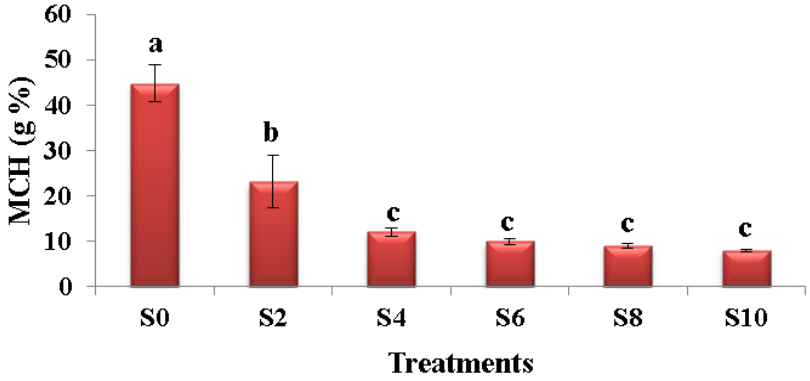


Figure 18: Comparative MCH (g %) content in blood of shubunkin goldfish *C. auratus* (L.) in different salinity levels at the completion of the experiment

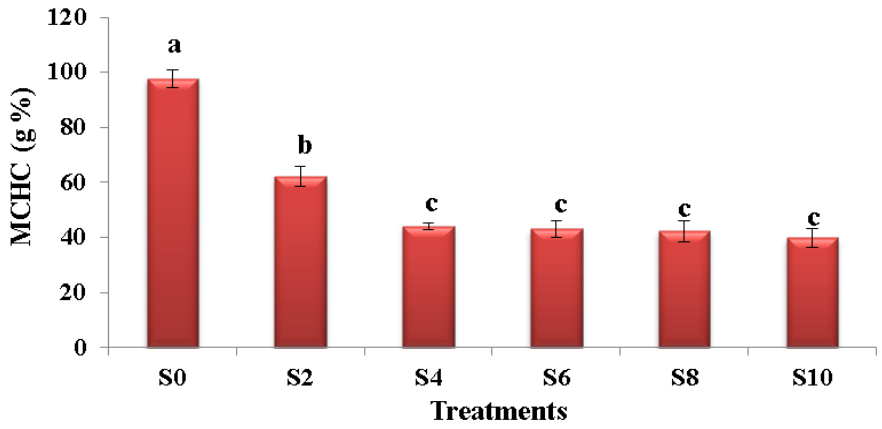


Figure 19: Comparative MCHC (g %) content in blood of shubunkin goldfish *C. auratus* (L.) in different salinity levels at the completion of the experiment

PCV or haematocrit during stress results from loosing good quantity of water (dehydration) because of difference between the internal and external environmental ionic composition. Due to osmotic pressure, water get released in external environment (higher salinity) leading to increasing PCV percentage. Sultan (2007) reported increase in PCV as first stage of acclimation to salt water and the value get back to preliminary level after some time, which varies from species to species. The results get further confirmed with earlier studies carried out by gradual transfer of grass carp (*Ctenopharyngodon idella*) to salt concentration of 4, 8 and 12 ppt (Al-Khshali 2011) and gradual (7.1, 9.1 and 10.9 ppt) and sudden transfer (10.9 and 12.5 ppt) of *Barbus sharpeyi*, leading to increased PCV, due to increase in Na^+ uptake by RBC, reflected in terms of PCV count along with plasma dryness. However, some of the earlier studies also showed opposite trend as noticed by Morgan and Iwana (1991) in juvenile fall chinook salmon (*O. tshawytscha*), PCV decreased with increasing salt concentration, while Ahmed *et al* (2004) reported no difference in PCV values of *Liza abu*, when transferred from freshwater to saline water of 7 ppt, but further transfer to 15 ppt showed reduction in PCV values. These differences can be attributed to differences in said studies with respect to species and size of fish, salinity levels and exposure period and ionic composition of water used. The increase in RBC is the result of increasing oxygen consumption owing to increased energy requirement (Martinez *et al* 2002 and Al-Hilali and Al-Khshali 2016).

Exposure to stress along with reduced dissolved oxygen concentration causes the release of catecholamines resulting in stimulation of splenic red blood cells production. This situation

causes increase in red blood cells along with PCV percentage (Kasim 1983 and Hosseini *et al* 2011). Further, increase in oxygen demand during stressed conditions forces kidneys to detect hypoxia leading to increased movement of red blood cells (Di Giulio and Hinton 2008 and Khabbazi *et al* 2015) The increase in RBC is mostly coupled with increased level of Hb, as Hb is the protein carried by RBC and playing a major role in respiration. Moreover, the increase in Hb concentration has always been considered as a vital indicator of any environmental change (Bani and Haghi 2009). The increase in Hb concentration has been reported by Hafez and Oryan (2002) in common carp exposed to high concentration of NaCl, in koi carp exposed to 12 ppt inland saline water (Sharma *et al* 2017) and in *C. auratus* exposed to temperature stress (Houston and Rupert 1997). However, in the present study, Hb showed decreasing trend with increasing salinity. Hb decreased significantly in S2 (2ppt), afterwards, the decrease was gradual but not significant. This trend can be explained in relation to poor quality of RBC resulting in abnormal form carrying inadequate amount of hemoglobin. This type of situation results in large numbers of red blood cells being destroyed, which can lead to anemia and moreover, this can be considered as an adaptive mechanism towards salinity stress. The findings w.r.t. increased RBC and decreased Hb levels in the present study need further investigation.

White blood cells (WBC) count in fish is a good indicator of physiological stress (Svobodovà *et al* 2001). The increase in WBC in the present study, with increasing salinity up to 10 ppt, could be attributed to the increase of WBC immigration across plasma (Gomes *et al* 2003). It can also be explained in terms of immunity reaction due to exposure of fish to salinity stress, resulted in increased cortisol, along with RBC and WBC as a defense mechanism in fish for getting back to balance state of ions and water to achieve internal stability. Further, the effect of salinity on blood characteristics is more prominent in young fish as compared to fingerlings (Akinrotimi *et al* 2012). Stimulation of immune system in response to tissue damage due to varied stress situations like exposure to pollutants or heavy metals also results in increased WBC count in number of fish species like *Heteropnuestes fossilis, Channa punctatus, Tinca tinca* and *Clarias batrachus* (Dhanekar *et al* 1985, Shah and Altindaǵ 2005 and Maheswaran *et al* 2008).

Blood indices viz., MCV, MCH and MCHC decreased significantly ($p \leq 0.05$) with increasing salinity. Fluctuations in haematological parameters with significant decreased values of MCV, MCH and MCHC may be due to stressful effect of exposure of various conditions

including environmental changes in freshwater fishes, as reported by many researchers (Shalaby 2001 and Vutkuru 2005). All these fluctuations in blood parameters may be attributed to the defense action against stress conditions through stimulation of physiological conditions especially erythropoiesis (Vinodhini and Narayanan 2009 and Abarghoei *et al* 2015).

4.2.6 Biochemical parameters

The mean biochemical parameters in blood serum of fish including total proteins, glucose, albumin and globulin were analyzed and albumin/globulin (Alb/Glb) ratio was calculated at the completion of the experiment (Table 13; Figure 20)

4.2.6.1 Blood glucose

Among different treatments, mean blood serum glucose (g dl^{-1}) of fish was 98.03, 44.63, 41.04 43.40, 61.50 and 191.20 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S10>S0>S8>S2>S6>S4). The blood serum glucose level decreased significantly ($p \leq 0.05$) from S0 to S6, showed significant increase in S8 as compared to S6 (but significantly lower than S0). The highest salinity treatment i.e. S10 (10ppt) showed abrupt and significant increase ($p \leq 0.05$) in blood serum glucose.

4.2.6.2 Total Protein

Among different treatments, mean blood serum protein (g dl^{-1}) of fish was 4.24, 3.75, 1.97, 2.85, 3.74 and 9.10 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S10>S0>S2=S8>S6>S4). The total protein in blood serum of fish decreased significantly ($p \leq 0.05$) up to S4, showed significant increase in S6 and S8 as compared to S4 (but significantly lower than S0). The highest salinity treatment i.e. S10 (10ppt) showed abrupt and significant increase ($p \leq 0.05$) in total blood serum protein.

4.2.6.3 Albumin

Among different treatments, mean blood serum albumin (g dl^{-1}) of fish was 1.88, 1.09, 0.45, 1.12, 1.15 and 1.13 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S0>S8=S10=S6=S2>S4). The albumin level in blood serum of fish decreased significantly ($p \leq 0.05$) up to S4, showed significant increase in S6-S10 as compared to S4, but significantly lower than S0.

4.2.6.4 Globulin

Among different treatments, mean blood serum globulin (g dl^{-1}) of fish was 2.35, 2.65, 1.52, 1.73, 2.59 and 7.97 in S0, S2, S4, S6, S8 and S10 respectively and the difference among

treatments were significant (S10>S2=S8>S0>S6=S4). The globulin level was significantly higher ($p \leq 0.05$) in S10 (10ppt) as compared to control (S0) and other salinity treatments. The globulin level was significantly lower ($p \leq 0.05$) in S0 (0ppt) as compared to all the salinity treatments except S6 (6ppt). However, globulin level did not revealed any particular trend.

4.2.6.5 Alb/Glb ratio (g dl^{-1})

Among different treatments, mean blood serum Alb/Glb ratio (g dl^{-1}) was 0.79, 0.41, 0.29, 0.74, 0.44 and 0.14 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S0=S6>S8=S2≥S4≥S10). The Alb/Glb ratio (g dl^{-1}) in blood serum of fish decreased significantly ($p \leq 0.05$) up to S4, increased significantly in S6 (insignificant from S0), and again decreased with increasing salinity from 8-10 ppt (S8-S10).

Table 13: Biochemical parameters in blood serum of shubunkin goldfish *C. auratus* (L.) in different salinity levels at the completion of the experiment

Biochemical Parameters (g dl^{-1})	TREATMENTS*					
	S0	S2	S4	S6	S8	S10
Glucose	$98.03^{b} \pm 0.44$	$44.63^{d} \pm 0.39$	$41.04^{f} \pm 0.35$	$43.40^{e} \pm 0.38$	$61.50^{c} \pm 0.27$	$191.2^{a} \pm 0.49$
Protein	$4.24^{b} \pm 0.04$	$3.75^{c} \pm 0.05$	$1.97^{e} \pm 0.03$	$2.85^{d} \pm 0.04$	$3.74^{c} \pm 0.04$	$9.10^{a} \pm 0.06$
Albumin	$1.88^{a} \pm 0.02$	$1.09^{b} \pm 0.02$	$0.45^{c} \pm 0.02$	$1.12^{b} \pm 0.04$	$1.15^{b} \pm 0.05$	$1.13^{b} \pm 0.02$
Globulin	$2.35^{c} \pm 0.03$	$2.65^{b} \pm 0.07$	$1.52^{d} \pm 0.05$	$1.73^{d} \pm 0.06$	$2.59^{b} \pm 0.09$	$7.97^{a} \pm 0.08$
Alb/Glb ratio	$0.79^{a} \pm 0.01$	$0.41^{b} \pm 0.01$	$0.29^{bc} \pm 0.02$	$0.74^{a} \pm 0.13$	$0.44^{b} \pm 0.03$	$0.14^{c} \pm 0.005$

*** S0 = 0 ppt, S2= 2ppt, S4 = 4ppt, S6 = 6ppt, S8= 8 ppt, S10= 10ppt**
Values are Mean ± S.E. ($p \leq 0.05$)
Values with same subscripts (a,b...f) in a row do not differ significantly ($p \leq 0.05$)

In the present study, serum blood glucose and total proteins decreased significantly ($p \leq 0.05$) up to 8 ppt (S8), with abrupt and significant rise in 10 ppt (S10). There was significant decrease and increase in albumin and globulin levels with increasing salinity up to highest level i.e. 10 ppt, whereas in accordance to increased values of globulins, Alb/Glb ratio decreased significantly up to 4 ppt, with sudden increase in S6, afterwards again decreased significantly ($p \leq 0.05$) in S8 and S10.

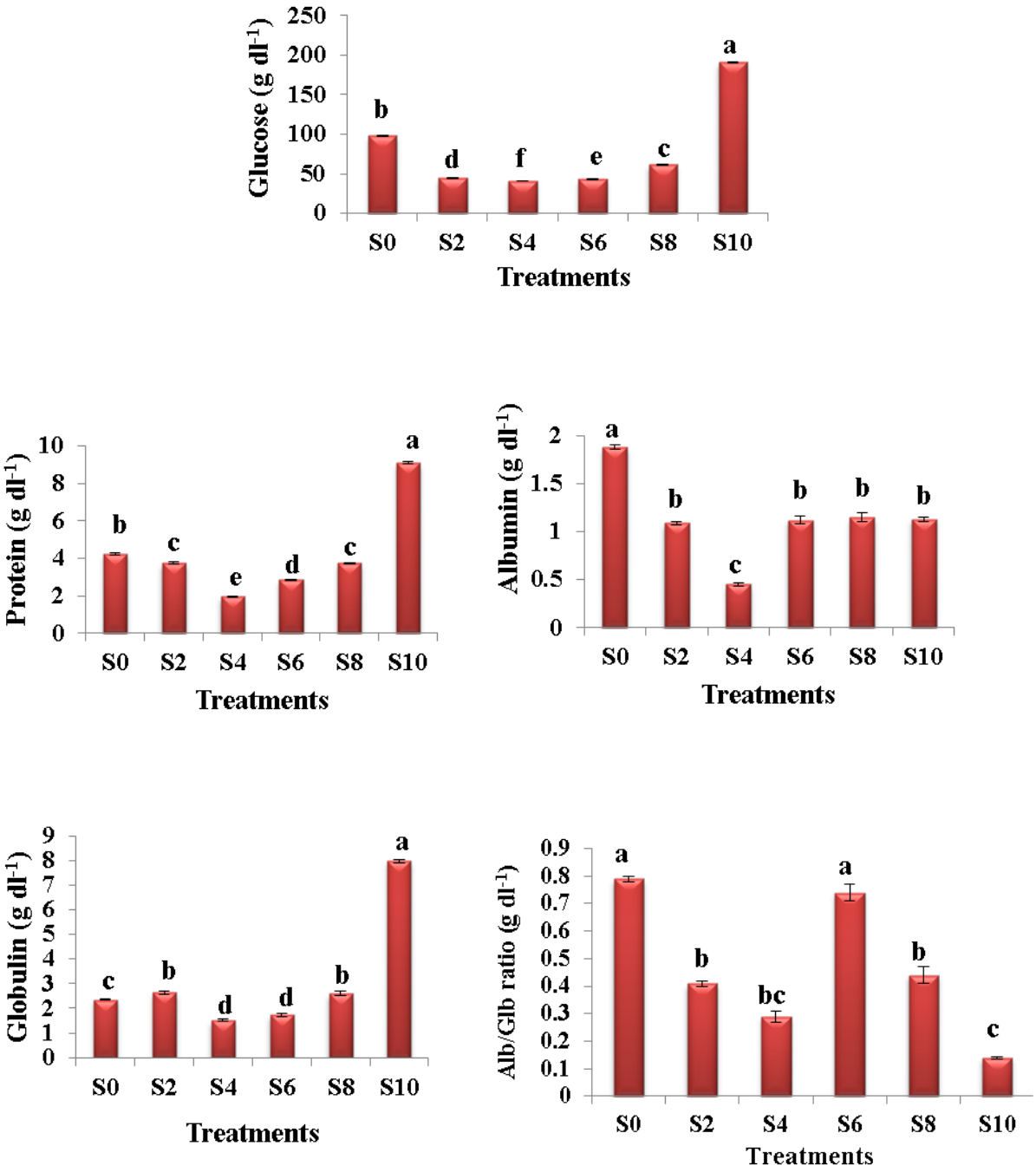


Figure 20: Comparative Glucose (g dl^{-1}), Total protein (g dl^{-1}), Albumin (g dl^{-1}), Globulin (g dl^{-1}) and Alb/Glb (g dl^{-1}) ratio in blood serum of shubunkin goldfish *C. auratus* (L.) in different salinity levels at the completion of the experiment

High salinity is one of the stress causing factor for both freshwater and marine fish. It affects fish in three dimensions. In primary response, sympathetic nervous system is stimulated for the release of catecholamines and plasma cortisol. In secondary responses, these hormones activate the release of glucose into the blood (Mommensen *et al* 1999) for energy production, heart rate, gill activity and metabolic rate. In the tertiary response, these changes in blood physiology cause reduction in growth, survival and disease resistance. Haematology or blood chemistry parameters are used as indicators of physiological stress response in fish (Lerman *et al* 2004, Koeypudsa *et al* 2007 and Kavya *et al* 2015). Salinity stress induced hormonal response can be observed in terms of high rate of glucogenolysis activity to meet the high energy demand and thus it results in increase level of blood glucose and reduced glycogen level in liver (Gelis 2004 and Kavya *et al* 2015) for few minutes to days, which can decrease over time until depleted (David *et al* 2005).

In the present study, the glucose level was measured after 120 days of experimental period, during which fish must be habituated towards salinity stress and decrease in glucose is linked to the depletion of reserve energy due to prolonged stress conditions. According to West *et al* (1993), during peak activity, glucose use can increase by almost 30-fold. However, it is also possible that fish exposed to prolonged chronic stress like in present study might suffer substrate depletion that led to decrease in blood glucose. A decrease in glucose in response to salinity stress up to 8 ppt in the present study, is linked to depletion of energy reserves due to osmoregulation. Few of the previous studies in this regard, depicted varied results showing both, an increase (Assem and Hanke 1979 and Bashamohideen and Parvatheswararao 1972) and decrease (Soengas *et al* 1991) in glucose value during salinity adaptation.

There appears to be high glucose demand in order to supply the energy by osmoregulatory mechanism (Plaut 1998), where upon glyconeogenesis even increases the use of glucose, could mass the plasma glucose increase prompted by cortisol. Sarma *et al* (2012) too observed difference in blood glucose level in *C. batrachus* exposed to salinities of 4 and 8 ppt. Blood glucose level was increased by 29.11% at 4 ppt and decreased by 28.8% at 8 ppt, compared to control. However, there were no significant differences in blood glucose level between control and 4 ppt and between 4 and 8 ppt. In this study also, low glucose level at 8 ppt could be attributed to higher utilization of glucose (Martinez-Alvarez *et al* 2002) and might be

associated with reduced appetite of the fishes at higher salinity (Usher *et al* 1991 and Plaut 1998). Likewise in the present study, decreased glucose level at higher salinities may result from energy utilization to cope up the salinity stress along with reduced appetite. However, abrut and significant rise in glucose level at 10 ppt revealed inability of fish to adapt higher salinity (10 ppt) conditions resulting in metabolic breakdown of freshwater fish (Mangat and Hundal 2014).

Changes in concentration of blood serum total protein and its components may be used as clinical indicator in assessment of the fish health, stress status and body conditions of particular species and it is an important non-specific immune parameter (Magnadottir 2006). In the present study, there was decrease in total serum protein, upto 8 ppt, which could be caused by alterations in amino acid metabolism (Hopkins *et al* 1995) resulting in breakdown of proteins. Pakhira *et al* (2015) and Dobšíkov *et al* (2009) reported decreasing trend of serum protein due to transportation stress indicating breakdown of proteins in rohu and common carp respectively. Few of the earlier studies also showed decline in total serum protein, after exposure to salinity. Shahkar *et al* (2015) found decline in plasma protein during increase of salinity in juvenile ship sturgeon (*A. nudiventris)*. Imanpoor *et al* (2012) too reported decreased serum protein with increase in salinity. Huang *et al* (2006) noted that as environmental salinity increases, energy consumption increases, which is provided through glucose and lipid through metabolism. Thus, when the sufficient energy is not available in the form of glucose, proteins would be utilized as energy source. Likewise, in the present study, fish has adapted itself to increasing salinity through the use of glucose for energy consumption and thus in response to osmoregulation mechanism; proteins were used in addition to carbohydrates.

However, some of the earlier studies by Küçük *et al* (2013) and Kavya *et al* (2015) observed increase in toal serum protein with increasing salinity *O. aureus* and *Notopterus notopterus* exposed to 12 ppt and 1.6 ppt respectively.

In the present study, blood serum protein decreased upto 4 ppt (1.97 g dl^{-1}), with little increase in 6 ppt (2.85g dl^{-1}) and 8 ppt (3.74g dl^{-1}), with abrut and significant rise 10 ppt (9.10g dl^{-1}). Protein components i.e. albumin and globulin also showed somewhat similar (decrease up to S4 and then increase) trend like total protein. Abrupt increase in serum protein, albumin and globulin levels are associated with stronger innate immune responses (Wiegertjes *et al* 1996) indicating activation of immune system (Siwicki *et al* 1994). Enhanced protein coupled with

decreased Alb/Glb ratio at higher salinity indicated abruptive defensive mechanisms. Reduction of Alb/Glb ratio in fish exposed to salinity up to 4 ppt stress is of significance for the protective mechanism (Yin *et al* 2011). The results can be explained with the justification that the adaptive capacity of gold fish is around 4 ppt with a state of homeostasis, which is also indicated in terms of fish growth at same salinity (4 ppt) w.r.to isotonic condition.

4.2.7 Antioxidant parameters

The effect of salinity was studied on antioxidant parameters in haemolysate of goldfish at the completion of the experiment in terms of superoxide dismutase (SOD), lipid peroxidation (LPO) and glutathione reductase (GR) (Table 14; Figures 21-23).

4.2.7.1 Superoxide dismutase (SOD)

Among different treatments, mean SOD (U mg^{-1} Hb) activity in haemolysate was 0.15, 0.20, 0.22, 0.50, 0.40 and 0.47 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S6=S10=S8>S4=S2=S0). The SOD activity increased significantly ($p \leq 0.05$) with incresase in salinity with maximum value in S6 and thereafter it remained stable.

4.2.7.2 Lipid peroxidation (LPO)

Among different treatments, mean LPO (nmol MDA G Hb^{-1}) activity in haemolysate was 0.10, 0.14, 0.40, 2.07, 3.22 and 4.43 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S10>S8>S6>S4=S2=S0). The LPO activity in fish increased significantly ($p \leq 0.05$) up to the highest salinity level i.e. 10 ppt (S10).

4.2.7.3 Glutathione reductase (GR)

Among different treatments, mean GR (Mm l^{-1}) activity in haemolysate was 2.01, 2.49, 3.03, 3.41, 3.92 and 4.29 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S10=S8>S6>S4>S2>S0). The GR activity in fish increased significantly ($p \leq 0.05$) up to the highest salinity level i.e. 10 ppt (S10).

Table 14: Antioxidant parameters in haemolysate of shubunkin goldfish *C. auratus* (L.) in different salinity levels at the completion of the experiment

Antioxidant Parameters	TREATMENTS*					
	S0	S2	S4	S6	S8	S10
SOD (U mgHb^{-1})	$0.15^{b}\pm0.04$	$0.20^{b}\pm0.05$	$0.22^{b}\pm0.02$	$0.50^{a}\pm0.04$	$0.40^{a}\pm0.05$	$0.47^{a}\pm0.08$
LPO (nmol MDA G Hb^{-1})	$0.10^{d}\pm0.01$	$0.14^{d}\pm0.01$	$0.40^{d}\pm0.03$	$2.07^{c}\pm0.54$	$3.22^{b}\pm0.01$	$4.43^{a}\pm0.46$
GR (Mm l^{-1})	$2.01^{e}\pm0.18$	$2.49^{d}\pm0.02$	$3.03^{c}\pm0.12$	$3.41^{b}\pm0.04$	$3.92^{a}\pm0.18$	$4.29^{a}\pm0.05$

*** S0 = 0 ppt, S2= 2ppt, S4 = 4 ppt, S6 = 6ppt, S8= 8 ppt, S10= 10ppt**
Values are Mean ± S.E. ($p \leq 0.05$); Values with same subscripts (a,b...f) in a row do not differ significantly ($p \leq 0.05$)
SOD-Superoxide dismutase, LPO-lipid peroxidation and GR-glutathione reductase

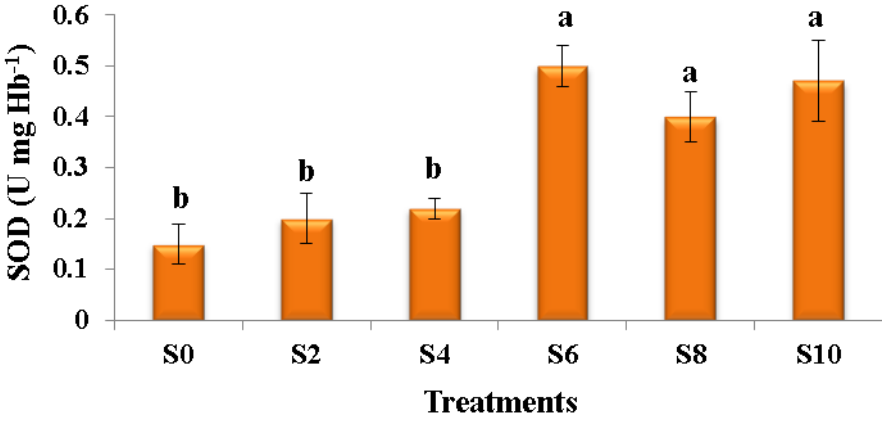


Figure 21: Comparative SOD (U mg Hb^{-1}) activity in haemolysate of shubunkin goldfish *C. auratus* (L.) in different salinity levels at the completion of the experiment

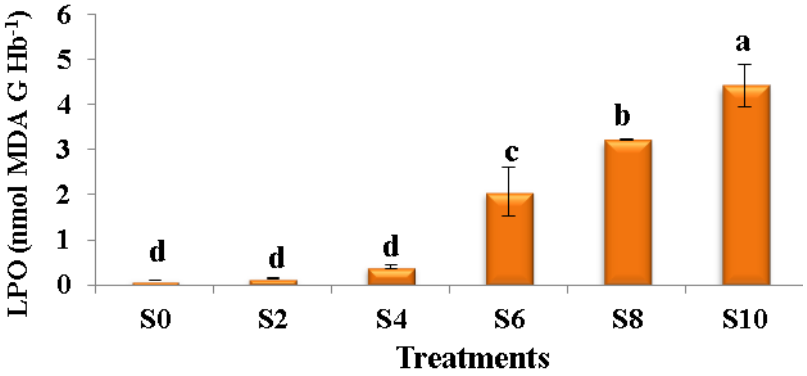


Figure 22: Comparative LPO (n Mol MDA g Hb^{-1}) activity in haemolysate of shubunkin goldfish *C. auratus* (L.) in different salinity levels at the completion of the experiment

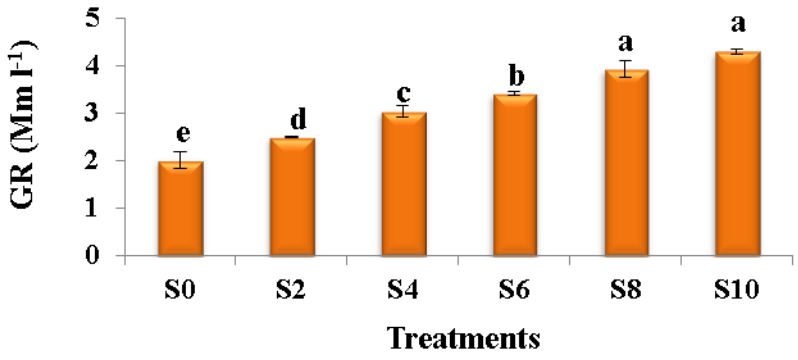


Figure 23: Comparative GR (Mm l^{-1}) activity in haemolysate of shubunkin goldfish *C. auratus* (L.) in different salinity levels at the completion of the experiment

Antioxidant parameters are considered as biomarkers to know the health status of the fish (Gülçin *et al* 2009). Results of the present experiment showed significant increase ($p \leq 0.05$) in values of all the parameters in all the treatments. Significantly higher values for SOD was observed in S6 (insignificant from S8 and S10), whereas values for LPO and GR were significantly higher in S10. Oxidative stress is the result of over production of reactive oxygen species (ROS) as compared to endogenous protection provided by antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione s- transferase and redox sensitive thiol compound, reduced glutathione (GR). Salinity changes leads to physiological and behavioural responses (Lushchak 2011) such as osmoregulatory demands. There is evidence that the stress associated by change in salinity causes an increase in the production of ROS (Liu *et al* 2007), which can be explained as oxidative stress (Martinez-Alvarez *et al* 2002). Further, significant increase in antioxidant enzymes indicated physiological response of fish towards salinity stress (Saglam *et al* 2014) and is probably due to triggring of defense system against oxidative stress.

Among the enzymes involved in anti-oxidative defence, SOD, GPx and catalase are studied to a greater extent (Karbownik and Reiter 2000). SOD converts superoxide anions into H_2O_2, which is then further degraded to H_2O by catalase or GPx. During stress acclimation to any environmental condition, various reactive oxygen species are generated in a process named as respiratory burst activity. This respiratory burst activity of phagocytes has been used frequently as an indicator (Sahoo and Mukherjee 2002 and Sahoo *et al* 2005) of stress condition. The results in terms of antioxidant status vary from species to species with respect to size, age, growth rate, stage of sexual maturity, type of diet, processing and storage time of diet as well as

environmental stressors (disease, water quality such as temperature, pH and salinity as well) along with levels of environmental toxicants (Gouillou-Coustans and Kaushik 2000, Birnie-Gauvin *et al* 2017). The antioxidant defence has the ability to augment under increasing environmental salinity showing that free radicals get distributed, while transfer of fish from freshwater to saline water indicating their active role in adaptation. A causal relationship was observed between an osmotic challenge and oxidative stress in freshwater fish, Sturgeon (*Acipenser naccarii*), which is able to tolerate marine salinity (Martinez-Alvarez *et al* 2002). After gradual acclimation to fully marine water (34 ppt), sturgeon displayed increased activities of SOD, CAT, GPx along with intensified lipid peroxidation in plasma. Although, still not very clear, but there is intimate direct link between high salinity and ROS generation (Carlstrom *et al* 2009), which can lead to oxidative stress and protein damage (Kwon *et al* 2009).

Fish like all other aerobic organisms, generate endogenous reactive oxygen species (ROS) and other oxidants during aerobic metabolism and energy production in the mitochondria. Under normal physiological status, the antioxidant defense systems including SOD, CAT and GST can be induced by slight oxidative damage as a compensatory response and thus the oxygen reactive species (ROS) can be removed to protect the organism from oxidative damage (Livingstoone 2001). These ROS can trigger oxidative damage to proteins, nucleic acid and lipids (Atli and Canli 2007), if not controlled by antioxidant enzymes. Increase in SOD, GR and LPO at higher salinities (S8-S10) in the present study can be co-related with salinity induced stress resulting from an imbalance between the production of reactive oxygen species and the antioxidant defenses (Birnie-Gauvin *et al* 2017).

In addition to SOD, reduced glutathione (GSH) or glutathione reductase (GR) is one of the important thiol molecule protecting cells from toxins in the form of free radicals. It has been reported that the levels of glutathione (GSH and GSSG) and glutathione redox ratio can be used as an indicator of thiol status during oxidative stress in fish (Stephensen *et al* 2002). Further, the lipid peroxidation process affects biomolecules associated with the membrane such as membrane bound proteins or cholesterol and may be of particular importance in fish, as their membranes contains a higher degree of polyunsaturated fatty acids (Halliwell and Gutteridge 1999). Generally, a high antioxidant enzyme activity indicates that there is a large amount of free radicals awaiting elimination (Anderson *et al* 1998 and Ross *et al* 2001), hence, the rising

activities of antioxidant enzymes (SOD, GR, LPO) indicate that the accumulation of free radicals has reached a peak, need to be managed, otherwise it can cause severe oxidant damage to the body cells (Winston and Gi Giulio 1991). Thus, the living organisms including fish (exposed to multiple environmental factors) have evolved effective antioxidant self-defense mechanism in order to maintain its homeostasis and act against oxidative stress, so the rising of antioxidant enzymes activity could effectively minimize the damage (Yin *et al* 2011), otherwise the body will suffer.

It is pertinent to mention here that, most of the studies related to alterations in antioxidant enzymes status in fish are related to effect of pollutants and seasonal variations with special reference to temperature changes (Birnie-Gauvin *et al* 2017). Lushchak and Bagnyukova (2006 a,b) has given detailed findings of temperature induced oxidative stress and associated antioxidant enzyme status (SOD, GST and GR) in gold fish, depicting stress in terms of elevated levels of all these enzyme at higher temperature.

As temperature dependent organisms, most fishes must routinely cope with fluctuations in environmental temperature and in the metabolic rate and consequently with oscillations in ROS level. Therefore, ROS generation, oxidation rates and antioxidant status are directly related to ambient temperature or metabolic activity. The values of antioxidant enzymes also vary according to changing wheather conditions. The increase of these enzymes in summer compared to other seasons is probably related to higher ambient water temperature and therefore to the oxygen consumption and reactive oxygen species generation during the warm period followed by enhanced anti-oxidant capacity in fish (Wilhelm Filho *et al* 2001).

Not much work has been done explaining salinity stress responses in freshwater fish w.r.to antioxidant enzymes activities, which are otherwise very useful markers in various stress conditions. This particular area need extensive research, so as to know the optimum salinity tolerance limit of the particular freshwater fish spcies to take up their culture in diverse environmental conditions with special reference to salinity.

4.2.8 Histopathological studies

Histopathological studies were carried out at the completion of the 120 days experimental period to know the alteration in gills, kidneys and liver of the gold fish reared at different salinities. The alterations w.r.to different organs are given in Plates IV-VI.

Environmental stress triggers the hypothalamic- pituitary – interregnal axis, which helps fish to adapt to environmental changes (Mommsen *et al* 1999). This result in increase in ACTH and cortisol, which alter the fish metabolism and physiology and under chronic conditions, organ morphology get affected (Harper and Wolf 2009). Histopathological analysis is a very sensitive and crucial parameter in determining cellular changes that may occur in target organs, such as gills, liver, kidney, intestine and muscle etc. During the present study, fish was under chronic salinity stress for 120 days, resulting in multiple metabolic and physiological changes, which are also prominent in terms of histological alterations in the major organs (gills, liver and kidney). The histological alterations were observed even at lowest salinity treatment S2 (2 ppt), which get severe with increasing salinity to the extent, that can be explained as physiological breakdown at S8 (8 ppt) and S10 (10 ppt).

4.2.8.1 Histopathological alterations in Gills

Gills are the organ, which are responsible for efficient gaseous exchange, due to presence of their epithelium with large surface area. Gills are also responsible for regulating the exchange of salt and water and thus play a major role in the excretion of the nitrogenous waste products, mainly ammonia (Mumford *et al* 2007). As per normal gill histology, the gill arch bears double rows of paired primary lamellae (PL), which in turn has a series of secondary lamellae (SL) located perpendicular to the PL. Numerous chloride cells (salt secreting) are present at proximal part of PL, Whereas, epithelial and pillar cells are present on SL. Under normal (freshwater) conditions, all these structures are intact to perform respiratory functions. Salinity stress to the freshwater gold fish in the present study showed remarkable changes in gill structure, which starts gradually in S2 (2 ppt) with lamellar bending and thinning of inter lamellar region to fusion of SL, Talengiactasia, Hyperplasia, Lamellar fusion, blood congestion and complete degeneration of SL in highest salinity treatment i.e S10 (10 ppt). All these changes indicated the response of fish to the changing salinity (WinKaler *et al* 2001).

In parallel to the observations of present investigation, Azevedo *et al* (2015) in one of the study w.r.to histological changes in gills of nile tilapia exposed to different levels of salinity also showed alterations in the form of inflammation, hyperplasia, lamellar fusion, epithelial lifting, talengiactasia and aneurysms etc. All these alterations were pronounced in higher salinity treatments (21 ppt) as compared to lower salinity (7 ppt) and thus are directly associated with increasing salinity as the major stressor (Schwaiger *et al* 1997). Raštović *et al* (2013) too observed high frequency of hyperplasia and lifting of gill epithelium, when common carp was reared in stream water (having discharge of surrounding households lead to water quality fluctuations) as compared to well water. Further, irreversible alterations such as fusion and necrosis of branchial epithelium (Poleksic *et al* 1994) along with hyperaemia and aneurism were also prominent after six month of rearing. In the present experiment, to cope up with increased pH and decreased DO, due to increase salinity, gills need to adapt to the changing water quality (Fernandes and Mazon 2003) and in this type of situation, hyperplasia and lifting of epithelium are the most common alterations found in the gill histology (Mallatt 1985). These changes are the fastest and easiest adaptations to changing water quality (salinity in present study) with the purpose of decreasing the respiratory surface and increasing diffusion distance (Sollid and Nilsson 2006). Hassan *et al* (2013) too observed hyperplasia of the gill epithelium, fusion of secondary lamellae and necrosis in tilapia and these changes were more prominent at higher salinity (20 and 35 ppt) as compared to lower salinity (5 ppt). Alterations like epithelial lifting, hyperplasia and hypertrophy of the epithelial cells along with fusion of secondary gill lamellae are examples of defense mechanisms, since these results in increase of distance between external environment and the blood, hence, serve as barrier to the entrance of contaminants (Fernandes and Mazon 2003, Akaishi *et al* 2004 and Butchiram *et al* 2009). All these alterations not only results in reduced secretary and excretory functions of the gills (Tilak *et al* 2006), but possibly lead to destruction of gill structure resulting in asphyxia, the most probable cause of death (only 60 % fish survival in present study at highest salinity of 10 ppt). Further, the severity of gill alterations also indicated that the fish was approaching their tolerance limit to salinity resulting in osmoregulatory failure (Lawson and Anetekhai 2011) again leading to mortality (6-10 ppt in the present study).

4.2.8.1 Histopathological alterations in Kidneys

The primary function of kidney in fish is osmotic regulation of water and salts rather than excretion of nitrogenous wastes (executed by gills) as in mammals. In freshwater fish, kidney must conserve salt and eliminate excess water, which is accomplished by a high glomerular filtertion rate, reabsorption of salts in the proximal tubules and dilution of urine in the distal convoluted tubules. The major histological alterations in gold fish kidney during increasing salinity conditions were observed in the form of degeneration of glomerular and distal convoluted tubules (S2), which get more severe leading to complete degeneration of glomerulus and emptying of Bowman's capsule along with hypertrophy of renal tubules, haemorrhages and mononuclear cellular infiltration in higher salinity treatments (S6, S8 and S10 ppt). Similar alterations were observed by Raštović *et al* (2013) in common carp exposed to water having high pH and low DO (stream water), but were comparatively mild (Silva and Martinez 2007).

In their study, the only moderate change observed was decrease in the tubular lumen (Camargo and Martinez 2007). The teleostan kidney is one of the first organs to be affected by contaminants in the water (Thophon *et al* 2003). In the present study too, kidney structure was altered severely indicating direct effect of salinity. The major change in the form of lumen dilation referred as hydropic swelling (Peebuaa *et al* 2006). Shrinking and degeneration of glomerulus along with increased amount of edematous fluid as observed in the present study were also recorded in previous studies, where fish were exposed to contaminants and pollutants (Abdelhamid and El-Ayouty 1991 and Hadi and Alwan 2012).

4.2.8.1 Histopathological alterations in Liver

The histology of fish liver differs from the mammalian in that, there is far less tendency of the hepatocytes to form distinct cords or lobules and the typical portal triads are not obvious. Distinct endothelial cell lines sinusoids, which are irregularly distributed between the polygonal hepatocytes with prominent central spherical nucleus and a densely stained nucleolus (Figueiredo-Fernandes *et al* 2007). Liver is the major organ associated with detoxification and biotransformation process (Van der Oost *et al* 2003), and is one of the most affected organ due to contaminants and pollutants (Rodrigues and Fanta 1998). Hepatocytes are expected to be primary target of aquatic contaminants, thus acting as excellent biomarker of stress conditions.

The vacuolization of hepatocytes observed in the present study indicate imbalance between the rate of synthesis of substances in the parenchymal cells and the rate of their release into the systemic circulation (Gingerich 1982). Pacheco and Santos (2002) described increased vacuolization of hepatocytes as a signal of degenerative process, indicating metabolic damage, which can be due to higher salinity exposure of 8 and 10 ppt. Further, According to Mollendroff (1973), vacuole formation can be considered as defense mechanism against substances injurious to hepatocytes, so as to prevent these substances from interfering with the biological activities of these cells.

In addition to this, there was increase in density of melanomacrophage aggregates in the parenchymal tissue of liver at 6 ppt in the present study, indicated degenerative and necrotic processes (Pacheco and Santos 2002). Finally cellular degeneration, complete blood congestion in portal vein, haemorrhages and necrosis at S8 (8 ppt) and S10 (10 ppt) can be explained as inhibition of synthesizing DNA needed for the growth and maturation of liver under abnormal conditions (Byczkowski 1976 and Sanad *et al* 1997).

It is pertinent to mention here that, most of the previous studies w.r.to histological alterations in different organs of fish were carried out to see the effect of chemical, xenobiotics, and pollutants or environmental variations in terms of water quality with special reference to temperature (Velmurugan *et al* 2009, Prashanth 2011, Hadi and Alwan 2012, Ahmadmoradi *et al* 2012, Banaee *et al* 2013, Butchiram *et al* 2013, Sharma and Tamot 2013, Gobinath and Ramanibai 2014, Okogwu *et al* 2015, Drishya *et al* 2016 and Sultana *et al* 2016). However most of the changes and abnormalities observed in the major organs during chronic salinity stress of 120 days in the present study are similar to the previous findings carried out with different stressors (chemicals, pollutants, environmental variations etc.). The information is lacking w.r.to effect of salinity on freshwater fish with special reference to histological alterations. The focused research in this area need to be done, so that most acceptable salinity range can be given for particular fish species in addition to other biomarkers (haematology, plasma ionic strength, oxidative stress etc.) indicating stress level in fish. The present study on freshwater gold fish reared under different salinity regimes (0-10 ppt) depicting histological alterations of gills, kidney and liver can be considered as base line study, for conducting elaborative studies in future.

4.2.9 Colouration studies

The effect of different salinity levels was also observed on colour development in terms of carotenoid content of fish muscle and skin along with digital analysis of skin with colour analyzer.

4.2.9.1 Carotenoid analysis of fish skin and muscle

Among different treatments, the mean carotenoid content (μg g^{-1}) at the initiation of the experiment was 35.80, 35.36, 36.05, 36.05, 35.48 and 35.51 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were insignificant. At the completion of the experiment, mean carotenoid content in different treatments was 49.33, 37.15, 36.90, 36.45, 38.41 and 35.58 in S0, S2, S4, S6, S8 and S10 respectively with significantly higher values ($p \leq 0.05$) in S0 and S2 (S0 = S2 > S4= S6 =S8 = S10) (Table 15; Figure 24).

4.2.9.2 Digital colour analysis

Digital parameters of skin (dorsal, ventral and lateral regions) were measured at the completion of the experiment in terms of L (luminosity), a* (balance between red/green) and b* (balance between yellow/blue) values. From L, a* and b* values, colour indices i.e. chroma and hue value were calculated (Table 16-17).

4.2.9.2.1 L a* b* colouration

At the completion of the experiment, mean L, a* and b* values in mid dorsal region were 59.91, 44.73, 47.70, 45.30, 43.53 and 45.71; 11.90, 1.82. 2.57. 1.62, 1.38 and 2.59; 16.27, 5.42, 15.48, 26.40, 8.33 and 6.56 in S0, S2, S4, S6, S8 and S10 respectively. The difference for L and b* values were insignificant ($p \leq 0.05$) among different treatments, whereas values for a* were significantly higher in S0 (S0>S10=S4=S2=S6=S8) as compared to all the salinity treatments. The mean values for L and b* were maximum in S0 and S6 respectively.

Among different treatments, mean L, a* and b* values in mid ventral region were 59.21, 48.80, 50.88, 46.81, 47.11 and 44.57; 16.91, 1.27, 2.62. 1.87, 3.60 and 1.38; 21.90, 2.61, 10.29, 6.13, 12.51 and 6.72 in S0, S2, S4, S6, S8 and S10 respectively. The differences for L and a*

Table 15: Carotenoid (µg g^{-1}) content in skin and muscle of shubunkin goldfish, *C. auratus* (L.) in different salinity levels at the initiation and completion of the experiment

Time of Observation	TREATMENTS*					
	S0	S2	S4	S6	S8	S10
0 day	35.80^{a}± 0.69	35.36^{a}±0.31	36.05^{a}±0.32	36.05^{a}±0.47	35.48^{a}±0.42	35.51^{a}±0.31
120 day	49.33^{a}± 2.60	37.15^{a}±0.27	36.90^{b}±0.42	36.45^{b}±1.67	38.41^{b}±1.69	35.58^{b}± 1.28

*** S0 = 0 ppt, S2= 2ppt, S4 = 4 ppt, S6 = 6ppt, S8= 8 ppt, S10= 10ppt**
Values are Mean ± S.E. (p≤0.05)
Values with same subscripts (a,b...e) in a row do not differ significantly (p≤0.05)

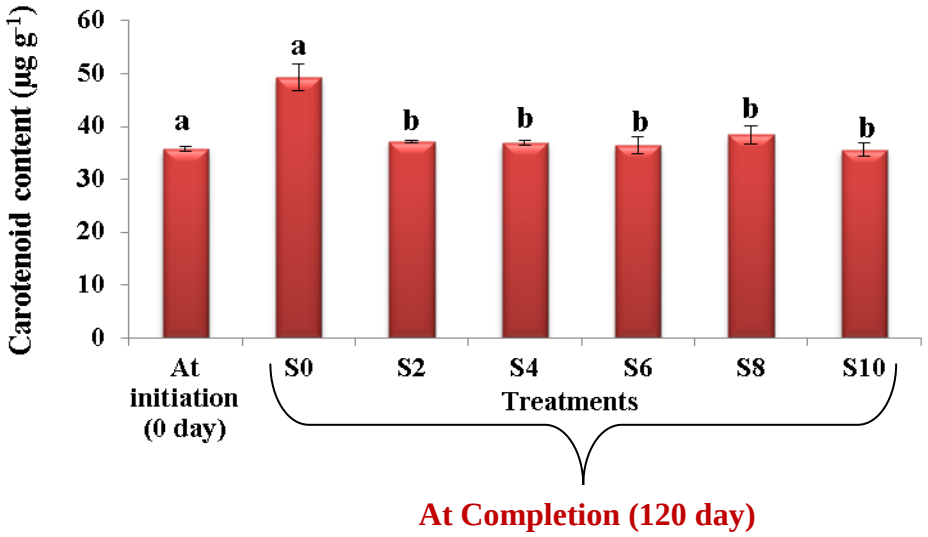


Figure 24: Comparative Carotenoid (µg g^{-1}) content in skin and muscle of shubunkin goldfish, *C. auratus* (L.) in different salinity levels at the initiation and completion of the experiment

values were insignificant (p≤0.05) among different treatments, whereas values for b* (S0≥S8=S4=S10=S6≥S2) differ significantly (p≤0.05).

Among different treatments, mean L, a* and b* values in lateral regions were 61.99, 47.96, 50.01, 41.77, 43.21 and 42.04; 30.78, 2.16, 2.08, 1.65, 4.86 and 1.45; 23.95, 2.73, 8.13, 2.38, 18.71 and 6.97 in S0, S2, S4, S6, S8 and S10 respectively. The differences for L (S0≥S4=S2≥S8=S10=S6), a* (S0≥S8=S2≥S4=S6=S10) and b* (S0≥S8≥S4=S10≥S6=S2) were were significant with maximum value for all the parameters in S0 (0 ppt).

Among different treatments, mean L, a* and b* values of three regions (dorsal, ventral and lateral) were 60.37, 47.16, 49.53, 44.62, 44.61 and 44.10; 19.86, 1.75, 2.42, 1.71, 3.28 and 1.80; 20.70, 3.58, 11.30, 11.63, 13.18 and 6.75 in S0, S2, S4, S6, S8 and S10, respectively. The differences for all the three parameters were significant ($p \leq 0.05$) among different treatments with significantly higher values in S0 (Table 17, Fig 25).

4.2.9.2.2 Chroma and hue

At the termination of the experiment, mean chroma and hue values in mid dorsal region were 61.48, 44.77, 47.77, 45.33, 43.55 and 45.83; 3522.64, 2565.66, 2737.49, 2597.70, 2495.53 and 2626.27 in S0, S2, S4, S6, S8 and S10 respectively. The differences for chroma and hue values were insignificant ($p \leq 0.05$) among different treatments, with maximum mean chroma and hue values in S0.

Among different treatments, mean chroma and hue values in ventral region were 63.64, 48.82, 50.95, 46.85, 47.25 and 44.61; 3646, 2797, 2919, 2684, 2707 and 2556 in S0, S2, S4, S6, S8 and S10 respectively. The differences for chroma ($S0 \geq S4 \geq S2=S8=S6=S10$) and hue ($S0 \geq S4 \geq S2=S8=S6=S10$) values were significant ($p \leq 0.05$) among different treatments with significantly higher value for both parameters in S0.

Among different treatments, mean chroma and hue values in lateral region were 72.20, 48.01, 50.05, 41.80, 43.49 and 42.11; 4136, 2750, 2868, 2395, 2492 and 2413 in S0, S2, S4, S6, S8 and S10 respectively. The differences for chroma ($S0 \geq S4=S2 \geq S8=S10=S6$) and hue ($S0 \geq S4=S2 \geq S8=S10=S6$) values were significant ($p \leq 0.05$) among different treatments with significantly higher value for both parameters in S0.

Among different treatments, mean chroma and hue values of three regions (dorsal, ventral and lateral) were 65.77, 47.20, 49.59, 44.66, 44.76 and 44.18; 3768.2, 2704.2, 2841.5, 2558.9, 2550.5 and 2531.8 in S0, S2, S4, S6, S8 and S10, respectively. The differences for mean chroma values were significant ($p \leq 0.05$) among different treatments with significantly higher values in S0, whereas, the differences were insignificant ($p \leq 0.05$) for hue values with maximum value in S0 (Table 17, Fig 26).

Table 16: Digital colouration parameters (La*b*) and Colour indices (chroma and hue) from different body regions of shubunkin gold fish, *C. auratus* (L.) in different salinity levels at the completion of the experiment

La*b* colouration /colour indices	TREATMENTS*					
	S0	**S2**	**S4**	**S6**	**S8**	**S10**
	Dorsal region					
L*	$59.91^{a}\pm10.12$	$44.73^{a}\pm2.52$	$47.70^{a}\pm16.81$	$45.30^{a}\pm1.05$	$43.53^{a}\pm0.68$	$45.71^{a}\pm3.22$
a*	$11.9^{a}\pm2.50$	$1.82^{b}\pm0.48$	$2.57^{b}\pm1.11$	$1.62^{b}\pm0.68$	$1.38^{b}\pm0.39$	$2.59^{b}\pm1.49$
b*	$16.27^{a}\pm6.51$	$5.42^{a}\pm2.82$	$15.48^{a}\pm7.46$	$26.40^{a}\pm22.69$	$8.33^{a}\pm7.52$	$6.56^{a}\pm4.70$
Chroma	$61.48^{a}\pm9.18$	$44.77^{a}\pm2.51$	$47.77^{a}\pm16.84$	$45.33^{a}\pm1.07$	$43.55^{a}\pm0.69$	$45.83^{a}\pm3.23$
Hue	$3522.64^{a}\pm526.5$	$2565.66^{a}\pm144.1$	$2737.49^{a}\pm965.3$	$2597.70^{a}\pm616.2$	$2495.53^{a}\pm397.4$	$2626.27^{a}\pm185.5$
	Ventral region					
L*	$59.21^{a}\pm6.12$	$48.80^{a}\pm3.45$	$50.88^{a}\pm7.84$	$46.81^{a}\pm2.72$	$47.11^{a}\pm1.14$	$44.57^{a}\pm1.65$
a*	$16.91^{a}\pm11.22$	$1.27^{a}\pm0.18$	$2.62^{a}\pm0.77$	$1.87^{a}\pm0.64$	$3.60^{a}\pm0.45$	$1.38^{a}\pm0.89$
b*	$21.90^{a}\pm8.40$	$2.61^{b}\pm1.10$	$10.29^{ab}\pm4.60$	$6.13^{ab}\pm2.85$	$12.51^{ab}\pm5.10$	$6.72^{ab}\pm3.92$
Chroma	$63.64^{a}\pm5.84$	$48.82^{b}\pm3.46$	$50.95^{ab}\pm7.87$	$46.85^{b}\pm2.74$	$47.25^{b}\pm1.14$	$44.61^{b}\pm1.67$
Hue	$3646^{a}\pm335.1$	$2797^{b}\pm198.2$	$2919^{ab}\pm451.07$	$2684^{b}\pm157.4$	$2707^{b}\pm658.6$	$2556^{b}\pm961.5$
	Lateral region					
L*	$61.99^{a}\pm6.12$	$47.96^{ab}\pm3.45$	$50.01^{ab}\pm7.84$	$41.77^{b}\pm2.72$	$43.21^{b}\pm1.14$	$42.04^{b}\pm1.65$
a*	$30.78^{a}\pm11.22$	$2.16^{ab}\pm0.18$	$2.08^{b}\pm0.77$	$1.65^{b}\pm0.64$	$4.86^{ab}\pm0.45$	$1.45^{b}\pm0.89$
b*	$23.95^{a}\pm8.40$	$2.73^{b}\pm1.10$	$8.13^{bc}\pm4.60$	$2.38^{b}\pm2.85$	$18.71^{ab}\pm5.10$	$6.97^{bc}\pm3.92$
Chroma	$72.20^{a}\pm5.84$	$48.01^{ab}\pm3.46$	$50.05^{ab}\pm7.87$	$41.80^{b}\pm2.74$	$43.49^{b}\pm1.14$	$42.11^{b}\pm1.67$
Hue	$4136^{a}\pm335.1$	$2750^{ab}\pm198.2$	$2868^{ab}\pm451.07$	$2395^{b}\pm157.4$	$2492^{b}\pm658.6$	$2413^{b}\pm961.5$

*** S0 = 0 ppt, S2= 2ppt, S4 = 4 ppt, S6 = 6ppt, S8= 8 ppt, S10= 10ppt**

Values are Mean ± S.E. ($p\leq0.05$); Values with same superscripts (a,b,c…..f) in a row do not differ significantly ($p\leq0.05$)

Table 17: Mean digital colouration parameters (La*b*) and Colour indices (chroma and hue) from different body regions of shubunkin gold fish, *C. auratus* (L.) in different salinity levels at the completion of the experiment

Colour Parameters	TREATMENTS*					
	S0	S2	S4	S6	S8	S10
L*	60.37^{a}±0.83	47.16^{bc} ±1.24	49.53^{b}±0.94	44.62^{c}±1.49	44.61^{c} ±1.25	44.10^{c} ±1.08
a*	19.86^{a}±5.64	1.75^{b}±0.25	2.42^{b}±0.17	1.71^{b}±0.07	3.28^{b} ±1.02	1.80^{b}±0.39
b*	20.70^{a} ±2.29	3.58^{b} ±0.91	11.30^{ab}±2.18	11.63^{ab} ±7.46	13.18^{ab} ±3.01	6.75^{b}±0.11
Chroma	65.77^{a}±3.27	47.20^{b}±1.23	49.59^{b}±0.95	44.66^{b} ±1.49	44.76^{b} ±1.24	44.18^{b}±1.09
Hue	3768.2^{a} ±187.3	2704.2^{a} ±70.59	2841.5^{a} ±54.04	2558.9^{a} ±85.65	2550.5^{a} ±79.34	2531.8^{a} ±62.74

*** S0 = 0 ppt, S2= 2ppt, S4 = 4 ppt, S6 = 6ppt, S8= 8 ppt, S10= 10ppt**

Values are Mean ± S.E. (p≤0.05); Values with same superscripts (a,b,c…..f) in a row do not differ significantly (p≤0.05)

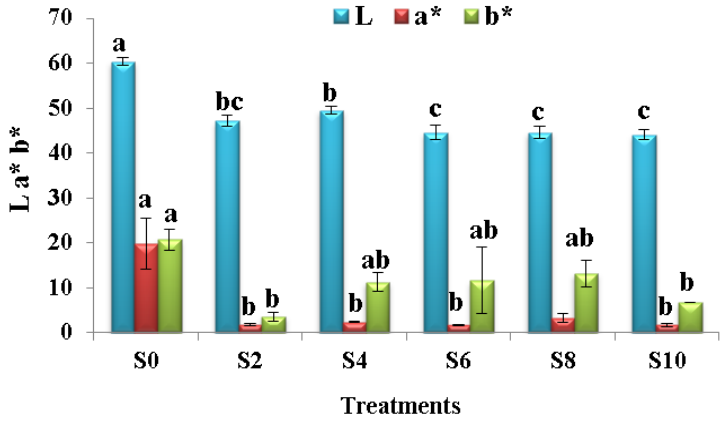


Figure 25: Comparative La*b* values from skin of shubunkin goldfish, *C. auratus* (L.) in different salinity levels at completion of the experiment

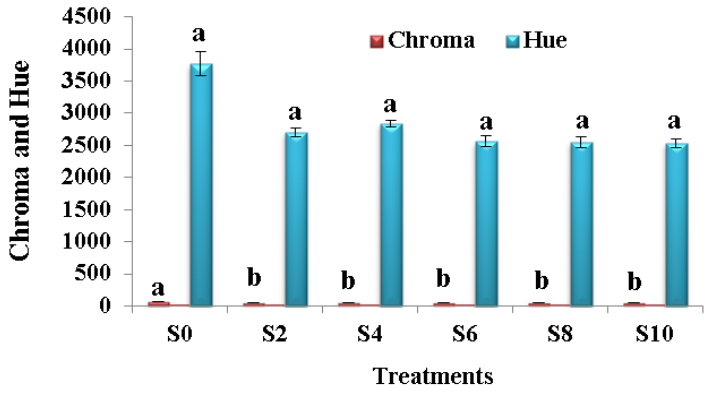


Figure 26: Comparative choma and hue values from skin of shubunkin goldfish, *C. auratus* (L.) in different salinity levels at completion of the experiment

In the present study, the visual observation of skin colour (Plate VII) revealed remarkable departure from normal body pigmentation with increasing salinity (2-10 ppt). At higher salinity (8-10 ppt), colour was bleached with lesser brightness as compared to control (0 ppt) and S2 (2 ppt). Maintenance of normal colour up to 2 ppt salinity in present study indicated the fish were able to maintain their normal body metabolism. The departure from normal colour at higher salinity of 10 ppt (S10) indicates gradual breakdown of metabolic and physiological activity. Lawson and Alake (2011) too observed similar results in gold fish exposed to different salinities. Normal gold colour was maintained up to 5 ppt up to four days and thereafter a change to bleached yellow colour was observed, which was restored to normal gold colour between days 9 to 14. However, in the present study, the fish was under prolonged salinity stress (120 days), resulting in physiological changes which must have affected the pigmentation pattern of the skin resulting in dull colouration coplued with reduced survival and growth.

Colouration in ornamental fish is the result of interactions of the nervous system and hormonal control, which affect the pigment granules of the chromatophore cells in fish body to be transported leading to intense colouration and can also aggregate resulting in light colouration. Depending upon inheritance, different individuals in the same population can have different deposition abilities. Further, fish age, size, internal neuroendocrine systems, androgenic hormones along with environmental conditions also influences pigment deposition including temperature, illumination, pollutants, low dissolved oxygen, high ammonia and salinity changes also influences carotenoid deposition and can change fish body colouration (Qiufen *et al* 2012).

Overall results in terms of survival, growth, stress responses (haematological, biochemical, antioxidant and histopathological) and colouration studies on shubunkin gold fish revealed that, although fish was capable of adapting and growing under salinity conditions up to 4 ppt in inland saline water, but 2 ppt salinity can be considered safe w.r.to overall performance of fish. Hence, it can be concluded that freshwater ornamental shubunkin gold fish, *Carassius auratus* (L.) can be reared for longer periods in inland saline water by maintanaing the salinity ≤ 2 ppt. However, based on the present study, carried out under lab conditions, field trials need to be conducted for better understanding of effect of salinity (inland saline water) coplued with multiple factors under natural conditions.

Chapter V

SUMMARY

Short term (Experiment I for 10 days) and long term (Experiment II for 120 days) experimental studies were conducted to assess the effect of salinity on survival, growth, colouration, stress responses and histopathological alterations in freshwater ornamental shubunkin goldfish, *Carassius auratus* (L.). Inland saline water was collected from salt affected areas of village Shajrana, district Fazilka (Punjab) and diluted with freshwater (S0) to prepare different experimental salinities viz., 2 ppt (S2), 4 ppt (S4), 6 ppt (S6), 8 ppt (S8), 10 ppt (S10). Inland saline water (stock) having salinity of 15 ppt and 12 ppt utilized for experiment I and experiment II respectively, was analysed for its physico-chemical properties w. r. t. temperature, pH, salinity, electrical conductivity (EC), total alkalinity (TA), total hardness (TH), ammonical nitrogen (NH_3-N), and ionic composition in terms of cations [Calcium (Ca^{2+}), magnesium (Mg^{2+}) Sodium (Na^{+}) and Potassium (K^{+})] and anions [chloride (Cl^{-}) and Sulphate (SO_4^{2-})].

Both the experiments were carried out in glass aquaria (50 litre capacity). All the aquaria were properly cleaned before stocking of the fish. Experimental aquaria were filled with water having different salinities (0-10 ppt). Each aquarium was supplied with continuous oxygen supply through aerator. Before initiation of the experiment, experimental fish were conditioned for one month in cemented pools in outdoor conditions. After proper conditioning, fish were acclimatized (gradual increase in salinity @ 1 ppt at 1-hr interval) and distributed @ 10/treatment randomly into six (one control and five experimental) treatments (0, 2, 4, 6, 8, 10 ppt) in triplicate in the month of June 2017 for experiment I to be carried out for 10 days and in the month of August 2017 for experiment II to be carried out for 120 days (4 months) from August 2017 to November 2017. At the time of stocking (for both the experiments I and II) the initial length and weight of fish varied from 6.5-8.5 cm and 4.5-8.5 g respectively. During experiment I, fish were fed once a day with commercial diet (OPTIMUM) @ 0.5 – 1.5% fish body weight, whereas during experiment II, fish were fed twice a day, daily up to satiation with the same commercial feed throughout the experimental period.

During experiment I, observations were recorded w.r.t fish survival, physico-chemical parameters (temperature, pH, EC, TA, TH, NH_3-N and ionic composition - Ca^{2+}, Mg^{2+}, Na^{+}, K^{+},

Cl^- and SO_4^{2-}) and fish behavior, daily. Colouration studies in terms of digital colour analysis (L, a* b* values and colour indices i.e. chroma and hue) of skin were analysed and calculated at the completion of the salinity tolerance test.

During experiment II, observation were recorded w.r.t. physico-chemical parameters (temperature, pH, DO, EC, TA, TH, NH_3-N and ionic composition - Ca^{2+}, Mg^{2+}, Na^+, K^+, Cl^- and SO_4^{2-}), fish behavior, fish survival and fish growth (total body length, body weight) at fortnightly intervals. Haematological responses, biochemical parameters, antioxidant parameters and histopathological studies were carried out at the completion of the experiment (120 day). Colouration studies in terms of carotenoid content were carried out at initiation and completion of the experiment, whereas digital colour analysis (L a* b* values and colour indices i.e. chroma and hue) were carried out only at completion of the experiment

Experiment I - Salinity tolerance test: Effect of salinity on survival of freshwater gold fish, *Carassius auratus* (Linn.) var. Shubunkin

5.1 Physico-chemical parameters of inland saline water (stock)

Mean temperature and salinity of inland saline water (stock) at the time of analysis was 28.0 ^oC and 15 ppt, respectively. Ionic profile of water sample revealed Na^+ and Cl^- as dominant cation and anion, respectively.

5.1.1 Physico-chemical parameters of water with different salinities

The water quality parameters viz. temperature, pH, EC, TA, TH, NH_3-N and ionic composition - Ca^{2+}, Mg^{2+}, Na^+, K^+, Cl^-, SO_4^{2-} varied from 29.0 to 29.90 ^oC, 6.51 to 8.26, 0.54 to 13.41 mS cm^{-1}, 255 to 418 mgl^{-1}, 285 to 1295 $CaCO_3$ mgl^{-1}, 0.125 to 0.285 mgl^{-1}, 45.31 to 230.8 $CaCO_3$ mg l^{-1}, 59.50 to 291.25 $CaCO_3$ mg l^{-1}, 54.60 to 765.31 mg l^{-1}, 6.90 to 44.50 mg l^{-1}, 22.05 to 1280 mg l^{-1} and 8.20 to 146.50 mg l^{-1}, respectively in different treatments (S0-S10). Except temperature, all other parameters increased significantly ($p \leq 0.05$) with increasing salinity.

5.1.2 Survival of fish

At the completion of survival test, fish survival (10 days) was 100 % in control (S0) and S2 (2 ppt), 96.66 % in S4-S8 (4-8 ppt), and 93.33 % in S10 (10 ppt). The fish survival during and at completion of salinity tolerance test did not differ significantly.

5.1.3 Fish behaviour

During salinity tolerance test, normal swimming activity of the fish was observed up to highest salinity i.e. 10 ppt for first 2 days, as the fish was gradually acclimatized to the salinity stress. However, from 4th day onward, fish showed low swimming activity in S4 and from 3rd day onward in S6-S10. Fish was found to be sluggish in S8 and S10 from 6th day onward. In terms of feeding behaviour, response of fish diverted from high appetite to low appetite in S4-S10 from 5th day onwards. Fish was able to adapt easily up to 6 ppt, whereas at 8 and 10 ppt (S8 and S10), low activity to sluggishness along with low appetite indicated gradual drifting away from normal physiological responses.

5.1.4 Colouration studies

Digital parameters of skin (dorsal and ventral region) was measured at the completion of the salinity tolerance test in terms of L (luminosity), a* (balance between red/green) and b* (balance between yellow/ blue) values. From L a* b* values, colour indices i.e. chroma and hue values were calculated.

5.1.4.1 L a* b* colouration

Among different treatments, mean L, a* b* values in dorsal region were 41.43, 44.95, 54.40, 54.18, 44.02 and 44.14; 1.37, 3.19, 1.83, 5.20, 5.53 and 0.87; 2.63, 5.73, 6.25, 20.56, 16.33 and 5.15 in S0, S2, S4, S6, S8 and S10 respectively. The differences for L, a* and b* values were insignificant ($p \leq 0.05$) among different salinity treatments. Among different treatments, mean L, a* and b* values in ventral region were 52.88, 47.45, 46.07, 49.57, 43.14 and 47.04; 1.75, 0.98, 0.36, 3.86, 7.68 and 0.52; 14.27, 4.62, 7.70, 16.85, 19.25 and 2.36 in S0, S2, S4, S6, S8 and S10 respectively. The differences for L and b* values were insignificant ($p \leq 0.05$) among different salinity treatments, whereas, values for a* (S8 > S6 > S0=S2=S10=S4) were significantly ($p \leq 0.05$) different.

5.1.4.2 Chroma and hue

Among different treatments, mean chroma and hue values in dorsal region were 41.47, 45.06, 54.46, 54.55, 44.44 and 44.18; 2376, 2582, 3120, 3125, 2546 and 2531 in S0, S2, S4, S6, S8 and S10 respectively. The difference for chroma values were insignificant ($p \leq 0.05$) among

different treatments, while values for hue were significantly ($p \leq 0.05$) higher in all salinity treatments in comparison to S0 (0ppt). Among different treatments mean chroma and hue values in ventral region were 52.92, 47.49, 46.08, 49.76, 43.83 and 47.05; 3032, 2721, 2640, 2851, 2511 and 2696 in S0, S2, S4, S6, S8 and S10 respectively. The difference for chroma values were insignificant ($p \leq 0.05$) among different treatments, while for hue (S0=S6=S2=S10≥S4=S8) values differences were significant ($p \leq 0.05$) among different salinity treatments. In the present study, the digital observation of skin colouration does not show any remarkable departure from normal body pigmentation, during short duration of 10 days exposure to increasing salinity levels.

5.2 Experiment II: To study survival, growth, colouration and stress responses of freshwater gold fish, *Carassius auratus* (Linn.) var. Shubunkin reared at selected salinity (inland saline water)

5.2.1 Physico-chemical parameters of inland saline water (stock)

Mean temperature and salinity of inland saline water (stock) at the time of analysis was 29.33 ^{o}C and 12 ppt, respectively. Ionic profile of water sample revealed Na^{+} and Cl^{-} as dominant cation and anion, respectively.

5.2.2 Physico-chemical parameters of water with different salinities

The water quality parameters viz. temperature, pH, DO, EC, TA, TH, NH_3-N and ionic composition - Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, Cl^{-} and SO_4^{2-} varied from 14.2 to 30.5^{O}C, 7.14 to 8.99, 5.50 to 9.52, 0.64 to 18.91 mS cm^{-1}, 232 to 336 $CaCO_3$ mgl^{-1}, 305 to 2930 $CaCO_3$ mgl^{-1}, 0.010 to 0.298 mgl^{-1}, 82.40 to 302.70 $CaCO_3$ mgl^{-1}, 63.8 to 914.50 $CaCO_3$ mgl^{-1}, 14.5 to 685.80 mgl^{-1}, 0.50 to 91.20 mgl^{-1}, 39.90 to 3010.51 $mgl^{-1}$and 2.32 to 121.69 mgl^{-1} respectively in different treatments (S0-S10). Except temperature all other parameters increased significantly ($p \leq 0.05$) with increasing salinity, while DO decreased significantly with increasing salinity.

5.2.3 Survival and growth of fish

5.2.3.1 Survival of Fish

During experiment II, fish survival was 100 % in S0 and S2 for experimental period of 120 and 90 days respectively, whereas for rest of the salinity treatments, gradual mortality was observed from 45th day onwards. At the completion of the experiment, fish survival was 100 %

in S0 (0ppt), 93.33% in S2 (2ppt), 86.66% in S4 (4 ppt), 80% in S6 (6 ppt) and S8 (8 ppt); and and 60 % in S10 (10 ppt) and the differences were significant.

5.2.3.2 Fish Growth

The growth in fish was assessed in terms of total body length (TBL) and body weight (BW) at fortnightly intervals during 120 day experimental period. At the completion of the experiment, growth parameters i.e. total body length gain (TLG), net weight gain (NWG) and specific growth rate (SGR) of fish for each treatment were calculated.

Among different treatments, the mean final total body length (cm) and mean body weight (g) of fish was 8.11, 8.19, 8.17, 8.23, 8.01 and 7.76; 7.98, 7.28, 7.38, 7.01, 5.37 and 5.16 in S0, S2, S4, S6, S8 and S10, respectively, which revealed significant ($p \leq 0.05$) effect of increasing salinity from 0-10 ppt on fish growth. The differences for growth parameters in terms of TLG (S2=S0≥S6=S4>S8=S10), %TLG (S2=S0≥S6=S4>S8>S10), NWG (S0≥S2≥S4=S6>S8=S10), %NWG (S0≥S2=S6≥S4>S8=S10) and SGR (S0≥S2≥S4=S6>S8=S10) were significant ($p \leq 0.05$), are indicative of negative impact of salinity on fish growth. Fish performane in terms of all the growth parameters revealed ≤ 2ppt salinity as comfortable level for long term rearing. Further, there was remarkable decline in growth at higher salinities of 8 and 10 ppt (negative values in S8 and S10).

5.2.4 Fish behavior

Fish behaviour in terms of swimming activity, feeding responses and morphological characters like colouration, body fragility and presence of mucus on skin were recorded at fortnightly intervals. The results indicated stressful condition of fish reared in higher salinity levels of 8 ppt (S8) and 10 ppt (S10). However, deviations from normal responses (low activity, sluggishness, low appetite, skin discolouration, high fragility and excessive mucus) were also seen at 6 ppt (S6) after experimental period of 45 days. These results are in accordance to the decreased fish survival and growth in S8 and S10.

5.2.5 Haematological parameters

The mean haematological parameters of fish including Haemoglobin (Hb), Red Blood Cell (RBC), White Blood Cells (WBC), Packed Cell Volume (PCV), Mean Corpuscular Volume

(MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Content (MCHC) were analyzed/ calculated at the completion of the experiment

Among different treatments, mean Hb (g %) of fish was 5.83, 4.50, 3.50, 3.56, 3.53 and 3.60 in S0, S2, S4, S6, S8 and S10 respectively and the values for control (S0) was significantly higher as compared to all the treatments (S0>S2=S10=S6=S8=S4), however, differences for all the salinity treatments were insignificant; mean RBC ($\times 10^6/mm^{-3-1}$) of fish was 1.31, 2.10, 2.90, 3.56, 3.86 and 4.46 in S0, S2, S4, S6, S8 and S10 respectively and the differences among treatments were significant (S10≥S8≥S6>S4>S2>S0); mean WBC ($\times 10^3/mm^{-3-1}$) of fish was 3.32, 3.40, 4.04, 4.83, 6.19 and 6.28 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S10=S8>S6≥S4≥S2=S0); mean PCV (%) of fish was 5.96, 7.23, 7.90, 8.26, 8.33 and 9.03 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S10≥S8=S6=S4>S2>S0); mean MCV (μm^3) of fish was 45.85, 36.50, 27.28, 23.33, 21.61 and 20.36 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S0≥S2≥S4≥S6=S8=S10); mean MCH (g %) of fish was 44.85, 23.17, 12.09, 10.01, 9.11 and 8.03 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S0>S2>S4=S6=S8=S10); mean MCHC (%) of fish was 97.72, 62.09, 44.24, 43.07, 42.37 and 39.88 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S0>S2>S4=S6=S8=S10).

Haematological parameters in terms of RBC, WBC and PCV showed increasing trend with increasing salinity (S0 to S10) and the differences among treatments were significant. However, Hb and haematological indices i.e. MCV, MCH and MCHC showed significant decrease up to S10. The increase and decrease of different parameters was significant but gradual, which can be due to low temperature coupled with increasing salinity conditions for longer duration (120 days), resulting in stress, which must have regulated the physiological responses gradually, so as to reach internal homeostasis.

5.2.6 Biochemical parameters of fish

The mean biochemical parameters in blood serum of fish including total proteins, glucose, albumin and globulin were analyzed and albumin/globulin (Alb/Glb) ratio was calculated at the completion of the experiment

Among different treatments, mean blood serum glucose (g dl^{-1}) of fish was 98.03, 44.63, 41.04 43.40, 61.50 and 191.20 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S10>S0>S8>S2>S6>S4); mean blood serum protein (g dl^{-1}) of fish was 4.24, 3.75, 1.97, 2.85, 3.74 and 9.10 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S10>S0>S2=S8>S6>S4); mean blood serum albumin (g dl^{-1}) of fish was 1.88, 1.09, 0.45, 1.12, 1.15 and 1.13 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S0>S8=S10=S6=S2>S4); mean blood serum globulin (g dl^{-1}) of fish was 2.35, 2.65, 1.52, 1.73, 2.59 and 7.97 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S10>S2=S8>S0>S6=S4); mean blood serum Alb/Glb ratio (g dl^{-1}) was 0.79, 0.41, 0.29, 0.74, 0.44 and 0.14 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S0=S6>S8=S2≥S4≥S10).

In the present study, serum blood glucose and total proteins decreased significantly ($p \leq 0.05$) up to 8 ppt (S8), with abrupt and significant rise in 10 ppt (S10). There was significant decrease and increase in albumin and globulin levels with increasing salinity up to highest level i.e. 10 ppt, whereas in accordance to increased values of globulins, Alb/Glb ratio decreased significantly up to 4 ppt with sudden increase in S6, afterwards again decreased significantly ($p \leq 0.05$) in S8 and S10.

5.2.7Anti-oxidant parameters

The effect of salinity was studied on antioxidant parameters in haemolysate of goldfish at the completion of the experiment in terms of superoxide dismutase (SOD), lipid peroxidation (LPO) and glutathione reductase (GR).

Among different treatments, mean SOD (U mg^{-1} Hb) activity in haemolysate was 0.15, 0.20, 0.22, 0.50, 0.40 and 0.47 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S6=S10=S8>S4=S2=S0); mean LPO (nmol MDA G Hb^{-1})

activity in haemolysate was 0.10, 0.14, 0.40, 2.07, 3.22 and 4.43 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S10>S8>S6>S4=S2=S0); mean GR (Mm l^{-1}) activity in haemolysate was 2.01, 2.49, 3.03, 3.41, 3.92 and 4.29 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were significant (S10=S8>S6>S4>S2>S0).

Results of the present experiment showed significant increase ($p \leq 0.05$) in values of all anti-oxidant parameters with increase in salinity (S0-S10). Significantly higher values for SOD is observed in S6 whereas significantly higher values for LPO and GR were observed in S10.

5.2.8 Histopathological studies

Histopathological studies were carried out at the completion of the 120 days experimental period to know the alteration in gills, kidneys and liver of the gold fish reared at different salinities.

5.2.8.1 Histopathological alterations in gills

Salinity stress to the freshwater gold fish in the present study showed remarkable changes in gill structure, which starts gradually in S2 (2 ppt) with lamellar bending and thinning of inter lamellar region to fusion of SL, Talengiactasia, Hyperplasia, Lamellar fusion, blood congestion and complete degeneration of SL in highest salinity treatment i.e S10 (10 ppt). All these changes indicated the response of fish to the changing salinity

5.2.8.2 Histopathological alterations in kidneys

The major histological alterations in gold fish kidney during increasing salinity conditions were observed in the form of degeneration of glomerular and distal convoluted tubules (S2), which get more severe leading to complete degeneration of glomerulus and emptying of Bowman's capsule along with hypertrophy of renal tubules, haemorrhages and mononuclear cellular infiltration in higher salinity treatments (S6, S8 and S10 ppt).

5.2.8.3 Histopathological alterations in liver

The major histological alterations in gold fish liver during increasing salinity conditions were observed in the form of vacuolization of hepatocytes, increase in density of melanomacrophage aggregates in the parenchymal tissue along with cellular degeneration,

complete blood congestion in portal vein, haemorrhages and necrosis at S8 (8 ppt) and S10 (10 ppt) were indicated of degenerative and necrotic processes.

During the present study, fish was under chronic salinity stress for 120 days, resulting in multiple metabolic and physiological changes, which are also prominent in terms of histological alterations in the major organs (gills, liver and kidney). The histological alterations were observed even at lowest salinity treatment S2 (2 ppt), which get severe with increasing salinity to the extent, that can be explained as physiological breakdown at S8 (8 ppt) and S10 (10 ppt).

5.2.9 Colouration studies

The effect of different salinity levels was also observed on colour development in terms of carotenoid content of fish muscle and skin along with digital analysis of skin with colour analyzer.

5.2.9.1 Carotenoid analysis of fish skin and muscle

Among different treatments, the mean carotenoid content ($\mu g\ g^{-1}$) at the initiation of the experiment was 35.80, 35.36, 36.05, 36.05, 35.48 and 35.51 in S0, S2, S4, S6, S8 and S10 respectively and the difference among treatments were insignificant. At the completion of the experiment, mean carotenoid content in different treatments was 49.33, 37.15, 36.90, 36.45, 38.41 and 35.58 in S0, S2, S4, S6, S8 and S10 respectively, with significantly higher values ($p \leq 0.05$) in S0 and S2 (S0 = S2 > S4= S6 =S8 = S10)

5.2.9.2 Digital analysis of skin

Digital parameters of skin (dorsal, ventral and lateral regions) were measured at the completion of the experiment in terms of L (luminosity), a* (balance between red/green) and b* (balance between yellow/blue) values. From L, a* and b* values, colour indices i.e. chroma and hue value were calculated.

At the completion of the experiment, mean L, a* and b* values in mid dorsal region were 59.91, 44.73, 47.70, 45.30, 43.53 and 45.71; 11.90, 1.82. 2.57. 1.62, 1.38 and 2.59; 16.27, 5.42, 15.48, 26.40, 8.33 and 6.56 in S0, S2, S4, S6, S8 and S10 respectively. The difference for L and b* values were insignificant ($p \leq 0.05$) among different treatments, whereas values for a* were significantly higher in S0 (S0>S10=S4=S2=S6=S8) as compared to all the salinity

treatments; mean L, a* and b* values in mid ventral region were 59.21, 48.80, 50.88, 46.81, 47.11 and 44.57; 16.91, 1.27, 2.62. 1.87, 3.60 and 1.38; 21.90, 2.61, 10.29, 6.13, 12.51 and 6.72 in S0, S2, S4, S6, S8 and S10 respectively. The differences for L and a* values were insignificant ($p \leq 0.05$) among different treatments, whereas values for b* (S0≥S8=S4=S10=S6≥S2) differ significantly ($p \leq 0.05$); mean L, a* and b* values in lateral regions were 61.99, 47.96, 50.01, 41.77, 43.21 and 42.04; 30.78, 2.16, 2.08, 1.65, 4.86 and 1.45; 23.95, 2.73, 8.13, 2.38, 18.71 and 6.97 in S0, S2, S4, S6, S8 and S10 respectively. The differences for L (S0≥S4=S2≥S8=S10=S6), a* (S0≥S8=S2≥S4=S6=S10) and b* (S0≥S8≥S4=S10≥S6=S2) were significant with maximum value for all the parameters in S0 (0 ppt). Among different treatments, mean L, a* and b* values of three regions (dorsal, ventral and lateral) were 60.37, 47.16, 49.53, 44.62, 44.61 and 44.10; 19.86, 1.75, 2.42, 1.71, 3.28 and 1.80; 20.70, 3.58, 11.30, 11.63, 13.18 and 6.75 in S0, S2, S4, S6, S8 and S10, respectively. The differences for all the three parameters were significant ($p \leq 0.05$) among different treatments with significantly higher values in S0.

At the completion of the experiment, mean chroma and hue values in mid dorsal region were 61.48, 44.77, 47.77, 45.33, 43.55 and 45.83; 3522.64, 2565.66, 2737.49, 2597.70, 2495.53 and 2626.27 in S0, S2, S4, S6, S8 and S10 respectively. The differences for chroma and hue values were insignificant ($p \leq 0.05$) among different treatments, with maximum mean chroma and hue values in S0; mean chroma and hue values in ventral region were 63.64, 48.82, 50.95, 46.85, 47.25 and 44.61; 3646, 2797, 2919, 2684, 2707 and 2556 in S0, S2, S4, S6, S8 and S10 respectively. The differences for chroma (S0≥S4≥S2=S8=S6=S10) and hue (S0≥S4≥S2=S8=S6=S10) values were significant ($p \leq 0.05$) among different treatments with significantly higher value for both parameters in S0; mean chroma and hue values in lateral region were 72.20, 48.01, 50.05, 41.80, 43.49 and 42.11; 4136, 2750, 2868, 2395, 2492 and 2413 in S0, S2, S4, S6, S8 and S10 respectively. The differences for chroma (S0≥S4=S2≥S8=S10=S6) and hue (S0≥S4=S2≥S8=S10=S6) values were significant ($p \leq 0.05$) among different treatments with significantly higher value for both parameters in S0. Among different treatments, mean chroma and hue values of three regions (dorsal, ventral and lateral) were 65.77, 47.20, 49.59, 44.66, 44.76 and 44.18; 3768.2, 2704.2, 2841.5, 2558.9, 2550.5 and 2531.8 in S0, S2, S4, S6, S8 and S10, respectively. The differences for mean chroma values were significant ($p \leq 0.05$) among

different treatments with significantly higher values in S0, whereas, the differences were insignificant ($p \leq 0.05$) for hue values with maximum value in S0

5.3.1 Conclusions

Overall results of the present study (experiment I and experiment II) revealed that

- Freshwater ornamental gold fish, *C. auratus* (L.) var. Shubunkin can tolerate salinity (w.r.to survival and behaviour) up to 6 ppt during short period of salinity exposure (10 days), whereas during longer salinity exposure (120 days) fish showed tolerance to ≤ 4 ppt.
- Fish growth was significantly affected in all the salinity treatments (2-10ppt), with negative values for all the growth parameters at higher salinity levels (8 and 10ppt).
- Significant increase (RBC, WBC and PCV) and decrease (Hb, MCV, MCH and MCHC) up to highest salinity level (10ppt) in haematological parameters indicated physiological adaptations in fish, so as to achieve internal stability or homeostasis.
- Biochemical parameters in terms of significantly decreased level of glucose and total protein up to 8 ppt and abrupt increase in 10 ppt; decreased and increased levels of albumin and globulin in all the salinity treatments, with decreased Alb/Glb ratio up to 4 ppt, increase in 6 ppt and then decrease up to 10 ppt, are also indicative of regulatory mechanism under salinity stress.
- Significantly higher antioxidant parameters in terms of SOD, LPO and GR up to 10 ppt further revealed prolonged exposure of salinity as stress factor for freshwater fish
- Histological alterations in gills, kidneys and liver of fish were observed even at salinity of 2 ppt, and these get severe with further increase in salinity to the extent, that can be explained as physiological breakdown.
- Carotenoid and digital colour analysis revealed remarkable departure from normal body pigmentation with increasing salinity from 2-10 ppt. Maintenance of normal body colour only up to 2 ppt is indicative of normal body metabolism

Overall results in terms of survival, growth, stress responses (haematological, biochemical, antioxidant and histopathological) and colouration studies on shubunkin gold fish revealed that, although fish was capable of adapting and growing under salinity conditions up to

4 ppt in inland saline water, but 2 ppt salinity can be considered safe w.r.to overall performance of fish. Hence, it can be concluded that freshwater ornamental shubunkin gold fish, *Carassius auratus* (L.) can be reared for longer periods in inland saline water by maintaining the salinity ≤ 2 ppt. However, based on the present study, carried out under lab conditions, field trials need to be conducted for better understanding of effect of salinity (inland saline water) coupled with multiple factors under natural conditions.

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