Dedication

This work is dedicated to the memory of my late father, Sayed Rajab, who passed on a love for reading and respect for education. May Allah bless his soul and grant him the highest levels of paradise….ameen.

Copyright Statement

The effect of dietary chromium(III) on growth and carbohydrate utilization in mirror and common carp (*Cyprinus carpio* L.)

by

Arafat Rajab Ahmed

A thesis submitted to the University of Plymouth

in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

Department of Biomedical and Biological Sciences
Faculty of Science and Technology

February 2012

The effect of dietary chromium(III) on growth and carbohydrate utilization in mirror and common carp (*Cyprinus carpio*) L.

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Abstract

The aim of feed formulation in aquaculture is to supply a suitable diet that provides nutritional requirements at relatively low cost. Carbohydrates are the most economic energy source for animals compared to protein and lipid; however, fish have limited capacity for dietary carbohydrate utilization. Trivalent chromium is an essential micronutrient for carbohydrate metabolism in vertebrates. The primary objective of this thesis was to enhance understanding of the effects of organic and inorganic forms of Cr on carbohydrate utilization, growth performance, gene expression and activity of specific key liver enzymes in carp *Cyprinus carpio*. In addition, effects of dietary Cr on body composition, Cr tissue content, blood cells DNA damage, and tissue histopathology (liver and gut) were evaluated. The first experiment (Chapter 3) tested levels of dietary Cr (0, 0.2, 0.5, 1.0, 1.5, 2.0 mg Cr kg^{-1} as Cr chloride) to determine Cr requirement; the second experiment (Chapter 4) compared bioavailability of different forms of Cr (Cr chloride, Cr picolinate, and Cr yeast); and the third experiment (Chapter 5) evaluated different levels of Cr yeast (0.5, 1.0, and 2.0 mg Cr kg^{-1}) on utilization of a starch or dextrin-based diet. A Cr supplementation of 0.5 mg Cr kg^{-1} (regardless of form of Cr) produced highest growth performance; whereas 2.0 mg Cr kg^{-1} did not differ from control. The 0.5 mg Cr kg^{-1} also enabled carp to utilize complex carbohydrates (e.g., starch) and did not affect final body composition. Only 2.0 mg Cr kg^{-1} caused DNA damage in blood cells and tissue damage (liver and gut histopathology). Cr content in whole body increased with dietary Cr, but Cr did not affect hexokinase gene

expression. Overall, results indicate that Cr can improve growth performance of carp and that Cr supplementation can enhance utilization of carbohydrates in fish feed.

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Fish husbandry and the experiments of this thesis were in accordance with the UK Animal Scientific Procedures Act, 1986 (under the UK Home Office project licence #30/2644 and personal licence #30/8746). In addition, the study was also fully approved by the University of Plymouth Ethical Review Committee.

Acknowledgments

I would like to express my sincere gratitude to my first supervisor Prof. Simon J Davies for his advices, encouragement and supervision of the research project and for seeing this thesis through to its conclusion. I would like to extend my sincere thanks to my second supervisor Prof. Awadhesh Jha for his guidance and encouragement throughout my study.

Many thanks and appreciation go to the Ministry of Higher Education and Scientific Research-Republic of Iraq for providing the financial support which made my PhD study in the United Kingdom possible. Many thanks to the Iraqi cultural attaché in London for their kind support. Special thanks to Dr. John Moody for his valuable assistance in enzyme assay and a big thanks to Dr. Andrew Fisher and Dr. Mike Foulkes for their help with ICP analysis. I am also grateful to Dr. Ted Henry for providing assistance whenever I asked for it. Sincere thanks are due to Liz Folland, Natalie Sweet, Andrew Atfield, Angela Harrop and Benjamin Eynon for the technical support where needed. I also wish to thank my colleagues within the Aquaculture and Fish Nutrition Research group for their invaluable assistance and encouragement. I would also like to express my sincere appreciation to the PhD students at the School of Biomedical and Biological Sciences (my colleagues and friends) for their practical help during the laboratory work and for many useful scientific discussions. I would to express my gratitude to all those people, though their names are not listed, who supported me in one way or another

Finally, I would like to express my heartfelt thanks to my beloved mother for her blessing, sisters, and brothers for more than I could ever repay.

Author’s declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee. This study was fully funded by the Ministry of Higher Education and Scientific Research of Republic of Iraq.

Relevant scientific seminars and conferences were attended at which work was presented and papers have prepared for publication:-

Publications:

- **Arafat R. Ahmed,** A. John Moody, Andrew Fisher, Simon J. Davies (2012). Growth Performance and Starch Utilization in Common Carp (*Cyprinus carpio* L.) in Response to Dietary Chromium Chloride Supplementation. Journal of Trace Element in Medicine and Biology. DOI: 10.1016/j.jtemb.2012.05.006

- **Arafat R. Ahmed**, Awadhesh N. Jha, Simon J. Davies (2012). The Efficacy of Chromium as a Growth Enhancer for Mirror Carp (*Cyprinus carpio* L): An Integrated Study Using Biochemical, Genetic and Histological Responses. Journal of Biological Trace Element Research. DOI: 10.1007/s12011-012-9354-4.

- **Arafat R. Ahmed**, Awadhesh N. Jha and Simon J. Davies (2012). The Effect of Dietary Organic Chromium on Specific Growth Rate, Tissue Chromium Concentrations, Enzyme Activities and Histology in Common Carp, *Cyprinus carpio* L. Journal of Biological Trace Element Research. DOI: 10.1007/s12011-012-9436-3.

- **Arafat R. Ahmed** and Simon J. Davies (2010). Chromium: the forgotten mineral that plays an essential role in the utilization of carbohydrates in mirror carp diets. International Aquafeed, 13(6):34-35.

Presentations and conferences attended:

Posters

- The role of chromium on growth performance, glucose utilization, and carbohydrate metabolism in common carp (*Cyprinus carpio*). The 3rd Annual Marine Institute Conference: Spirit of Discovery, December, 2010, University of Plymouth, UK.
- The efficacy of chromium as a growth enhancer for mirror carp (*Cyprinus carpio*). The 8th Marine Biological Association Postgraduate Conference, May 2011, Queen's University, Belfast, Northern Ireland.

Oral presentations (Seminars):

- Chromium: An often forgotten mineral-How it can improve aquaculture. Alltech's 26th International Animal Health & Nutrition Symposium, May 2010, USA.
- The effect of chromium on growth and carbohydrate assimilation in common and mirror carp (*Cyprinus carpio*), Seminar, May 2011, Plymouth University, UK.
- The role of chromium on growth and carbohydrate metabolism in common carp (*Cyprinus carpio*). The 8th Marine Biological Association Postgraduate Conference (Oral presentation), May 2011, Queen's University Belfast, Northern Ireland.

Word count of main body of thesis: 36.493

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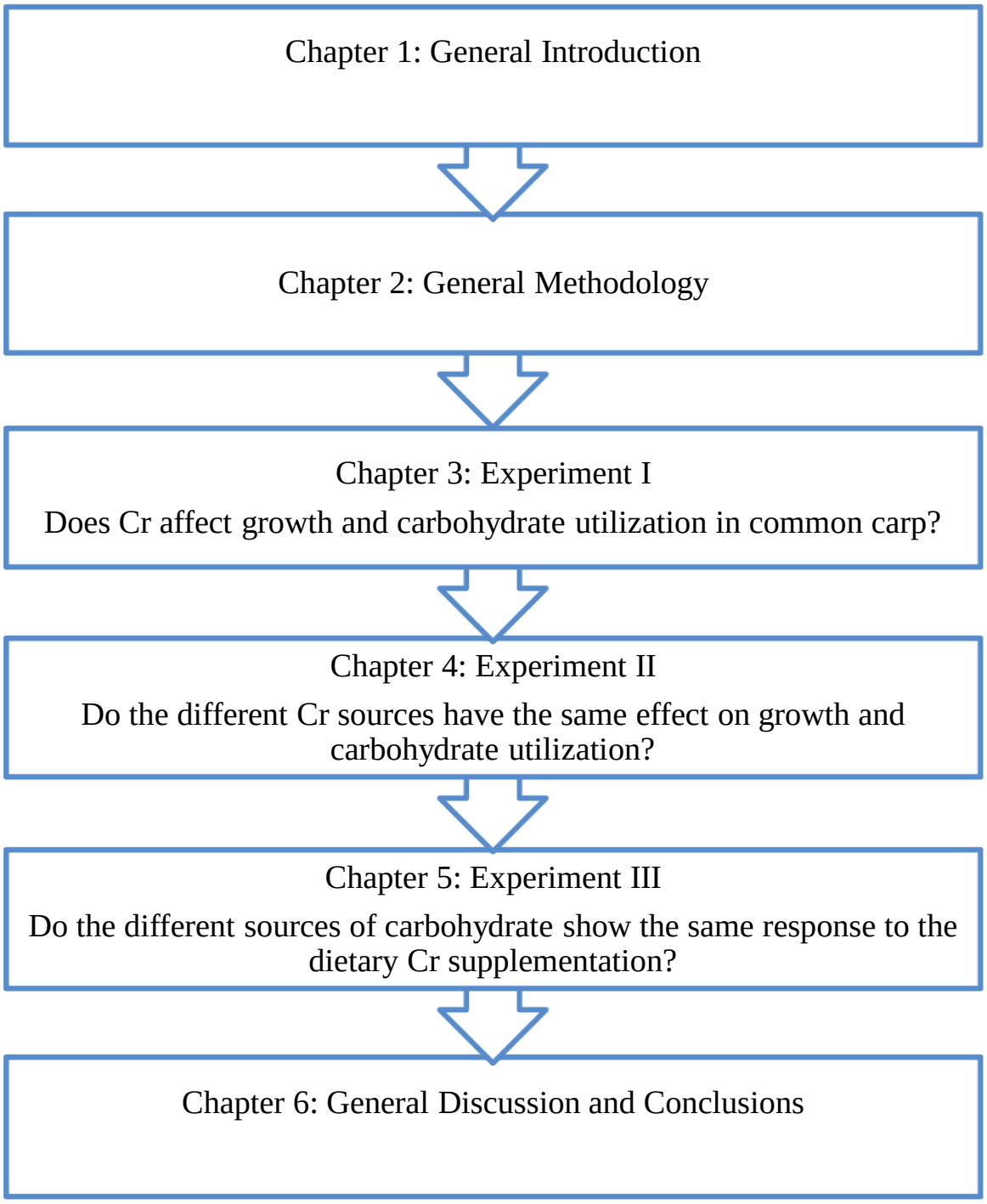


Thesis outline and the main aim of each experiment.

Chapter 1

General introduction

Chapter 1: General introduction

1.1 Aquaculture and fish nutrition

The term Aquaculture is used to describe the science and business of producing aquatic organisms including fish, molluscs, crustaceans, and aquatic plants in fresh, brackish, and marine environment for human consumption. Aquaculture is considered a part of fisheries science with some differences between the two terms 'fisheries and aquaculture' in respect of management and development (Pillay and Kutty, 2005). The aims of aquaculture are to increase production levels of the aquatic organisms above that occurring in natural ecosystems and to provide a stable source of high quality protein to meet the increasing demand for food production. In 2007, aquaculture sector produced 43% of various aquatic animals and plants and the production was dominated by shellfish, herbivorous and omnivorous fish (Bostock et al., 2010). In 2008, aquaculture continued to show a growing trend and the total production of food for human (including finfishes, crustaceans, molluscs, and other aquatic animals) reached 52.5 million tonnes (FAO, 2010). At the present, aquaculture industry has become a way of life, creates jobs and stimulates economic activities and money investment around the world. The aquaculture industry is one of the most vital strategies to improve the nutritional status and to increase the income of the populations in developing countries (Pillay and Kutty, 2005). Some people involved directly in the aquaculture sector as fishers or fish farmers or engage in different activities connected indirectly in fish farming such as net making, boat construction and maintenance, fish processing, marketing, and distribution; while others are involved in development, improvement, administration, and scientific research. It has been estimated 45 million fishers are employed part or full time in addition to 6 million occasional fishers were reported to the Food and Agriculture Organization in 2008 (FAO, 2010).

Over hundred years, human in different countries have developed the basic forms of cultivation and applied various aquaculture technologies and feeding strategies in order to control and stabilize production in small and large-scale of aquaculture projects. The aquaculture industry supplies significant amounts of healthy food for human globally and it has become the fastest growing food production sector in recent years. Figure 1.1 shows the world aquaculture production (quantity and value) in 2008 which reflect the economic activity of the aquaculture sector.

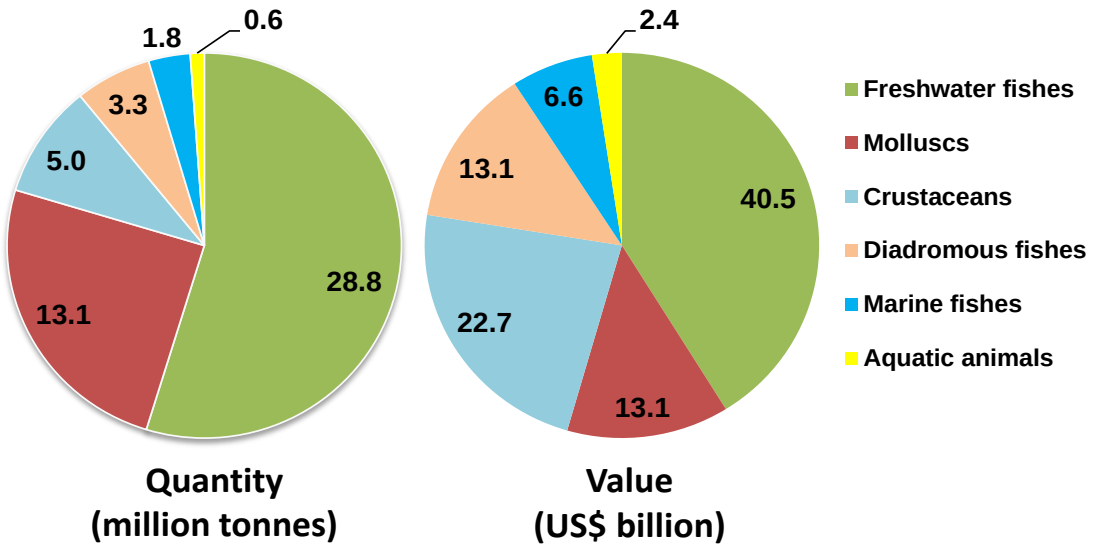


Figure 1. 1 World aquaculture quantity in 2008 represented as million tonnes and the value presented as US $ billion (FAO, 2010).

However, the expanding development of the aquaculture industry is associated with an increased demand in aquafeeds production (Stone, 2003). Similar to the other farmed animals, nutrition plays a key role in the total output of the aquaculture industry, fish health, growth, and final production. It has been reported that feed costs over 50% of an aquaculture project which significantly affect the total value of the economic return. Feed ingredients are selected based on their role to support the physiological activity of the animal, availability,

palatability, and cost (NRC, 2011). To meet the requirements of intensive feeds production, feed costs, and fish health; efficient and appropriate feed ingredients from sustainable sources are needed. Protein, oil, and carbohydrates are the major ingredients that are included in fish diets at defined levels to meet the requirements of each individual species. The protein source in most aquafeed is fish meal or 'industrial fish' (the most expensive component of fish diets) produced from the raw material of the total catch of fish which is not directly destined for human consumption. The global fish meal production seems to be limited and dependent on the total catch of wild fish. Apart from the high cost and limited production of dietary protein, the accumulation of the end-products of protein catabolism (ionized and non-ionized ammonia) lead to serious environmental impacts causing deterioration in water quality of the aquatic system (Jauncey, 1982a; Moon, 2001). In addition, there is an ethical aspect to using fish meal at high levels in diets for carnivorous fish with public concern and consumer pressure. Therefore, more attention has been paid to find alternative protein sources to fish meal.

Carbohydrates; the most abundant group of organic compounds in nature and the most economic component of animal feeds, are used efficiently by higher animals as energy sources and as precursors of more complex compounds (Berg et al., 2007). Starch is the main source of carbohydrate in pig diets, whereas ruminants are able to digest more complex carbohydrate such as cellulose and hemicellulose (Nafikov and Beit, 2007). In aquaculture feeds, carbohydrates (starches and sugars) are included to reduce feed costs and for their binding properties during feed manufacturing and the formulation of pellets (Pillay and Kutty, 2005). However, there are some restrictions concerning the limited capacity of fish to digest and utilize dietary carbohydrates (Wilson, 1994). Since diet cost is a critical factor in fish farming, numerous studies have been made to elucidate the mechanism and factors that influence carbohydrate metabolism such as insulin level in fish blood, key liver enzymes

activity and gene expression and to assess the potential of using carbohydrate as alternative non protein energy source (Furuichi and Yone, 1981; 1982b; Panserat et al., 2000a; Panserat et al., 2000b; Tan et al., 2006a; Enes et al., 2008a; Enes et al., 2010).

1.2 Carbohydrates: definition and classification

Carbohydrates are defined as compounds containing carbon, hydrogen and oxygen. These compounds include sugars, starch, cellulose, gums, and other substances. They make up most of the organic materials on earth because of their vital roles in all forms of life. The carbohydrates of animal feeds are classified according to their complexity as:

A. Monosaccharides or the simplest carbohydrates consist of 5 or 6 carbons with the general formula $C_5H_{10}O_5$ for the pentose, e.g. ribose and $C_6H_{12}O_6$ for the hexoses, e.g. glucose.

B. Disaccharides: consisting of two molecules of monosaccharaides with the general formula $C_{12}H_{22}O_{11}$ such as sucrose and maltose. In order to use disaccharides by animals, break them down to monosaccharaides is needed.

C. Polysaccharides or complex carbohydrates: are represent all of high molecular weight which formed by the aggregation of a long number of simple sugars units joined together by glycosidic linkages (Parker, 2000). Polysaccharides have the general formula $(C_6H_{10}O_5)n$, and the value of n may be 200 or more such as cellulose and lignin. Complex carbohydrates must be converted into simpler sugars to be absorbable by the intestine and transport to blood. In high animals; starch, dextrin, and disaccharides are hydrolysed to simple sugars by enzymes secreted in the digestive tract such as alpha-amylase. Beside the digestive enzymes, greater microbial activity is required for Polysaccharides digestion (Svendsen, 1974).

1.3 Carbohydrate metabolism

Carbohydrates are a vital source to build and fuel cells and provide an ideal substrate for energy reserves in animals. Excess carbohydrates are stored for later consumption as starch in plants and glycogen in animals (Wood, 1983). Animals possess the ability to control glycogen synthesis (glycogenesis) and glycogen breakdown (glycogenolysis). Glycogen phosphorylase kinase stimulates glycogenesis to produce glucose 1-phosphate. When glucose is plenty, glycogen synthase is activated and glucose 1-phosphate is used to increase the size of the glycogen molecule. Protein kinases and protein phosphatases regulate both glycogen synthase and glycogen phosphorylase kinase (Moyes and Schulte, 2008).

The gluconeogenesis pathway syntheses glucose from non-carbohydrate sources, such as amino acids and glycerol. It occurs in the liver and kidneys when dietary glucose is insufficient to meet energy needs by the body. In Gluconeogenesis, cells convert pyruvate to glucose and glycogen under a regulation of pyruvate carboxylase and fructose 1,6-bisphosphatase (Moyes and Schulte, 2008). On the other hand, glycolysis is the anaerobic enzymatic conversion of glucose to lactate or pyruvate, resulting in energy stored in the form of ATP. The glycolysis pathway consists of 10 principle steps (Figure 1.2). Different factors are involved in the regulation of glycolysis metabolic pathway such as the availability of substrate and the concentration of enzymes responsible for catalysing the irreversible reactions of glycolysis. These enzymes include hexokinase, phosphofructokinase, and pyruvate kinase. Hexokinase (EC 2.7.1.1) catalysing transfers phosphate group from ATP molecule to D-glucose to produce glucose-6-phosphate in the first step of glycolysis. High level of the latter product in blood inhibits the hexokinase activity and signal that glucose is no longer needed for energy or glycogen synthesis. The next steps of glycolysis are a series of enzymatic reactions to convert the glucose backbone to fructose. The other most vital enzyme in these reactions are phosphofructokinase (step 3) that catalyses the conversion of

fructose-6-phosphate to fructose-1,6-bisphosphate. The final step of glycolysis is catalysing by pyruvate kinase (step 10) to produce the ATP and pyruvate (Berg et al., 2007; Moyes and Schulte, 2008). The levels of these three vital enzymes are regulated by insulin secreted by pancreatic beta cells as a response to increases the concentration of glucose and amino acids in the blood.

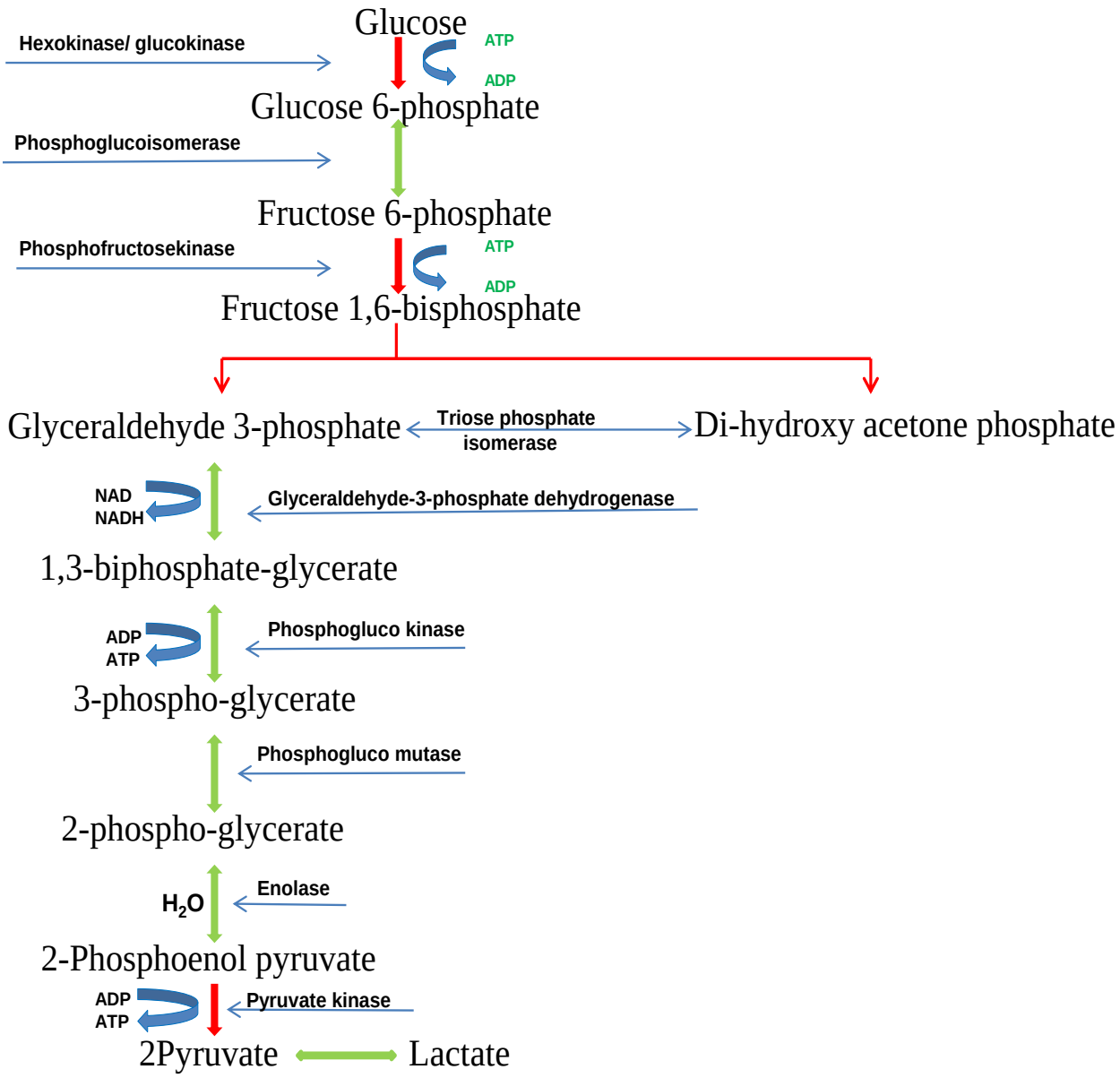


Figure 1. 2 The glycolysis pathway and the enzymes involved in different steps (the red arrows refer to irreversible steps in glycolysis pathway).

1.4 Carbohydrates in fish nutrition

The major ingredients of a typical fish diet can be classified as protein, lipids, and carbohydrates as well as minerals and vitamins (NRC, 1993). Carbohydrate are considered to be a major and essential source of energy for humans and animals and the most economical provider of nutrients which are required for growth, maintenance, and reproduction (Parker, 2000). Glucose is essential for energy metabolism, when dietary glucose is inadequate or when glycogen stores are limited, non-carbohydrate precursors will be catabolised for energy production and for synthesis of different metabolic compounds via gluconeogenesis pathway (Moyes and Schulte, 2008). It is known that fish have a relatively poor regulation over blood glucose levels with variation (between and within species) in their ability to digest dietary carbohydrate due to anatomical and functional differences of the gastrointestinal tract and other associated organs (Krogdahl et al., 2005). For example, it has been found that the ratio of intestine length to the total body length in carp is 1.8-2.0, the value being four times longer compared with those of rainbow trout and eels (Takeuchi et al., 2002). Accordingly, omnivorous, fresh water fish including carp can tolerate high levels of carbohydrates in their diets and show a capacity for protein sparing, tissue replacement and growth better than carnivorous species (Wilson, 1994; Stone, 2003). Different studies suggested different hypotheses to explain the poor utilization of dietary carbohydrates in fish which are:-

1- Limited presence of the most important digestive enzymes in the gastro intestine of different fish species. For instance, the amylase activity in the digestive tract of fish is generally lower than those of terrestrial animals (Takeuchi et al., 2002). Further, fish species differ greatly in terms of α-amylase activity which is only produced in the exocrine pancreas (Krogdahl et al., 2005). High values of the total amylase activity were detected in the digestive tract of carp (72.53 U mg^{-1}) compared with the value in the digestive tract of eel (0.06 U mg^{-1}) (Hidalgo et al., 1999).

2- The low capacity of glucose utilization could be due to reduced levels of insulin receptor tyrosine kinase in skeletal muscle. It has been reported that the number of receptors per unit weight of white skeletal of different fish species (brown trout, gilthead sea bream, tilapia, and carp) is low compared with mammals (Parrizas et al., 1994). It is worth mentioning that the same study demonstrated that carp muscle receptor numbers was much higher in comparison with rainbow trout. In another study, Gutiérrez et al. (2007) demonstrated that rainbow trout muscle tissue has been shown to have from 3-10 % of the insulin receptors per milligram of protein compared with those in the white or red skeletal muscle of rats. In addition, the insulin receptors binding capacity in trout was lower than that reported for mammals. In the same study, no difference in insulin receptor binding in skeletal muscle of trout fed different levels of carbohydrate (low and high) have been found. The main conclusion derived from the latter study is that higher glycemic levels observed in trout fed a high carbohydrate diet are not as a result of impaired binding of insulin to its receptors in skeletal muscles but there is another mechanism may be involved.

3- The rate of glucose uptake by the cells depends on the number of glucose transporters on the cell surface and the affinity of the transporters to glucose (Wright et al., 1989). GLUT-1 and GLUT-4 are kind of proteins belong to glucose transporters family transport glucose into muscle and fat cells in mammals. The number of GLUT-4 transporters in the plasma membrane increases in the presence of insulin which signals the fed status. Subsequently, insulin promotes the uptake of glucose by muscle and fat (Berg et al., 2007). It has been found that tilapia peripheral tissues contain a low level of GLUT-1 and devoid of GLUT-4 (Wright et al., 1989).

4- The low utilization of carbohydrate in fish may be attributed to an insufficiency of insulin level as reported in rainbow trout (Furuichi and Yone, 1982a). This theory seems

to be weak because another study claimed that the level of insulin in fish blood ranged between 0.2 and 5.0 mM and this concentration tends to be higher than the level found in mammals (Mommsen and Plisetskaya, 1991). In carp, Hertz et al. (1989) recorded a status of glucose intolerance despite the high level of insulin in the plasma. In this area, the biologically active insulin needs to be measured with more precision.

5- It has been suggested that the poor assimilation of dietary carbohydrate in fish is due to an insufficient regulation of glucose utilization via glycolysis and glucose production via gluconeogenesis (Enes et al., 2009). For example, the expression of the glucokinase enzyme that catalysis the first step of glycolysis to convert glucose into glucose-6-phosphate is highly induced in the liver of rainbow trout after feeding dietary carbohydrate (Panserat et al., 2000b). On the other hand, the gluconeogenesis enzyme (glucose-6-phosphotase) in the liver of the same species did not show any improvement in the activity or the expression after feeding dietary carbohydrate (Panserat et al., 2000a).

6- In general, fish have lower hexokinase activity (the glycolytic enzyme) and thereby poor capacity for glucose utilization (Wilson, 1994). It is worth to point out that hexokinase activity in fish does not stimulate by dietary carbohydrate source or level (Cowey et al., 1977; Tung and Shiau, 1991).

7- The structure of carbohydrate molecules may influence its attack by pancreatic secretion of amylase. For example, it has been established that the low digestibility of crude starch in fish resulting from the reduction in alpha-amylase activity duo to its absorption to the starch molecules which leads to inhibition in the starch hydrolysis process (Spannhof and Plantikow, 1983). A suitable degree of wet heat treatment may change the structure and increase the digestibility and bioavailability of dietary starch (Hemre et al., 2002; Stone, 2003).

Other factors appear to be associated with carbohydrate utilization in fish such as carbohydrate complexity and dietary inclusion level, metabolic enzyme activity, meal frequency, the environmental temperature and poikilothermic nature of fish compared to mammals and birds (Wilson, 1994; Hemre et al., 2002). Moreover, it has been shown that better growth performance were obtained from adding complex rather than simple sugars to fish diets indicating that fish are able to utilize poly carbohydrates (e.g. starch) better than monosaccharide (e.g. glucose) (Stone, 2003). In addition, the level of dietary carbohydrate may affect the activity of gluconeogenic and glycolytic enzymes. In a comparative study, Furuichi and Yone (1982b) investigated the effect of diets containing (0 or 10%) dextrin on hepatic enzymes related to carbohydrate metabolism in different fish species with different feeding habits. The results showed that the differences in the activities of enzymes were attributed to the differences in dietary dextrin concentration. Furthermore, diet ingredients could influence the ability of fish to utilize dietary carbohydrate. In this context, Panserat et al. (2002) investigated the mechanisms involved in poor metabolic utilization of dietary carbohydrates in rainbow trout (*Oncorhynchus mykiss*) by studying the effect of dietary lipids on the regulation of two hepatic enzymes (glucokinase and glucose-6- phosphatase). In the latter study, two groups of juvenile trout fed either a low (10%) or a high (25%) level of dietary lipids for 8 weeks. The results suggested that fish fed high level of dietary lipids had inefficient control of glycemia compared with fish fed the low level of lipids. The author claimed that the poor dietary carbohydrate utilization in rainbow trout may be related, partly, to increased hepatic glucose production under conditions of high dietary fat intake. Moreover, Tung and Shiau (1993) observed that body size influenced carbohydrate utilization in tilapia and that larger fish gained significantly more body weight, protein, and energy deposition than the smaller fish when fed the glucose diet and both groups (4.55 and 0.46) g utilized starch better than glucose throughout the experimental period (8 weeks). In addition, meal

frequency also seems to affect carbohydrate utilization in fish. In this aspect, Tung and Shiau (1991) fed tilapia with a diet containing 44 % of different carbohydrate sources (starch, dextrin, and glucose) for 8 weeks (two or six times a day). The results showed that fish fed six times a day gained more body weight than fish fed the same diet only two times a day.

It is important to distinguish between a tolerable and an optimal level of carbohydrate in fish diets. The first term refers to the level which does not reduce growth or increased mortality, whereas the second refers to that glucose being fully oxidized for energy production and subsequently spare protein for growth (Hilton et al., 1987). The optimal level of dietary carbohydrate for carp ranging between 30 and 40 %, 20% for sea bream and does not exceed 10 % for yellow tail (De Silva and Anderson, 1995). The ability of fish to utilized dietary carbohydrate is measured by glucose tolerance tests, where the individual animal is loaded with glucose and blood is drawn at intervals for glucose measurement to determine how quickly it is cleared from the blood. In human blood, glucose returns to a baseline level within 1 or 2 hours after feeding time but glucose clearance in fish is more slower and prolonged (De Silva and Anderson, 1995). In human and animal studies, the regulation and the capacity of glucose metabolism showed a remarkable degree of improvement by Cr supplementation which potentiates the action of insulin *in vitro* and *in vivo* experiments (Mertz, 1974). However, the potential advantages of Cr for fish need to be determined.

1.5 Minerals in fish nutrition

Minerals are inorganic elements play a critical role in various body functions and are necessary to sustain life and maintain optimal health for humans and animals. Depending on their physiological function, minerals can be divided into three categories as presented in Table 1.1.

Table 1.1 Categories of essential trace elements based on their physiological function (Hardy, 2001).

Bone/scale mineralization	
Calcium	Bone, scale, skin, muscle function
Phosphorus	Bone, scale, skin, phospholipids
Magnesium	Bone, scale, skin, muscle function
Specific physiological function	
Iron	Hemoglobin
Selenium	Glutation peroxidase
Iodine	Thyroid function
Sodium and potassium	Ionic balance
General physiological function	
Copper	Cofactor for enzyme activity
Manganese	Cofactor for enzyme activity
Zinc	Cofactor for enzyme activity

Depending on the amount needed, minerals are categorized into two groups:-

• ***Trace elements (micro-elements):*** this group of minerals is usually required in small amount. This category includes iron, copper, manganese, zinc, cobalt, molybdenum, chromium, vanadium, silicon, tin, arsenic, selenium, fluorine, iodine, aluminium, and nickel.

• ***Major elements (macro-elements):*** the element considered to be a macro element when the dietary requirement is more than 100 mg kg^{-1}. This group includes calcium, phosphorus, magnesium, potassium, sodium, chlorine, and sulphur (Davis and Gatlin, 1996).

Fish and terrestrial animals require the same macro and micronutrients in their diets to support the vital metabolic processes (Lovell, 1989). Calcium and phosphorus as macro-nutrient are the most important elements in exoskeleton, bone, scales, and teeth formation and responsible for maintaining the acid-base equilibrium (Watanabe et al., 1997). In addition calcium is required for blood clotting process (NRC, 1993) while phosphorous is a component of deoxyribonucleic acid (DNA), ribonucleic acids (RNA), nucleotide and act as a co-factor for different enzymes (Davis and Gatlin, 1996). Some trace elements serve as metalloproteins (e.g. iron in haemoglobin, copper in haemocyanin) or metalloenzymes (e.g. zinc serves as a metalloenzyme for carbonic anhydrase, carboxypeptidases, glutamic dehydrogenase and a variety of other enzymes) (NRC, 1980). Minerals are also playing as a co-factor or activator of enzymes (e.g. Manganese is considered to be an activator for different enzymes such as leucine-aminopeptidase and zinc activates the alkaline phosphatase enzyme) (Clark et al., 1987; Davis and Gatlin, 1996). Not all the elements are essential for the higher animals, but at least thirteen minerals may be essential for aquatic animals including fish and the deficiency in some of them leads to severe morphological abnormalities and different pathological symptoms (Kaushik, 1995). In this regards, zinc deficiency resulted in growth reduction, anorexia, and low zinc serum level in channel catfish (*Ictalurus punctatus*). Other symptoms have been observed in salmonids fed diets deficient in zinc such as fin erosion and poor egg hatchability (Jauncey, 1982a). The symptoms resulting from the deficiency of some elements such as Ca^{2+} is unlikely to happen because this element is normally taken up from the water. In addition, the commercial diet contains a relatively high level of Ca^{2+}, while the diet is the most important source of phosphorus than the water which is in general deficient in phosphorus (Steffens, 1989). Therefore, the diet phosphorus content should meet the requirement of each species (Watanabe et al., 1988). The requirement and deficiency symptoms for only seven trace elements have been determined

for some species while the other elements need more investigations. The most important trace elements for fish and their metabolic role are presented in Table 1.1.

Table 1. 1 Trace elements and their metabolic role in fish.

Trace elements	Function (Watanabe et al., 1997; Steffens, 1989)	Requirement	Species	Life stage	Reference
Copper	Metalloenzymes	2-3 mg kg^{-1}	Grouper (*Epinephelus malabaricus*)	Juveniles	(Lin et al., 2010)
Chromium	Regulator of carbohydrate metabolism	0.8 mg kg^{-1}	Grass carp	Fingerlings	(Liu et al., 2010)
Iron	Involved in haemoglobin pigment and respiratory system	150-160 mg kg^{-1}	Tilapia	Juveniles	(Shiau and Su, 2003)
Manganese	Organic matrix of bone	5.5-6.4 mg kg^{-1}	Yellow catfish (*Pelteobagrus fulvidraco*)	Juveniles	(Tan et al., 2012)
Molybdenum	involved in the structure of xanthine oxidase	No data available			
Selenium	An integral component of glutathione peroxidase	0.2-0.25 mg kg^{-1}	Channel catfish (*Ictalurus punctatus*)	Fingerlings	(Gatlin and Wilson, 1984)
Zinc	Metalloenzymes	17.0-17 mg kg^{-1}	Hybrid striped bass (*Morone chrysops* × *Morone saxatilis*)	Juveniles	(Buentello et al., 2009)

Compared to the trace elements, the biochemistry and metabolic role of macronutrients for fish and different animals are well known as presented in Table 1.2.

Table 1. 2 Macro elements and their metabolic role in fish.

Macro elements	Function (Steffens, 1989)	Requirement	Species	Life stage	Reference
Calcium	Plays a role in skeletal formation, muscles and neural activity, blood clotting, and vitamin D metabolism.	0.45%	Channel catfish	Juveniles	(Robinson et al., 1986)
Phosphorous	Skeletal formation and involved in enzymes metabolic processes.	0.4%	Channel catfish	Juveniles	(Wilson et al., 1982)
Magnesium	Involved in phosphorylation reaction and different enzyme systems.	0.54 g kg^{-1}	Guppy (*Poecilia reticulate*)	Freshwater aquarium fish species	(Shim and Ng, 1988)
Potassium	Plays a role in stimulating the neural and muscular system.	2-3 g kg^{-1}	Tilapia	Juveniles	(Shiau and Hsieh, 2001)
Sodium	Important for osmoregulation system.	1.5 g kg^{-1}	Tilapia	Juveniles	(NRC, 2011)

1.6 The bioavailability of minerals

For establishing dietary mineral requirements for humans and animals, definitive bioavailability data are needed which require high precision techniques. In metabolic research, using stable isotope tracers is an effective and valuable tool to obtain reliable information on mineral absorption and bioavailability, mineral turnover, pool size, and different aspects in minerals metabolism and nutrition studies (Turnlund, 2006). This analytical technology has been developed rapidly to be used in clinical diagnosis and studying the fate of the dietary trace element in humans due to the inherent safety of the stable isotope tracers compared with alternative radioactive substances (Koletzko et al., 1998; Turnlund, 1998). It has been suggested that most of the elements in nature have one or more isotope (radioactive or stable) forms which is identical in terms of the chemical structure with a slight difference only in weight. For example, the element carbon has three naturally occurring atomic forms which are 12C, 13C and 14C. These forms or isotopes are different in respect of the number of neurons found in the nucleus. 12C is the most abundant of the three forms and representing the common stable isotope of carbon. In this method which called the isotopic labelling technique, a small amount of isotope tracer (label) is incorporated into a sample material (the carrier) in order to follow the movement of that element of interest through a chemical, biological, or physical system. The tracer is identical in terms of the physiological, biological, and chemical properties of the tracee but has special features that make its detection in the system along with the tracee possible (Turnlund, 2006). Oral and intravenous doses can be traced and separated from endogenous elements in the body using various computers and modelling techniques (Turnlund, 1998). Isotope tracer administration, absorption, and secretion are presented in Figure 1. 3.

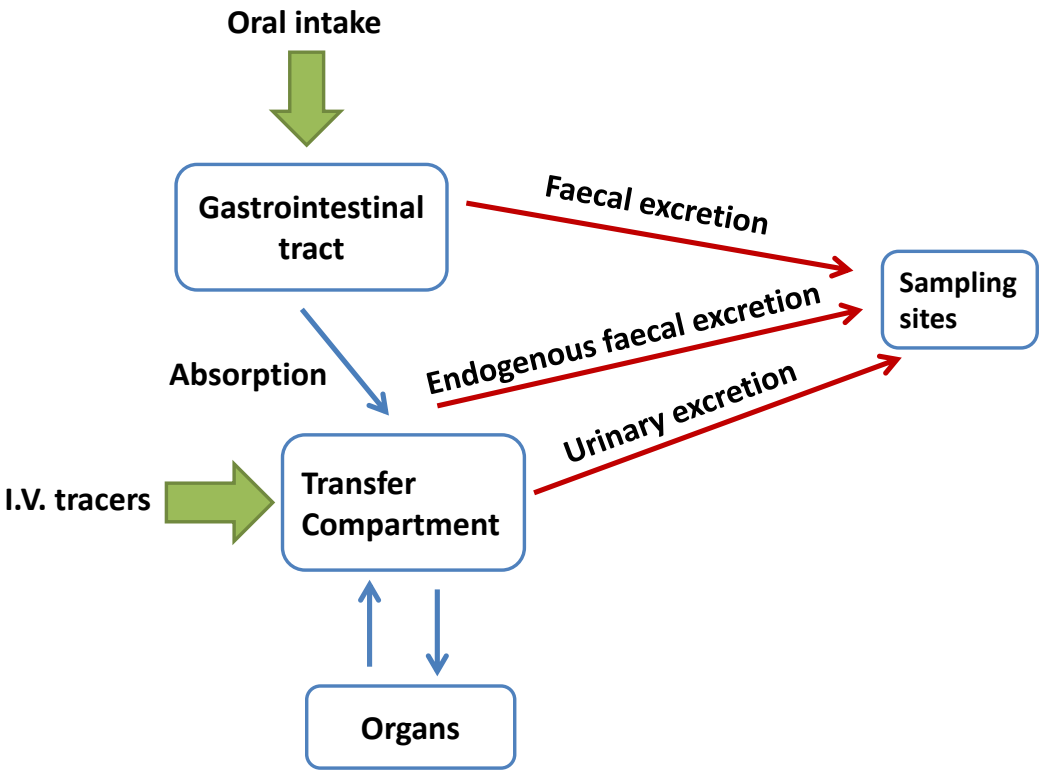


Figure 1. 3 The diagram showing the double stable tracer technique (one of the tracers administered orally and the second injected intravenously). Different samples are collected and analysed to study the distribution of an element inside the body.

Using tracers in animal studies started many years ago by Hahn et al. (1939) who applied this technique to investigate the metabolism of radioactive iron, absorption, transportation and utilization in dogs. However, the application of this technique in animal nutrition studies still limited due to the high cost of the isotope tracer which may cost several thousand dollars for small quantity of tracer element.

The bioavailability of the mineral for fish is highly affected by the chemical form of the compound used as a source of the supplement. The simple state or ionic forms are easily absorbed and subsequently considered to be more bioavailable for animal's physiological requirement such as Ca^{2+}, Mg^{2+}, and Mn^{2+}. However, some elements are able to form stable compounds that have poor solubility in water (Davis and Gatlin, 1996). The bioavailability is

associated with the intake level of the nutrient, concentration, valence state, and the ligands available in the gastrointestinal tract when the element is taken up by the animal (Lall, 2002). In addition, composition, digestibility, and particle size of the diet, chelators, inhibitors, physiological and pathological conditions of the fish tested and the environmental conditions are important factors affecting the biological action and mineral availability (Watanabe et al., 1997). The interaction and competition between different nutrients on the binding sites during the absorption and transport processes may reduce the chance of a certain element to be more available.

1.7 Difficulties in studying mineral requirement of fish

Studying the mineral requirements (especially trace elements) of fish is difficult because trace elements are required in very small amounts which make the formulation of purified experimental diet low in mineral content difficult to prepare. It is known that some feed stuffs such as fish meal (as a source of protein in practical diets for fish) contain significant amounts of minerals that may prohibit controlling those element levels in investigation with fish (Watanabe et al., 1988). Therefore, many investigators undertaking studies on mineral requirements of fish have been trying to minimize the element content in the basal diet by using alternative sources of protein or adding mineral premixes free from the element of interest. However, most of the basal diets employed for such requirement studies in aquatic animals contain a background level of the element tested. For example, Buentello et al. (2009) formulated different diets to study dietary zinc requirement of Hybrid striped bass, *Morone chrysops* × *Morone saxatilis* and bioavailability of two different zinc compounds. Despite dietary protein level being formulated from casein, gelatine, and crystalline amino acids to minimize the naturally occurring zinc content in the experimental diets, the chemical analysis showed that the basal diet contain 7.0 mg Zn kg^{-1}. In another study aimed to determine dietary potassium (K) requirement of juvenile hybrid tilapia (*Oreochromis*

niloticus × *O. aureus*), vitamin free casein was used as a source of protein in the diet and the mineral premix was free from K. However, the proximate analysis showed that the basal diet (un-supplemented control) contains 0.5 g K kg^{-1} (Shiau and Hsieh, 2001). Similarly, Delbert et al. (1984) investigated the dietary selenium requirement of fingerling channel catfish (*Ictalurus punctatus*). The ingredients included casein (vitamin free) and selenium–free mineral mix. The fluorescence spectrophotometry detected 0.06 mg Se kg^{-1} in the basal diet. However, it has been suggested that the bioavailability of trace elements from fish meal is low to fish. In this context, rainbow trout fed a fish meal diet without trace element supplementation in long term feeding experiment resulted in growth reduction and low feed efficiency in this particular study (Watanabe et al., 1980).

In addition to the diet as a source of minerals, ion exchange via the gills and skin of fish with the surrounding water increases the complexity of understanding dietary requirement and determination (Figure 1.4). For example, the dietary phosphorus requirement of channel catfish has been investigated by Wilson et al. (1982). These workers used a semi-purified diet with additional phosphorus-free mineral premix. However, the rearing water contained 0.04 ppm phosphorus. Recently, dietary copper requirement of juvenile yellow catfish (*Pelteobagrus fulvidraco)* has been determined by Tan et al. (2011). The analysis of the experimental diets showed that the control diet contains 2.14 mg Cu mg^{-1} and the concentration of Cu in the rearing water ranged from 0.6-1.0 µg L^{-1}. Moreover, studying the nutritional status of trace elements in fish requires precise information about the age, sex, health and physiological condition of the tested animal (Lall, 2002). To fully assess the health of the target animal after subjection to a dietary supplement, it is normally to measure the trace elements in different tissues such as liver, muscles, bone, and blood in order to make a full assessment of bioavailability and distribution. Despite the modern analytical techniques

that are involved in the trace element detection, there is still a problem in detecting such small concentrations in different tissues such as heart, spleen and brain.

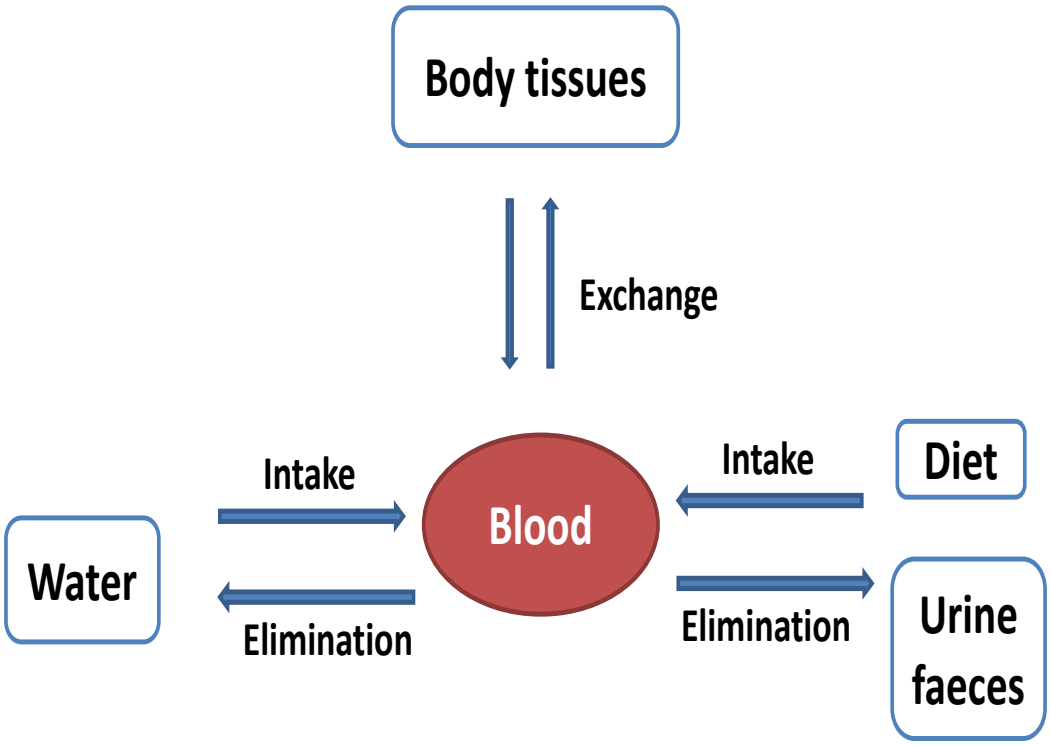


Figure 1. 4 Diagram illustrating the metabolism of minerals by fish. Modified from Steffens (1989).

1.8 The general proposal for trace elements effect

The general proposal for trace element function postulates that trace element levels below those required for optimum health may induce different structural abnormalities and functional disorders in fish. The severity of symptoms and signs of deficiency may be associated with the element's characteristics, sensitivity of organism tested, the degree and the duration of mineral deprivation as well as the speciation of the mineral. Elevating the amount of trace element administered to the requirement level will correct the functional disorders and maintain the homeostatic mechanisms. However, essential elements may become toxic when given in a high concentration and may cause severe pathological problems leading to death. Figure 1.5 illustrates the Bertrand's law of optimal nutritive

concentration of an essential trace element. The first part of the curve shows a state of deficiency which decreases as the concentration of the trace element increases. The plateau indicates a state of sufficiency of the element when the body function and growth performance is at its optimal level. When the element concentration exceed the ability of the animal to metabolize or excrete excesses, the element becomes toxic and death may occur at any point.

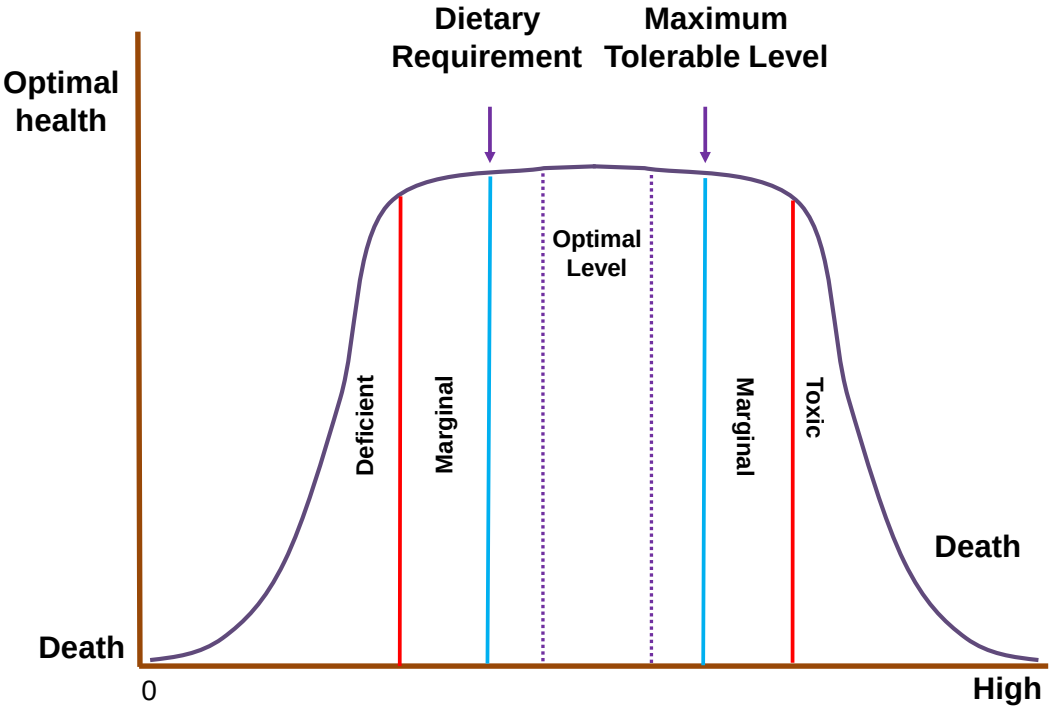
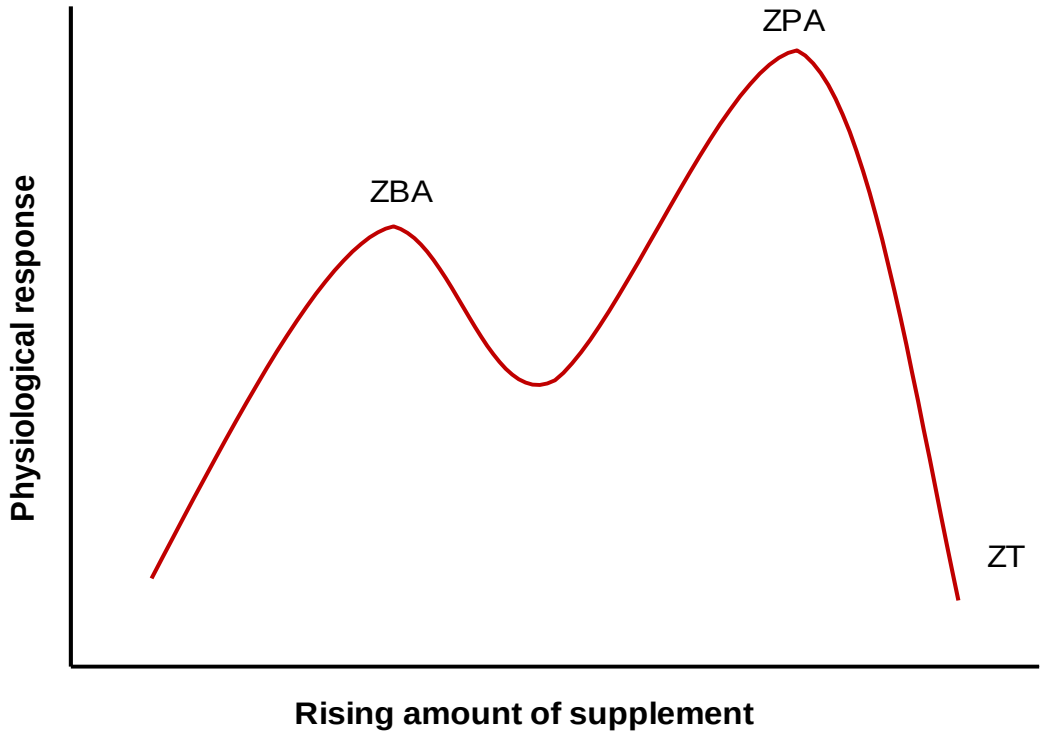


Figure 1. 5 Schematic illustration of Bertrand's law of optimal nutritive concentration of an essential trace element.

Venchikov (1974) proposed another model for trace element effects involving three zones of action. The first one is the biological zone in which the biological system will represents the correction of a deficiency symptoms and showing the optimal function in response to a certain amount of an element supplementation. Increasing the dose or supplement further may lead to a certain depression followed by a zone of a new increased activity in which the element acts as a drug not as an essential nutrient and thirdly the zone of toxicity and death (Figure 1.6).



ZBA= zone of biological action, ZPA= zone of pharmacodynamic action, ZT= zone of toxicity and death.

Figure 1. 6 Actions zones of a trace element depending on concentration. Modified from Venchikov (1974).

As mentioned previously, most of the trace elements have a key role in activating different hormones and enzyme systems with definitive information concerning the requirement, bioavailability, mechanism, deficiency symptoms, and toxicity feature. However, the real functions of some elements (e.g. chromium) in fish nutrition still need further research. One of the vital strategies used to modulate glucose utilization in humans, domesticated, and experimental animals is using minerals as a modifier of the assimilation processes. Chromium has been distinguished as an essential element regulates the normal carbohydrate metabolism in animals and man (Mertz, 1974b; Anderson, 1987; 1989; 1998;; Mertz, 1993; Vincent, 2007).

1.9 Chromium as a trace element

Chromium (Cr), the 24th element in the periodic table with an atomic weight of 51.99 has been found in almost all living organisms: humans, animals, and plants as well as in the air, soil and water (WHO, 1988). Cr has different oxidation states ranging from Cr^{2-} to Cr^{6+} but only two forms: trivalent Cr(III) and hexavalent Cr(VI) are found in nature (NRC, 2005). In the hexavalent state, Cr is a strong oxidizing agent, easily crosses biological membranes and reacts with proteins and amino acids (Pechova and Pavlata, 2007). These properties have made the hexavalent form of Cr more toxic than trivalent complexes. The final interaction of hexavalent Cr in the biological system results in reduction to the trivalent form (Lushchak et al., 2009b). The latter state has a poor capacity to cross cell membranes; however, it represents the most stable and active form of Cr which is tightly associated with protein, DNA and a variety of low molecular weight molecules (Snow, 1994). The biological availability of this element depends on its ability to form coordination compound and chelates (Watanabe et al., 1997) and the biological effect of Cr depends on the chemical nature of compound in which Cr is present (Robert et al., 2000). There are two categories of Cr compounds: organic and inorganic, studies suggested that Cr from dietary organic complexes such as Cr-Pic and Cr yeast (Bio-chrome) is absorbed more efficiently and has a greater biological effect than Cr from inorganic salts such as Cr chloride (Pechova and Pavlata, 2007). Dietary brewer's yeast was reported to be a rich source of the active form of Cr based on the activation of insulin to stimulate glucose utilization in rats (Steele et al., 1977). This element is responsible for activating some important enzymes such as lysozyme and is also required for stabilizing the structure of proteins and nucleic acids (NRC, 1974). Moreover, trivalent Cr acts as a cofactor of insulin hormone, enhances the ability of insulin to regulate glucose, protein and lipid metabolism (Anderson, 1997; Hertz et al., 1989; NRC, 1997a). Studies suggested that Cr can increase insulin binding, insulin receptor numbers and increase insulin receptor phosphorylation (Steele et al., 1977; Anderson, 1998). Another

study postulated that Cr modulates the insulin action by effecting insulin stability and degradation or via increasing its affinity for its receptors (Hertz et al., 1989).

1.10 The essentiality of chromium

Mertz (1974b) highlighted two terms which are widely used to describe the role of trace element in living system. The scientific term "essential" and the practical term "important". The author proposed that the former term refers to the degree of deficiency signs which are important in establishing the essentiality of a certain element; while the practical importance of an element "is judged by the relation of the individual requirement to the occurrence of the element in food, water, and air". The most important criteria for the essentiality can be summarised in several points as reported in some scientific publications (Mertz, 1974; Frieden 1984; Vincent, 2007) are:-

1. The essential element should exist in living cells.
2. It must be capable to interact with living system.
3. A reduction in the daily intake of the element below the required level causes a reduction in the biological function to suboptimal level.
4. The deficiency signs must be corrected by an adequate amount of supplementation.
5. For the essential element, a clear mechanism for absorption and transport should be known.

The deficiency of Cr in humans and animals is well documented in the scientific literature. For example, Anderson (1989; 1994) reported different signs of Cr deficiency in human included impaired glucose tolerance decreased insulin binding and decreased insulin receptors number and these signs were alleviated by Cr supplementation. Previously, Cr deficiency signs in mice and rat were induced in an experiment and corrected by oral Cr administration (Mertz and Roginski, 1969). For these reasons, Cr considered by many researchers as an essential trace element (Schwarz and Mertz, 1959; Mertz and Roginski,

1969; Anderson, 1997; Vincent, 2004). Despite that, the essentiality of Cr for humans still a subject of debate. In this regard; Nelson, (2007) reviewed the clinical and nutritional effects of Cr from human studies. The author classified Cr as a "nutritionally or pharmacologically beneficial element" but not essential because the biologically active form of Cr has been found effective and useful under a certain condition such as the stress and a definitive mechanism for Cr action has not been established yet.

1.11 Mechanism of chromium action

In 1957, Schwarz and Mertz isolated a compound from porcine kidney powder and later in 1959; the same material has been isolated from brewer's yeast and proposed as a biologically active form of Cr. This compound which named glucose tolerance factor (GTF) has been found to alleviated different symptoms (impaired lipogenesis, glycogen synthesis, glucose clearance rate and amino acid uptake) in rats fed diets containing Tourela yeast and deficient in trivalent Cr (Steele et al., 1977). The precise structure of the mentioned complex is not completely determined; however, it seems to contain nicotinic acid, amino acid glycine, glutamic acid and cysteine in addition to trivalent Cr (Steele et al., 1977). The structure of the GTF is presented in Figure 1.7.

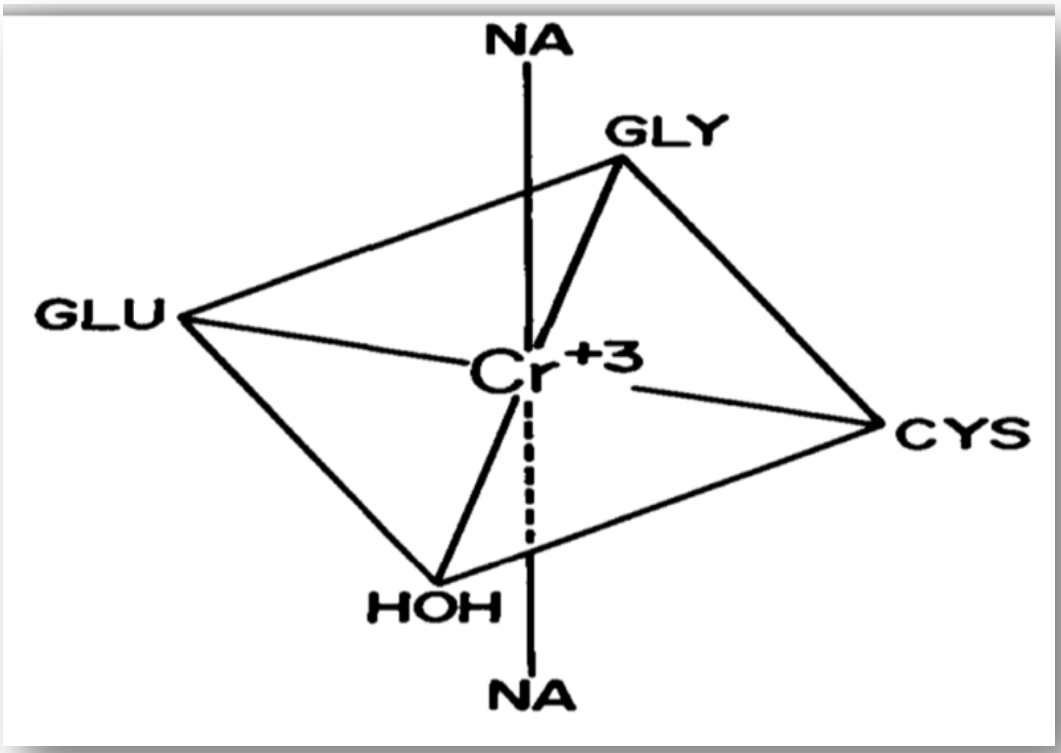


Figure 1. 7 The hypothetical structure of GTF (Steele et al., 1977).

After more than five decades of research, the biologically active form of Cr has still not been fully confirmed. Nevertheless, it is widely accepted that the naturally-occurring oligopeptide, low-molecular-weight Cr-binding substances (LMWCr), is the biologically active form of Cr which has been found to activate the insulin receptor kinase activity and subsequently improve glucose metabolism. This compound has been isolated from different animals but detected in high concentrations in bovine liver (Daveis and Vincent, 1997). In terms of the structure, LMWCr contains aspartate, glutamate, glycine and systine in addition to trivalent Cr (Vincent, 1999). From the description of the two compounds, GTF and LMWCr consist of the same amino acids and trivalent Cr; it seems that they refer to the same agent which potentiates the action of insulin. After absorption, Cr is transferred from blood to LMWCr molecule by a special carrier protein called transferrin. Later, transferrin receptors migrate to

the plasma membranes of insulin-sensitive cells. Cr is released and transferred from transferrin to LMWCr which exist in insulin-dependent cells as inactive form called apo-chromodulin. The binding of the latter form with chromic ion will produce the active form which called hollo-chromodulin that plays a critical role in insulin signal amplification system (Figure 1.8) (Vincent and Bennett, 2007).

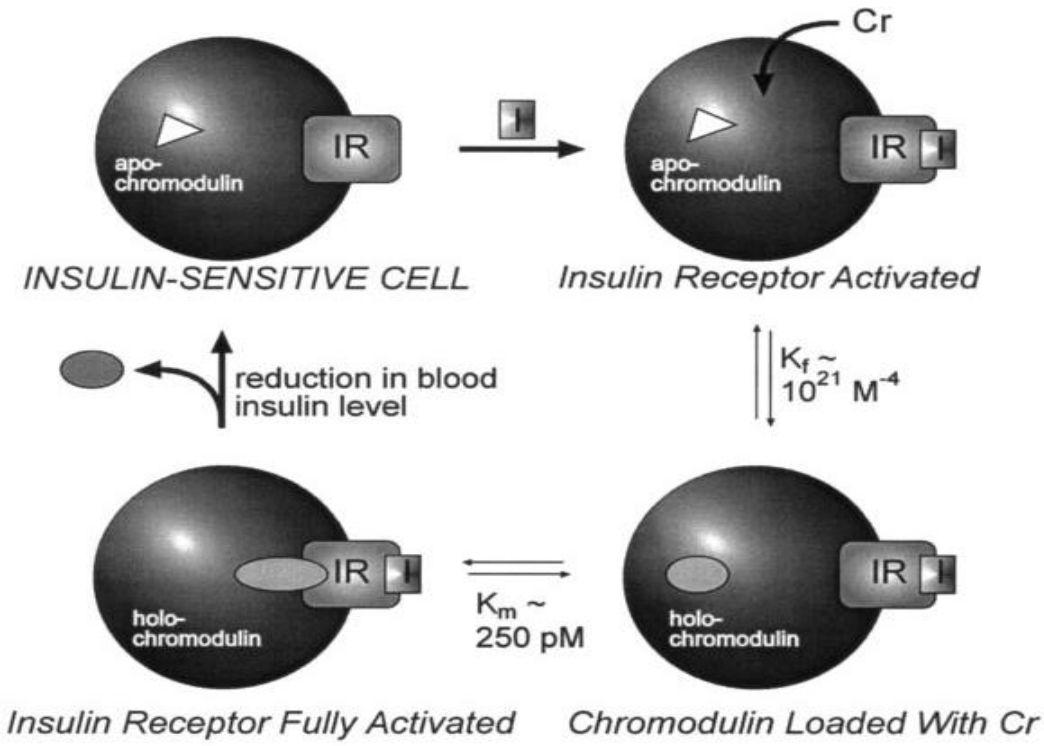


Figure 1. 8 Diagram illustrates the proposed mechanism for Cr and LMWCr potentiating the action of insulin. The source is (Cefalu and Hu, 2004).

1.12 Chromium intake, absorption and transport

Under the nutritional trials conditions, the primary route of trace element absorption is via the gastro intestinal tract. Exposure of a living organism to a foreign chemical for a period of time will lead to presence of the chemical within the cell, provided the cell membranes allow its translocation within the cells. Figure (1.9) represents the most important terms used in nutrition studies and the pathways of an element inside the human and animal body after the administration.

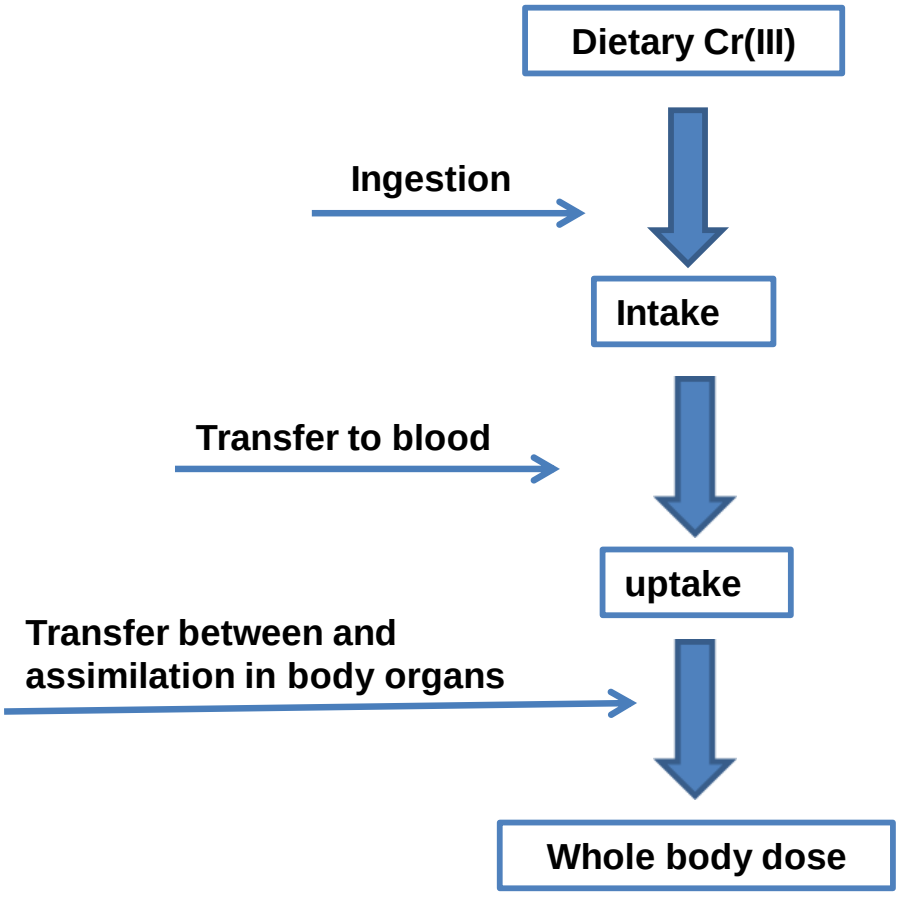


Figure 1. 9 Schematic representation of the pathways through which a chemical agent may pass during its presence in the animal body.

The efficient homeostatic mechanisms of living cells regulate the absorption and excretion processes. However, homeostatic mechanisms can fail under different conditions:

1. When the daily intake of an element is less than the amount excreted in faeces and urine, a state of deficiency will develop and certain diseases may appear.
2. Excesses amount of an element can be worse for health than deficiency. An excess amount of an element will lead to accumulation of an element in the body and eventual toxicity to cells.
3. A state of homeostatic mechanisms failure may occur due to hereditary diseases in which the transporting mechanisms for a trace element are inefficient and this case may lead to accumulation and certain disease (Schroeder, 1976)

Many chemicals are selectively absorbed combined with proteins or enzymes and transferred to the circulatory system immediately following the administration. Chemicals in the gastrointestinal tract can produce negative effects on the surface of

the mucosal cells such as necrosis. Similarly, oral administration of chemicals that are rapidly absorbed from the gastrointestinal tract, in theory, would expose the liver to concentrations of the chemical which may produce different changes in the tissue structure (Loomis and Hayes, 1996).

The absorption of Cr in the gastrointestinal tract depends on the form and valence state of Cr (WHO, 1988). In general, intestinal absorption of trivalent Cr is low in humans and animals ranging from 0.5 to 2.0% for inorganic salts (e.g Cr chloride) while the bioavailability of organic Cr complexes (e.g. Cr picolinate) have reported to have higher absorption potential (Pechova and Pavlata, 2007). The biologically active form of chromium, GTF, is water soluble and stable against variety of chemical treatments, binding of Cr ion to suitable ligands (e.g. organic acids) increases the stability of the metal against precipitation in the alkaline medium of the intestine and increases absorption efficiency of the organic form compared to the inorganic Cr compounds (Mertz, 1974a; WHO, 1988). Within the category of trivalent compounds, there are differences in the absorption efficiency resulting from a difference in the chemical form and structure. The low bioavailability of inorganic Cr is likely to be associated with the formation of non-soluble Cr oxide or due to the interference with ion forms of other minerals such as zinc and iron (Borel and Anderson, 1984). An *in vitro* study demonstrated that the small intestine of rats treated with Cr chloride was shown to be the most active area for dietary Cr absorption (Doisy et al., 1979). In addition, the level of plasma protein (transferrin and albumin) affects the absorption of trivalent Cr (Feng, 2007). It is worth mentioning that some studies suggested an inversely relationship between Cr absorption and Cr uptake (Vincent and Stallings, 2007). Once absorbed, Cr is distributed to various tissues of the body and the distribution of Cr associated with its chemical state and the dose administrated (NRC, 1974). The physiological regulation of chronic ion absorption and transport has not been clearly identified, but it is apparent that the absorption happens by passive diffusion process or by transport proteins in the intestinal mucosa (Ducros, 1992). Using the perfusion technique demonstrated that inorganic trivalent Cr was absorbed by a nonsaturable passive diffusion process (Dowling et al., 1989). After absorption, Cr is circulated in the blood bound to ß-globulin and transported into the cells bound to transferrin (the ferric ion caring protein) (Cefalu and Hu, 2004; Zefra-Stone et al., 2007). Figure 1.10 illustrates the proposed pathway for chromium in the human and animal body where the absorbed level of chromium is distributed by the circulatory system to the target organs.

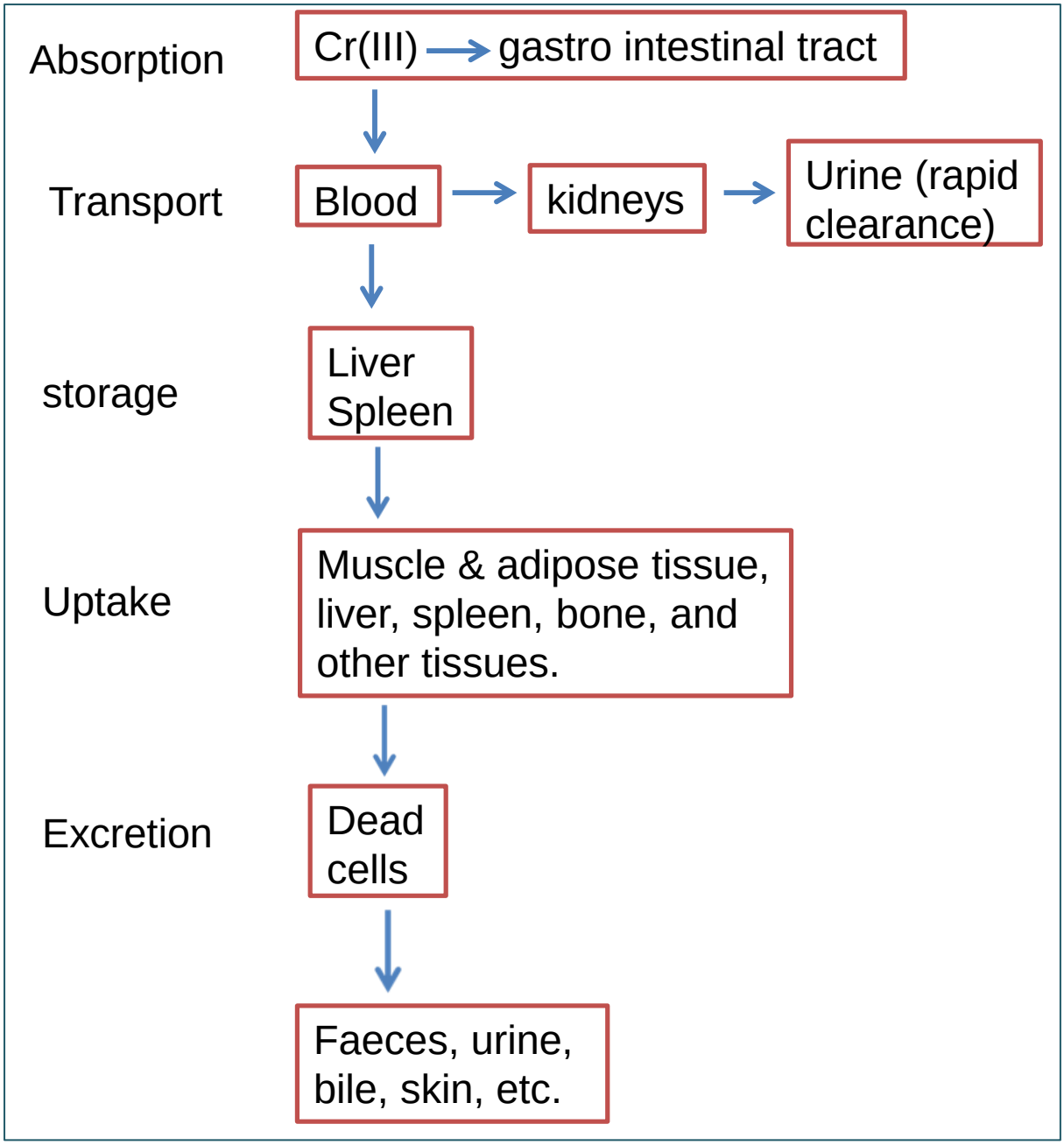


Figure 1. 10 Pathways for Cr(III) fate in the body of human. Modified from Lim et al. (1983).

1.13 Chromium accumulation

There are differences between tissues in terms of their ability to accumulate metals or toxicant. In addition, the accumulation process depends on the specific features of each metal. For example, cadmium as a metal-binding protein, metalothionein, accumulates in the kidney while accumulation in bone is a feature of lead (Woolley, 2003). The distribution studies of

chromium in fish have shown that Cr(III) is accumulated in different organs including opercula, muscles, and intestine, while the concentration level of Cr in the liver was under the detection level (Tacon and Beveridge, 1982). Different factors affect the accumulation of Cr in tissues such as the chemical form and valence, concentration, and route of entry (Yamaguchi et al., 1983). In addition, the accumulation of metals by small fish is higher than that of big fish due to various bioaccumulation processes (Nussey et al., 2000). Although the trivalent form is poorly absorbed, many studies have reported Cr accumulation in animal tissues after a period of diet administration (Tacon and Beveridge, 1982; Jain et al., 1994; Zha et al., 2009). Studies have shown that trivalent Cr treatment can result in higher Cr accumulation than treatment with hexavalent Cr because once the former is inside a cell it is slowly excreted (Stearns et al., 1995).

1.14 Chromium toxicity

Information about the tolerable level of chromium in vertebrates is limited. However, according to Bertrand's law, every element is toxic in high doses (Schroeder, 1976). In general, the term toxicity refers to the degree to which a substance can harm humans or animals. Toxic effect could be acute when involves harmful effects in an organism through a single or short-term exposure while the toxicity described as sub chronic when a substance has the ability to generate effects for more than one year but less than the lifetime of the exposed organism. The other degree of toxicity is chronic which produced after long-term of exposure to a toxic agent. A high level of absorbed element could induce metabolic or tissue toxicity or may produce complexes migrate from the cytoplasm to the nucleus and attack the DNA integrity (Schroeder, 1976, NRC, 1997). Different factors may increase the production of the reaction oxygen species (ROS), the chemically reactive molecules, leading to damage the sensitive structures of DNA, lipid and protein. Figure 1.11 demonstrates the most common factors that may induce DNA damage in living cells.

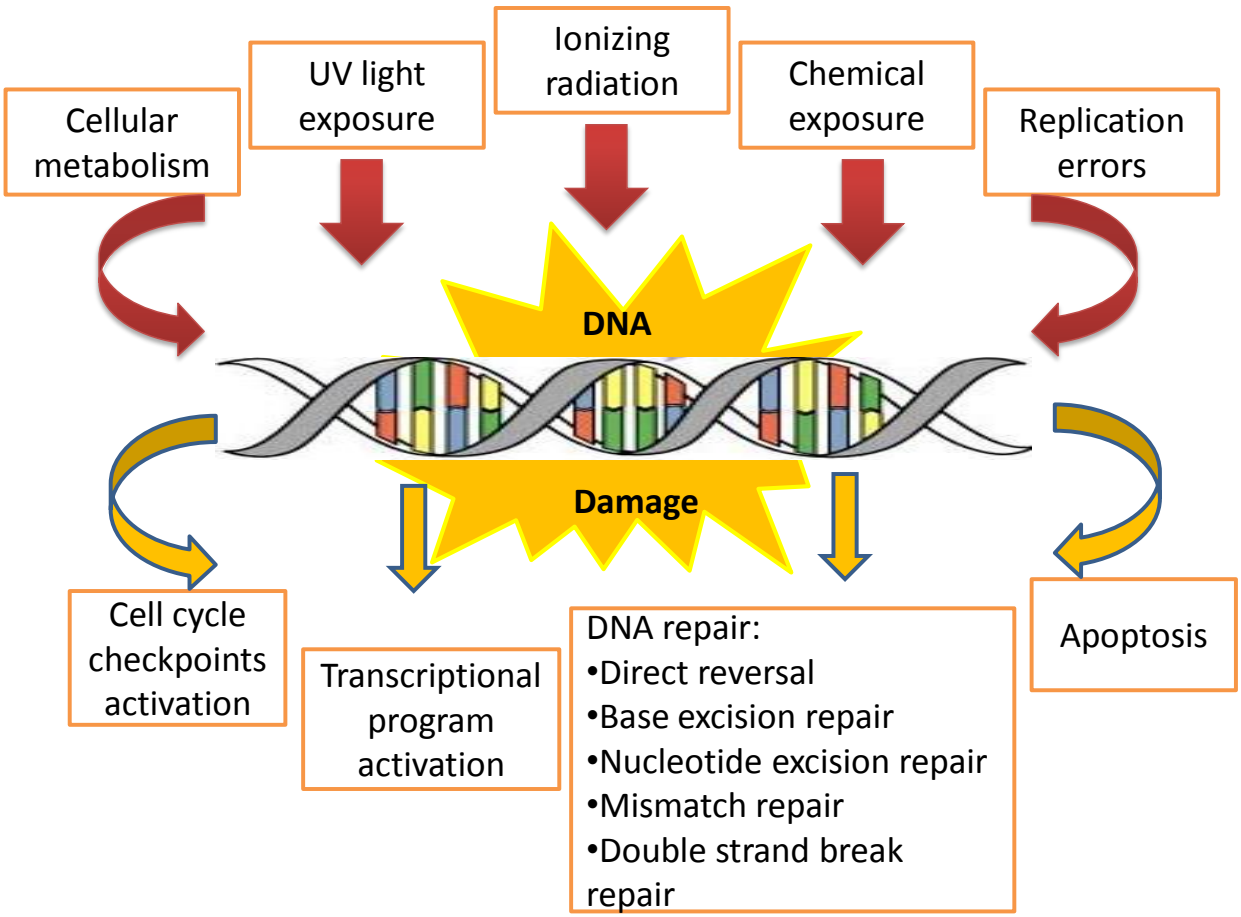
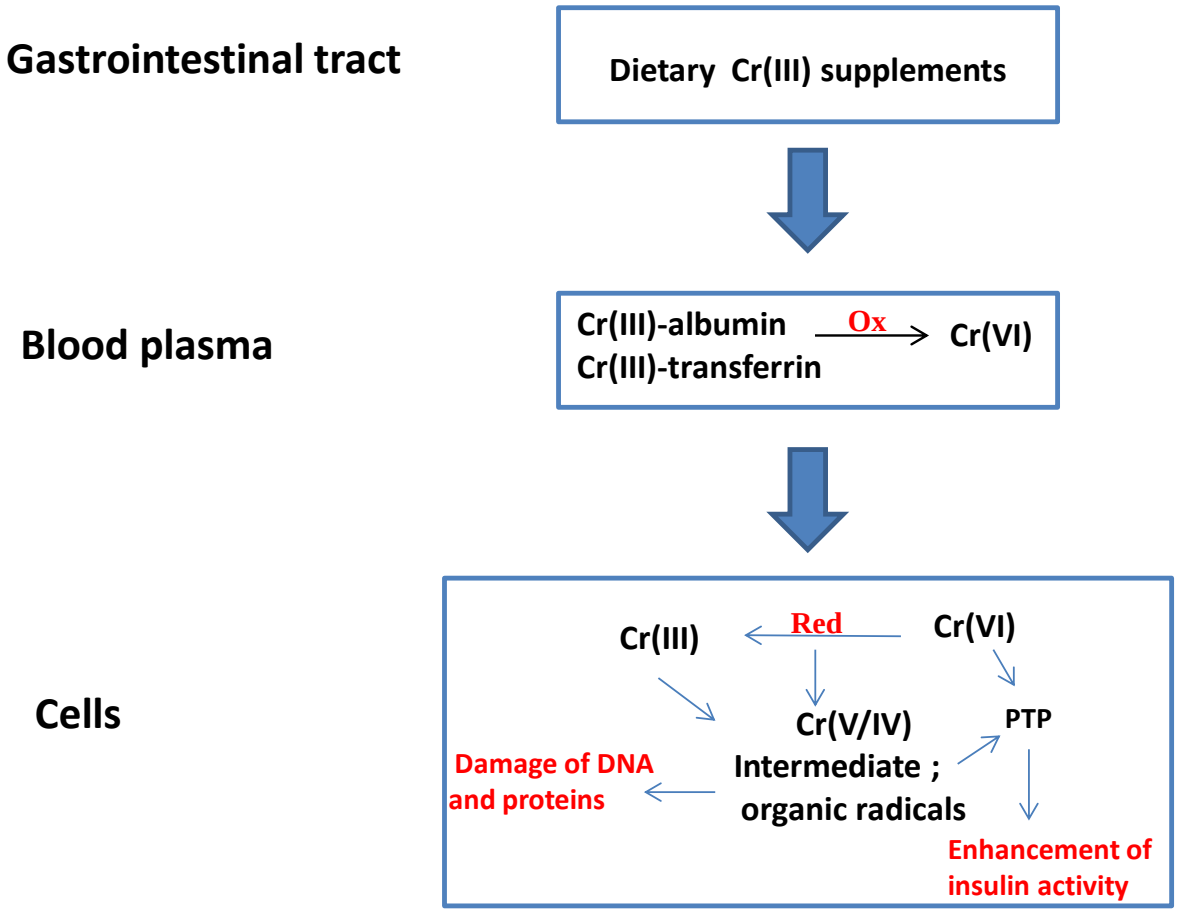


Figure 1. 11 Schematic representation of DNA damage sources and DNA damage response.

The genotoxicity of Cr is well documented by De Flora et al. (1990) in an extensive review which focused on the genotoxic effect of 32 Cr compounds involved in short term assays using different targets such as bacteria, Chinese hamster, mouse embryo and human lymphocyte. The conclusion of the review is that Cr has a potential to produce variety of genotoxic effects including DNA damage and mutations. These workers reported that the hexavalent Cr anion is considered more toxic than trivalent Cr cation because the former is dominant at physiological pH and easily taken up by the general anion channel protein. The mechanism of chromium genotoxicity is still unclear because of the complex intracellular metabolism of chromium. However, Snow (1991) reported that chromate ions are transported across cell membranes and are reduced intracellularly via reactive Cr(V) and Cr(IV)

intermediates to the stable form of chromium [Cr(III)]. During this process, oxidative DNA damage, chromium mediated crosslinks and mutations in number of target genes are generated. The effect of hexavalent Cr at 250 µM has been investigated in isolated lymphocytes of goldfish (*Carassius auratus*). The results showed that Cr toxicity was concentration-dependent and associated with the formation of reactive oxygen species (ROS) (Krumschnabel and Nawaz, 2004). Another study recorded DNA damage, necrosis of kidney cells, and growth reduction in Chinook salmon (*Oncorhynchus tshawytscha*) exposed to aqueous Cr concentrations ranged from 0 to 266 g l^{-1} (Farag et al., 2006). In general, the toxicity of Cr to fish is affected by species, body size, life stage and the environmental conditions such as the temperature, pH level, salinity and hardness (Holdway, 1988). In addition, the valence state of Cr is a critical factor determining its activity, stability, bioavailability, and toxicity features (NRC, 1997).

In human studies, the genotoxicity of Cr(III) supplement as chromium picolinate (CrPic) has been well investigated because the latter compound is the most popular supplement with a nutritional value for human (Vincent and Stallings, 2007). In this regard, CrPic has been found the most active form of Cr as a genotoxic in short term assays (Stearns, 2007). The proposal for the oxidative mechanism of biological activities of Cr(III) complexes is shown in Figure 1. 12.



PTP= protein tyrosine phosphatases; Ox= oxidation, Red= reduction

Figure 1. 12 Proposed oxidative mechanism of biological activities of Cr(III) complexes. Modified from Levina et al. (2007).

Recently, different studies have used the Single Cell Gel Electrophoresis or comet assay technique to evaluate the impact of trivalent Cr on different targets with different results reflecting the nature of Cr compound used, Cr concentration and the organism tested. To our knowledge, the toxic effects resulting from dietary trivalent Cr in fish have not been fully elucidated.

1.15 Chromium in human studies

Recently, intensive reviews have been made to demonstrate the mechanism of Cr action (Vincent, 1999, 2000b, 2004, 2007; Cefalu and Hu, 2004; Pechova and Pavlata, 2007). Moreover, many studies concentrated on the role of Cr in glucose and lipid metabolism in

humans. For example; Anderson, (1987) reviewed different studies with human in which glucose metabolism and glucose tolerance were improved after Cr supplementation. Similarly, Mertz (1993) reported that trivalent Cr compound supplementation improved glucose tolerance in 12 out of 15 controlled studies included individuals from different countries, the concentration of Cr compounds used ranging from 1 to 40 µmol day^{-1} and supplements equal to or more than 3 µmol have been found effective in some of these studies. Several investigations have suggested a potential association between Cr action and insulin signalling (Vincent and Bennett, 2007). Since the diabetes is common mellitus around the world with approximately 16 million of these patients in the USA, many studies have been funded by the American Diabetic Association, American Health Association, and American Chemistry Society to detect the real connection between Cr and diabetes (Vincent and Stallings, 2007). The USA Food and Drug Administration reported that 158 million Americans take some form of dietary Cr supplements (Stearns, 2007) and the nutritional supplement as CrPic was described as multimillion-dollar industry (Vincent, 2007). Further, it has been reported that Cr may affect lipid metabolism by affecting lipoprotein lipase activity (Barasi, 2003). In this context, Cr supplementation increases the high-density lipoprotein (HDL) (Riales and Albrink, 1981; Anderson, 1994) and decreases the low density lipoprotein (LDL) cholesterol, and triacylglycerols (Anderson and Kozlovsky, 1985; Lefavi et al., 1993). However, the response of the human body to Cr is associated with the amount, form of Cr supplementation, degree of glucose intolerance and the duration of the study (Anderson, 1998).

1.16 Chromium in animal studies

It has been suggested that Cr supplements may become an economical means to decrease fat and increase muscle tissue in pigs (Mertz, 1993). In this regard, 200 µg Cr kg^{-1} as CrPic in swine diets throughout the growing-finishing periods were effective in improving carcass composition by increasing lean tissue and decreasing fat tissue (Boleman et al., 1995; Mooney and Cromwell, 1997). More advantages have been reported for swine fed diets supplemented with CrPic or Cr chloride including decreased morbidity and enhancing the effectiveness of vaccine administration (Moonsie-Shageer and Mowat, 1993). The same effect has been observed in other livestock species fed 250 µg Cr kg^{-1} as Cr-Pic (Kitchalong et al., 1995). The same compound improved the fecundity in pigs and efficiency of production in swine (Lindemann et al., 1995). Another study suggested that Cr tripicolinate improved growth in growing horses (Ott and Kivipelto, 1999). In dairy cows, Cr supplementation enhanced resistance to the important production diseases such as mastitis (Burton et al., 1993). In laying Japanese quails (*Coturnl* × *oturnl* × *japanica*), different levels of CrPic (0, 200, 400, 800 or 1200 µg Cr kg^{-1}) resulted in increased body weight, feed intake, improved feed efficiency, egg quality, increased egg weight, egg shell thickness, and egg specific gravity (Sahin et al., 2002). Similarly, Ahmed et al. (2005) concluded that Cr chloride supplementation (0.2 mg kg^{-1}) enhanced growth performance and carcass characteristics in broiler chickens. On the contrary, different Cr compounds supplementation had no effect on growth and carcass composition of growing finishing pigs (Matthews et al., 2003) and did not affect the immune responses in cattle (Kegley et al., 1997) and swine (Van de Ligt et al., 2002). Again, the animals response to Cr supplementation in their diet may be associated with the amount, source of Cr, the physiological condition of tested animal, degree of glucose intolerance and the duration of study (NRC, 1997; Anderson, 1998).

1.17 Chromium in fish studies

The different aspects of Cr effect in fish nutrition are summarized in Table 1.3 Limited studies have been carried out on fish and the interest of these experiments was mainly oriented towards the effect of Cr on growth and final body composition. Most of these studies were performed on tilapia and rainbow trout with limited work undertaken with other species such as carp and gilthead sea bream using different Cr sources and levels with different experimental duration and diet ingredients composition.

A level of 1.0 mg Cr kg^{-1} as Cr chloride in diets for rainbow trout produced the best growth response but it was not significantly different from fish fed other diets supplemented with 2.0 or 6.0 mg Cr kg^{-1} (Tacon and Beveridge, 1982). Despite the high concentrations used in the latter study, the Cr carcass content did not show significant differences between the experimental groups. The same source of Cr at a level at 2.0 mg Cr kg^{-1} improved glucose utilization in carp after 6 hours of injection (Hertz et al., 1989). These workers used fish meal, soya bean meal and wheat, whole-milled to formulate the experimental diet. In a comparative study, Shiau and Chen, (1993) investigated the influence of different forms of Cr on the utilization of two sources of carbohydrate by juvenile hybrid tilapia (*Oreochromic niloticus* × *O. aureus).* The results suggested that supplementation of 2.0 mg Cr kg^{-1} as an inorganic form (Cr chloride or sodium dichromate) improved glucose utilization in fish. In addition, greater weight gain, food intake, protein retention, energy retention and body lipid content were observed in fish fed the starch compared to those fed the glucose diet. Similarly, a level of 2.0 mg Cr kg^{-1} from organic sources (Cr-Nic and CrPic) significantly improved glucose but not starch utilization in tilapia (Pan et al., 2002). Another form of organic Cr, Cr yeast, enhanced immune response in juvenile rainbow trout fed three semi-purified diets (Gatta et al., 2001). Recently, 0.8 mg Cr kg^{-1} as CrPic significantly promote growth performance in grass carp but the same source at 1.6 mg Cr kg^{-1} did not affect growth in

rainbow trout (Selcuk et al., 2010). Similarly, 2.0 mg Cr kg^{-1} as Cr-Pic did not affect growth and carbohydrate utilization in hybrid tilapia fed experimental diets formulated to contain 45 % glucose or dextrin (Pan et al., 2003).

To our present knowledge, there are about 13 documented studies addressing the role of Cr in fish nutrition and the results of these studies are conflicting. It is apparent from the preceding review and the results of different studies in Table 1.3 that Cr metabolism can be modified depending on the dietary Cr source and concentration, dietary carbohydrate source and level, in addition to the difference in fish species tested and the duration of the study.

Table 1. 3 The role of chromium in fish nutrition.

Species	Concentration of chromium (mg kg^{-1})	Duration of study (weeks)	Chromium source	Parameters	Results	Author(s)
Rainbow trout (*Salmo gairdneri*)	0, 1.0, 3.0 and 6.0	8	Cr Chloride	Growth performance and feed utilization	No significant differences between the treatments	(Tacon and Beveridge, 1982)
Tilapia (*Oreochromis niloticus*×*O. aureus*)	2.0	8	Cr Chloride	Weight gain, protein & energy deposition, liver glycogen content& body composition.	Dietary Cr increased weight gain, energy deposition and liver glycogen content.	(Shiau and Lin, 1993)
Tilapia	2.0	10	Cr Chloride, Sodium-di chromate and chromic oxide	Liver enzyme activity, growth, and carcass indices.	Cr improved glucose utilization; chromic oxide was more effective than other forms of Cr, no differences in HK activity between the experimental groups.	(Shiau and Chen, 1993)
Rainbow trout (*Oncorhynchus mykiss*)	0.5	12	Chelated Cr	Growth, protein and energy utilization, and blood glucose level.	No effect on final body weight, nitrogen and energy retention, Cr affected blood glucose clearance.	(Bureau et al., 1995)
Rainbow trout	1.5, 2.3, and 4.1	6	Cr yeast	Red and white blood cells count and serum lysozyme activity	Cr yeast modulated the immune response of rainbow trout	(Gatta et al., 2001a)
Gilthead seabream (*Sparus aurata*)	0.8 and 5.3	12	Cr yeast	Growth, feed conversion ratio, and carcass indices	No effect	(Gatta et al., 2001b)

Table 1. 3 continued

Tilapia	2.0	8	Cr-Nic Cr-Pic Cr-Meth Cr-Acetic acid	Growth, Fructose-1,6-phosphate and phosphfrucokinase activity	Cr-Pic and CrNic significantly affected glucose utilization and specific growth rate	(Pan et al., 2002)
Tilapia	2.0	8	Cr-Pic	Growth, liver G6PDH, 6PGDH and carcass indices,	No significant effect on growth, higher liver enzyme activities were observed in fish fed the dextrin than those fed glucose.	(Pan et al., 2003)
Rainbow trout	0.4, 0.8 and 1.6	8	Cr-Pic	Growth, serum glucose and cholesterol conc.	Serum glucose and cholesterol levels decreased with increasing dietary Cr supplementation	(Kucukbay et al., 2006)
Rainbow trout	1.6	8	Cr-Pic	Growth	No effect	(Selcuk et al., 2010)
Grass carp (*C. carpio*)	0, 0.2, 0.4, 0.8, 1.6, and 3.2	10	Cr-Pic	Growth, and blood biochemical parameters (glucose, insulin, cholesterol, triglyceride and HDL-C)	CrPic improved growth at a level 0.8 mg kg^{-1}	(Liu et al., 2010)
Tilapia	1.0 and 2.0	12	Cr yeast and Cr oxide	Growth, chemical composition, and glucose level.	Cr affected the final body composition	(Magzoub et al., 2010)
Tilapia	0.2, 0.4, 0.6, 0.8, and 1.2	12	Cr-Pic	Growth, body composition, and organs indices	No effect on growth, 0.4 mg kg^{-1} increased dry matter and crude protein of fish carcass	(Mahrim, 2012)

1.18 Choice of species for nutrition studies

The aims of aquaculture could be achieved through selection of species or strains with higher growth rates, superior feed conversion efficiencies, adaptable for different environmental conditions and greater resistance to diseases with high fecundity. In terms of the fecundity (as an example), Atlantic salmon (*Salmo salar*) and rainbow trout can achieve breeding status in their second year of life. Mature salmon and rainbow trout females produce about 1500 and (2000-12.000) eggs kg^{-1} body weight respectively (Lall, 2004). In carp, the female reach sexual maturity at 15 months when reared at 15 ºC and a healthy carp female produce about 150. 000 eggs per kg body weight (Billard, 1999; Pillay and Kutty, 2005). The common carp is one of the most successful species in aquaculture due to its features that can be summarized in several points:-

1. Carp and many species of Cyprinids have resilient to a wide range of environmental conditions. For example, an adult common carp can tolerate fluctuations in water temperature within the range of 1 to 35 ºC and can survive under conditions of anoxia (<1 mg O_2 l^{-1}) for a few hours in summer and for extended durations under the ice layer in winter. In addition, carp can tolerate a wide pH range from 5 to 9 (Billard, 1999).
2. Carp is an adaptable species that can live at high densities within small ponds, cages or tanks.
3. Carp have high fecundity and can spawn throughout the year with a fast growth rate and high production yields.
4. Carp is an omnivorous fish consumes any source of food from different levels of the trophic web such as water plants, benthic warms, insects and crustaceans (Parker, 2000).
5. Cyprinids are preferred as a food source in many countries around the world.

"A domestic animal can be defined as one that has been bred in captivity for purposes of economic profit to a human community that maintains total control over its breeding, organization of territory, and food supply" (Clutton-Brock, 1999). The common carp is one of the oldest cultured fish in the world and the first domesticated species because of its specific features that made it so popular for commercial culture (Pillay and Kutty, 2005). Culturing and breeding of carp started 4000 years ago in China and several hundred years ago in different regions of Europe like the Czech Republic, Germany, Hungary, as well as Russia and Ukraine. It is worth to mention that Japan and China are considered the pioneers in carp culture in Asia; however, carp farming started to expand recently in India, Indonesia and Vietnam due to the advantages mentioned above (Bakos and Gorda, 2001). It has been reported that the dominate species in world aquaculture production in 2008 were carp fish with highest outcome value as a major species group (FAO, 2010). On the other hand, it has been estimated that 31% of the total aquafeed production in 2008 was for carp as major farmed species (Bostock et al., 2010). Compared with other species, carp is capable of tolerating high levels of carbohydrate in its diet with relatively low protein content. Thereby, diets formulation for carp is economic and may support the environment in a more sustainable manner. Figure 1.13 shows the global compound aquafeed production in 2008 for culture species presented as percentage of total aquafeed production.

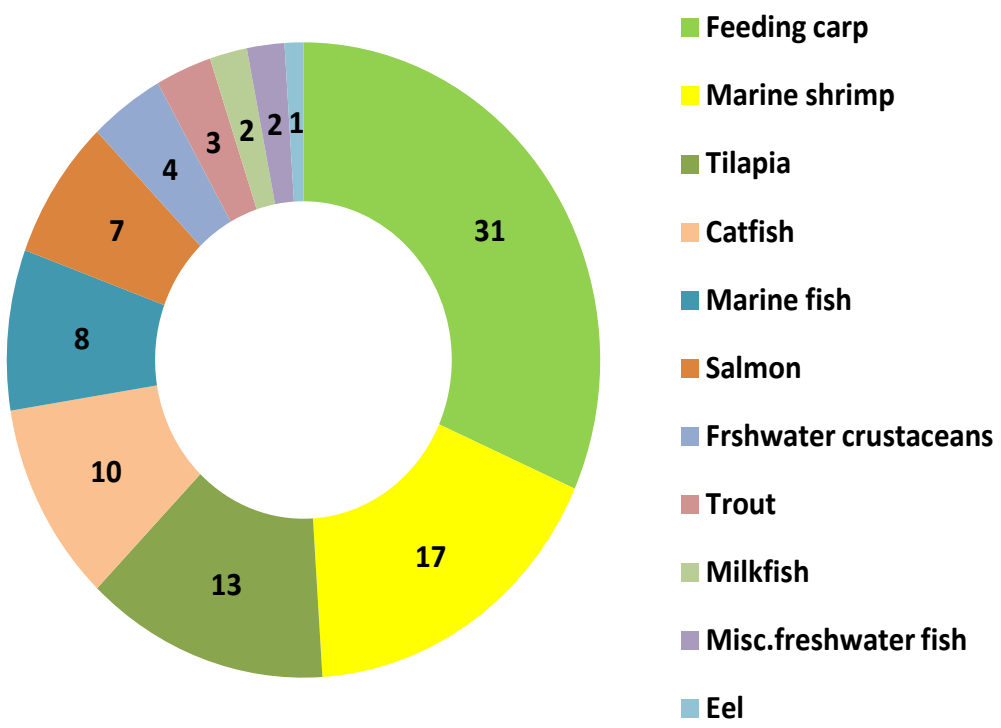


Figure 1. 13 Estimated global compounds aquafeed production in 2008 for major farmed species presented as percentage of total aquafeed production, dry feed basis (Tacon and Martin, in press).

Interestingly, NRC (2011) reported that the main consumer of prepared feeds is carp species which consumed approximately 42% of the total volume consumed. Since carp species has the capacity to utilize dietary carbohydrate, it has been chosen as an experimental animal to assess the effect of different levels and sources of Cr on growth performance and carbohydrate utilization in fish. In addition, very few studies have involved common and mirror carp in this kind of research. It is worth mentioning that common carp and mirror carp are similar in terms of genetics and biological features.-

There are three well known varieties of common carp which are:

• The orange coloured scale carp (*C. carpio* var. *flavipinnis*).

• The partially-scaled mirror carp (*C. carpio* var. *specularis*).

• The virtually scaleless leather carp (*C. carpio* var. *nudus*).

1.19 Nutrient Requirements of carp

In general, fish feeds should provide the energy and nutrients required for different metabolic functions such as growth, reproduction and fish health maintenance taking into account that deficiency or excess in any of the diet components may lead to adverse impacts. The basic nutrients required by fish include proteins, lipids, carbohydrates, vitamins, and minerals with variation in the requirements of each species. Different studies and reviews have provided guidelines for feeding carp species (Jauncey, 1982a; Satoh, 1991; Takeuchi et al., 2002; NRC, 2011).

1.19.1 Proteins

The natural diet of fish is typically high in protein content due to their limited capacity to assimilate dietary carbohydrate. The main function of dietary protein is therefore to provide the animal with the essential amino acids balance and nonessential amino acids nitrogen to support building production, tissue replacement and other metabolic processes (Jauncey, 1982a). The amino acids essential for growth are leucine, isoleucine, valine, threonine, phenylalanine, tyrosine, methionine, cysteine, tryptophan, arginine, histidine, and lysine (Billard, 1999).

The protein requirement is defined as the minimum level of protein needed to be sufficient for maximum growth production and to meet requirements for the indispensable amino acids (NRC, 1993). Typically, common carp require 10-12 g kg^{-1}BW day^{-1} of protein to produce the maximum growth rate which is quite similar to the requirement level of the other fish species (Satoh, 1991). It seems that different life stages are demand different protein levels in the production of common carp. For instance, the optimum level for fry and fingerlings ranges between 43 and 47 %, whereas fingerlings and sub adults require 37-42 % of dietary protein and 28-32 % for adult fish (NRC, 1977; 1981; 2011). In general, protein levels ranging between 30 and 35 % in carp diet found to be sufficient for the optimal growth

(Jauncey, 1982a). It is noteworthy that protein sources such as fish meal are the most expensive component in fish diet; thus, many studies have concentrated on seeking alternative energy sources to spare protein for growth (Carter and Hauler, 2000; Hu et al., 2008).

1.19.2 Lipids

The first main function of dietary lipids is to produce the metabolic energy (Adenosine Tri Phosphate) and the second function is to sustain the phospholipids structure and integrity of cellular membrane (Jauncey, 1982a). Carp as omnivorous and warm water fish can exploit lipid efficiently as dietary energy sources (Wilson, 1994). It has been estimated that a supply of 1% of both n-6 and n-3 fatty acids leads to better growth and efficiency in juvenile common carp (Takeuchi and Watanabe, 1977). The digestibility of lipids in fish is influenced by different factors such as the amount of fat in the diet, water temperature and the molecular structure of dietary lipids (Parker, 2000). Table 1.4 shows common carp requirements for the macronutrients.

Table 1. 4 Macronutrient requirements of common carp (Takeuchi et al., 2002).

Nutrient	Requirement
Protein	30 – 35 g 100 g^{-1}
Lipids	5 – 15 g 100 g^{-1}
Essential fatty acid	
Linoleate	1g 100 g^{-1}
Linolenate	1g 100 g^{-1}
Digestible energy	13 – 15 MJ kg^{-1}
	310 – 360 k cal
Carbohydrate as (starch)	30 – 40 g 100 g^{-1}

1.19.3 Vitamins

Vitamins are essential organic substances supplied by the natural food in extremely small amounts and many of these vitamins function as coenzymes in the metabolic pathway; therefore, dietary vitamin supplementation in fish diets is required to maintain optimal enzyme activities and key function of metabolism (Jauncey, 1982a). The vitamin requirement of common carp is associated with different factors (e.g. water temperature, storage method, diet ingredients and the various life stages of fish) (Satoh, 1991). It has been reported that juvenile and adult of common carp do not demand vitamin C in their diet because they can exploit D-glucose to synthesize ascorbic acid (Takeuchi et al., 2002). The stability of some vitamins may influenced by heat, temperature, moisture, pH level, lipid oxidation, and metal content (Satoh, 1991). Certain vitamins may be degraded due to the diet preparation technique and subsequent storage conditions; therefore, supplemental levels of vitamins are always supplied 2-5 times higher than the requirement level to maintain a safety reserve in fish diet formulation (Takeuchi et al., 2002). Vitamin requirements of common carp and their deficiency signs are summarized in Table 1. 5.

Table 1. 5 Vitamin requirements of common carp and deficiency symptoms (Jauncey, 1982b; NRC, 1993; 2011).

Vitamin	Requirement ($mg\ kg^{-1}$)	Deficiency signs
Thiamine	0.5	Poor growth, nervousness, and loss of skin colour.
Riboflavin	7	Anorexia, poor growth, haemorrhages in hepatopancreas, photophobia, kidney necrosis, disorientation.
Pyridoxine	6	Anorexia, ascites, ataxia, exophthalmia, convulsions, nervous disorders, anaemia, low hepatopancreatic transferase.
Pantothenicate	30	Anorexia, weight reduction, irritability, anaemia, haemorrhages in the skin, and lethargy.
Niacin	28	Anorexia, poor growth, poor survival haemorrhages in the skin, high mortality.
Biotin	1	Poor growth, erythrocyte fragility and fragmentation, lethargy, increased number of dermal mucous cells.
Choline	500	Poor growth, fatty hepatopancreas, vacuolization of hepatic cells.
Inositol	440	Anorexia, poor growth, dermatitis, loss of skin mucosa, skin erosion.
Para amino benzoic acid	200	No signs.
Vitamin A	400 IU	Anorexia, weight loss, exophthalmia, skin depigmation, twisted opercula, haemorrhagic fin and skin.
Vitamin E	100	Muscular dystrophy, exophthalmia, lordosis, kidney degeneration, and pancreatic degeneration.
Folic acid	No data	No signs.
Vitamin C	No data	Caudal fin erosion and deformed gill arches in larval stage, and poor growth.

1.19.4 Minerals

The importance of macro and micro elements for fish in general has been discussed previously in details; however, some mineral requirements and signs of deficiency have been identified specifically for carp species as listed in Table 1.6.

Table 1. 6 Mineral requirements of common carp and deficiency symptoms (Satoh, 1991).

Mineral	Requirement	Deficiency signs
Phosphorous	0.6-0.7%	Poor growth, skeletal abnormality, low feed efficiency, and high lipid content.
Magnesium	0.04-0.05%	Poor growth, sluggishness and convulsions, high mortality, high calcium content in bone.
Iron	150 mg kg^{-1}	Low specific gravity, hemoglobin content and hemoglobin values with low haematocrit values, abnormal mean corpuscular diameter.
Zinc	15-30 mg kg^{-1}	Poor growth, increased mortality, fins and skin erosion, low zinc and magnesium content in bone
Manganese	13.0 mg kg^{-1}	Poor growth, dwarfism with skeletal abnormalities, increased mortality, low content of calcium, magnesium, phosphorous, zinc and manganese in bone.
Copper	3 mg kg^{-1}	Poor growth
Cobalt	0.1 mg kg^{-1}	Poor growth

Despite the data mentioned above, limited information are available about different elements such as chromium, molybdenum, fluorine, and vanadium.

1.20 Conclusions, aims and research objectives

The longer term sustainability of the aquaculture industry could be achieved via reducing feed costs strategy. Studies have largely emphasised on finding suitable alternatives for the most expensive ingredient in fish feed (i.e. fishmeal) through substitution with low cost alternative sources, while ensuring that this substitution will not affect the normal growth and quality of the cultured animal. Carbohydrate is widely used to formulate the animal diets but for the reasons mentioned previously, fish have limited capacity to use dietary carbohydrate as a source of energy.

Chromium plays a key role in glucose metabolism by acting as a co-factor for insulin and its role in glucose metabolism has been reported for poultry and mammals. However, limited studies have been made on fish and the actual requirement was not determined for each species. None of the studies mentioned in the literature provided a holistic view concerning the effect of different levels and forms of Cr on the general fish health. Therefore, this study was designed to investigate the effect of organic and inorganic Cr on growth and starch and dextrin utilization in common and mirror carp.

Hypothesis

Overall, this thesis aimed to test the following hypotheses:

(a) Dietary chromium enhances growth and activity of key liver enzymes in common carp, *Cyprinus carpio,* at a certain level (Chapter 3).

(b) Different forms of chromium affect growth, body composition and have different effects on blood cells and gut and liver histopathology (Chapter 4).

(c) The source of carbohydrate affects body composition and the accumulation process in carp (Chapter 5).

The main objectives of this project were to:-

• Determine the dietary Cr requirement of common carp using the carp practical diet.

• Compare the bioavailability of organic and inorganic Cr.

• Study the effect of different levels of Cr on the utilization of different sources of carbohydrates (starch and dextrin).

• Examine the effect of Cr on the key liver enzymes, body composition, and Cr accumulation in liver, gut, and whole fish tissue.

• Investigate the impact of different levels and forms of Cr on the histological structure of the gut and liver tissues.

• Elucidate the effect of Cr on DNA structure.

• Study the effect of Cr on gene expression.

The hypotheses tested in each experiment are included in the relevant chapter.

Chapter 2

General materials and methods

Chapter 2: General materials and methods

2.1 Overview

This chapter embraces the main generic materials and methods as well as the products used for most ingredients. It provides details with regard to the experimental holding facilities, system used to hold carp, and also the basic design of feeding trials, general conditions and procedures. Aspects relating to the formulation and production of diets are presented such as the source of raw materials. The source of carp and their fundamental husbandry are mentioned as well as sampling of fish for tissues, organs, and blood.

The specific products for each trial in succeeding chapter are included separately.

2.2 Experimental diets

The experimental diets were produced at the Aquaculture and Fish Nutrition experimental laboratory of Plymouth University, UK. The diets were formulated to meet the nutritional requirements of carp species according to NRC (2011). The ingredients of the experimental diets were obtained from different sources as listed below:-

• Herring fish meal LT94 as a protein source in the diet was obtained from CC MOORE & Co. Ltd, Dorset, UK.

• Maize starch (S4126, Sigma-Aldrich, Poole, UK) was used as a source of carbohydrate.

• Pure sunflower oil (obtained from a local supermarket) was the source of lipid in the experimental diets.

• Pea protein and maize gluten were provided by Roquette Company, Frêres, France.

• *Vitamin/mineral premix (Premier Nutrition Products -PNP Ltd- Rugeley, Staffordshire, UK). The product contains: 121 g kg^{-1} calcium, vit A 1.000 µg kg^{-1}, vit D_3 0.100 µg kg^{-1}, vit E (as alpha tocopherol acetate) 7.0 g kg^{-1}, Copper (as cupric sulphate) 250.0 mg kg^{-1}, Magnesium 15.6 g kg^{-1}, Phosphorus 5.2 g kg^{-1}.

* Vitamin/mineral premix is Cr free

The quantity of the ingredients (g kg^{-1}) used to formulate the experimental diets are presented in Figure 2.1.

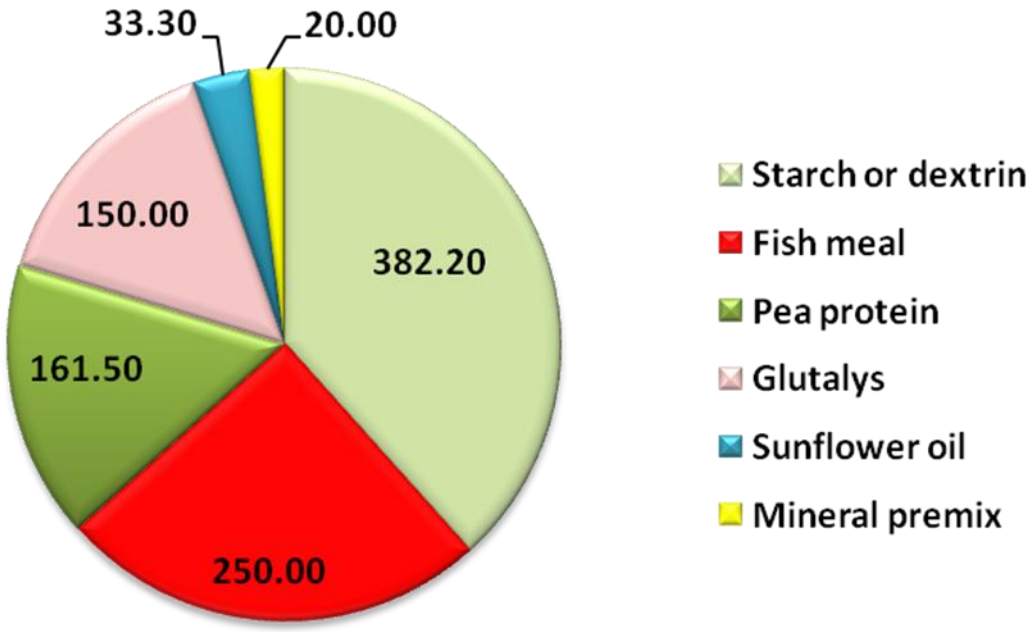


Figure 2. 1 The general ingredients used to formulate the experimental diets (g kg^{-1}).

The concentration of chromium in the ingredients of the diet was measured using an ICP-OES (Varian 725-ES, Australia) instrument as described in Cr analysis in the ingredients, diets and whole fish tissue section and the results are presented in Table 2.1.

Table 2. 1 The concentration of chromium in the diet ingredients presented as µg Cr kg^{-1}.

Ingredient	Cr concentration
Maize starch	312.11 ± 21.56
Dextrin	308.27 ± 15.42
Fish meal	329.32 ± 28.17
Gluten (glutalyse)	558.45 ± 42.80
Pea protein	468.25 ± 35.20

Samples were analysed in triplicate. Values are mean ±SE.

Despite that most ingredients contain a certain level of chromium as presented in Table 2.1 above; the bioavailability of this element from these sources is unknown. In addition, the level of chromium in the basal diet is changeable depending on the level of the included materials. In this study, the un-supplemented diet (with a background level of chromium) will be considered as a control diet; while other treatments will represent the level of chromium added. Other ingredients such as chromium compounds that used as sources of chromium in the experimental diets are mentioned in the relevant experimental chapters.

2.3 Diets preparation procedure

All the dry ingredients were weighed and gradually mixed together using a Hobart food mixer (Cater-Bake, UK) followed by the addition of the oil. Warm water was added to reach a consistency suitable for cold extrusion to form small size pellets (2 mm diameter) using a PTM Extruder system (Model P6, Plymouth, UK). Diets were air dried in an oven at 45 ºC for 24 hour. Later, each diet placed in plastic containers, labelled, and stored in dry and cold place until used. The formulations and proximate compositions of the experimental diets used in each individual experiment, along with details regarding the feeding practices, are described in the methodology of the appropriate chapter for each experiment.

During the acclimation period (4-6 weeks), fish were reared in five fibreglass tanks (71 L capacity) and fed 2% of their body weight twice a day a commercial diet -EWOS Sigma 50- which consist of 230 g kg^{-1} oil, 450 g kg^{-1} protein, 10 g kg^{-1} fibre, 100 g kg^{-1} ash, 10 000 IU kg^{-1} vitamin A, 1750 IU kg^{-1} vitamin D and 240 IU kg^{-1} vitamin E.

2.4 Experimental fish and husbandry

All experimental work involving animals was carried out in accordance with the 1986 Animals Scientific Procedures Act under the UK Home Office project licence number 30/2644 and personal licence number 30/8746.

The experimental animals used in this study were common carp (*C. carpio*) for experiment I and III, and mirror carp (*C. carpio*) for experiment II. Fish were obtained from Hampshire Carp Farm Fisheries (Hampshire, UK).

The water temperature was set at 25 ± 0.5 °C and there was daily monitoring of the pH and dissolved oxygen levels in water system using an HQ40d pH and dissolved oxygen multi-parameter meter (HACH Company, Loveland, USA) and weekly monitoring of NH3, NO2,- and NO3- using a Nutrient Analyser (SEAL AQ2 Analyser, Hampshire, UK).

The normal city mains water was used in the rearing system where the level of Cr is negligible; however, there was a regular measurement of the Cr concentration in water system using an ICP-OES instrument. The water quality was maintained by changing the mechanical filters and partially changing the water every day. A photoperiod of 12 h light and 12 h dark was used throughout the experiments.

The experimental system consists of 18 closed tanks (28 × 44 × 55 cm; 72 L capacity for each tank) and flow rate of water to each tank was 3 L min^{-1}. The tanks were thoroughly cleaned every week when the fish were removed for weighing. Figure 2.2 presents the design of the experimental system used in all experiments.

Figure 2. 2 The recirculation system used in all experiments (located at Plymouth University, Fish Nutrition research unit).

2.5 Fish weighing and sampling

Throughout the feeding trials, the fish in each tank were re-weighed every week collectively after one day of feed deprivation and daily ration adjusted accordingly (this was in general held at an average of 3% body weight per day). At the end of the experiment, the same weighing procedure has been applied for the final growth performance evaluation. The anaesthetisation of fish for sampling purposes was carried out using tricane methanesulfonate (MS-222) (Pharmaq Ltd. Hampshire, UK) at a concentration of 0.2 g l^{-1} and buffered with sodium bicarbonate to keep the pH at the neutral level.

After the experimental procedure, the fish were placed in aerated water taken from the experimental system and allowed to recover for a few minutes before being returned to the original tank in the experimental system. Where fish required to be sacrificed for samples collection (liver and gut tissues) they were first anaesthetized and then killed with a sharp blow on the head.

Tissue samples (livers and gut) were dissected out and placed in appropriate containers i.e. cryogenic tubes (for enzyme assay), glass containers (for histology procedure), or plastic tubes (for tissue Cr content) and immediately stored in a proper conditions as described in the appropriate section of the methodology chapter.

2.6 Growth performance calculations

Percentage weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), and protein efficiency ratio (PER) were calculated using the following formulae:-

1- % weight gain (WG):-

$$\%WG = \frac{FBW - IBW}{IBW} \times 100$$

FBW = final body weight of fish

IBW= initial body weight of fish.

2- Specific growth rate (SGR):-

Specific growth rate is defined as the increase in live weight per unit time. Growth of fish under the culture conditions where food is always available, my closely approximate an exponential curve.

$$SGR = \frac{(ln\ FBW\ -\ ln\ IBW)}{number\ of\ days} \times 100$$

ln FBW =natural log of the final body weight of fish

ln IBW= natural log of the initial body weight of fish

$$FCR = \frac{feed\ intake\ (g)}{wet\ weight\ gain\ (g)}$$

3- Feed conversion ratio (FCR):-

Feed conversion ratio is a numerical value used to measure the degree of gross utilization of food for growth in animals. The lower FCR is, the more efficiently the feed is being utilized by fish. It is usually described by the following equation:-

4- Protein efficiency ratio (PER):-

In this parameter, growth is used to measure the nutritive value of dietary protein.

$$PER = \frac{Weight\ gain\ (g\ wet\ fish)}{weight\ of\ protein\ fed\ (g\ crude\ protein\ fed)}$$

2.7 Proximate analysis of diets and whole fish tissues

Before starting each trial, sufficient amount of the experimental diets were sampled for proximate analysis. Also before starting each trial, 18 fish from the whole population were collected for initial body composition analysis. These fish have been divided into three groups (6 fish in each group) to be analysed as triplicate. At the termination, two fish per tank

(6 per treatment) were sampled to determine carcass composition. Chemical composition of the experimental diets and fish carcass were determined according to the standard techniques stated by the Association of Official Analytical Chemists (AOAC) (2002).

2.7.1 Moisture

For moisture content determination, feeds and whole fish carcass samples were thermally dried to constant weight in an oven at 110 °C for 24 hour. Specifically, 3.0g of the ground feed samples were placed into a pre-weighed dish and dried in an oven at 110°C. The whole fish samples weighed onto a pre weighed dish and dried in the oven as described above until constant weight was obtained. After removing the samples from the oven they were placed in a desiccator to cool and reweighed again.

The moisture content of the samples was calculated as follows:

$$Moisture\ (\%) = \frac{sample\ weight\ (g) - \ dried\ sample\ weight\ (g)}{sample\ weight\ (g)} \times 100$$

2.7.2 Crude protein

The Kjeldahl method was used to determine the nitrogen content of diets and whole fish samples. This standard procedure was applied using Kjeldatherm, Gerhardt (Germany). Briefly, 100-200 mg of sample was weighed into a Kjeldahl digestion tube and one catalyst tablet and 5ml concentrated sulphuric acid were added. Then, the tubes were placed into the digestion block. Changing the colour of samples to emerald green signals an end of the digestion step and the final result is ammonium sulphate solution. The tubes were removed from the block and left to cool inside a fume cupboard for at least 15 minutes. The second step is a distillation phase using the Vapodest 50_S Gerhardt (Germany). This step aims to produce ammonia gas by adding excess base to the acid digestion mixture. The final step is a titration to quantify the amount of ammonia in the receiving solution. All samples were

analysed in triplicate. Similarly, for each batch of samples, three casein standard and three blank tubes were prepared and analysed.

The protein content of the samples was calculated as:-

$$Protein\ (\%) = \frac{(sample\ titre - blank\ titre) \times\ 0.1 \times 14 \times 6.25}{sample\ weight\ (mg)}$$

Where: 0.1= molarity of acid, 14= relative molecular mass of nitrogen, 6.25 = animal protein: nitrogen constant.

2.7.3 Crude lipid

Crude lipid was estimated by the Soxtec method using petroleum ether on a Soxtherm System Gerhardt (Germany). Briefly, 2-3 g of ground sample was placed into a Soxtec extraction thimble. A Soxtec extraction cup was weighed and 140 ml of petroleum ether were added. The thimble was moved to the Soxtec System and boiled in the solvent for 30 minutes. After the extraction, the extraction cup was removed and placed into an oven at 110°C for solvent evaporation. After 15 minutes, the extraction cup was removed from the oven and left to cool. Crude lipid was quantified by reweighing the extraction cup. All samples were analysed in triplicate.

$$\%\ Fat = \frac{final\ weight\ of\ cup - initial\ weight\ of\ cup}{initial\ weight\ of\ samples} \times 100$$

2.7.4 Ash

The ash content of diets and fish tissues was determined by dry ashing approximately 2-3 g of sample in a porcelain crucible in a muffle furnace at 550 °C overnight. After ashing, the samples were removed from the furnace, cooled to room temperature in a desiccator and then reweighed.

The ash content was calculated as follows:

$$\%\ Ash = \frac{(weight\ of\ crucible\ + residue) - weight\ of\ crucible(g)}{sample\ weight\ (g)} \times 100$$

2.8 Cr analysis in whole fish tissue, ingredients and diets

Two fish per tank (6 per treatment) were collected to determine Cr carcass content. Dietary Cr analysis and the concentration of Cr in whole fish tissue were undertaken according to AOAC (2002) with slight modifications. Briefly, 0.5 g of dried, homogenized samples was weighed into a Tecator tube. Concentrated nitric acid and hydrochloric acid (4 ml of each) were added to each tube. The tubes were left at room temperature for two hours for the contents to digest slowly and then they were transferred to a Tecator hot block and heated to 200-250 ºC for 1.5 h. The blank and reference samples were prepared following the same procedure. The reference used in Cr analysis is DORM-2 (dogfish reference muscle) which is produced by the National research Council Canada, Ottawa, Canada. It has a certified value for Cr of 34.7 ± 5.5 mg kg^{-1}. After digestion, the cold samples were diluted to 25 ml in clean volumetric flasks. A stock solution of 10,000 mg L^{-1} Cr (Aristar, BDH, Poole, UK) where Cr was present as ammonium dichromate salt was used to prepare the standards by serial dilution. The concentration of chromium was determined using an ICP-OES instrument (Varian 725-ES, Australia) or an Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Thermo Scientific, X Series 2, Hemel Hempstead, UK), depending on the concentrations found in the samples.

Calculations

The results were calculated as follows:-

$$= \left[\frac{\text{The ICP result (µg/l)} \times \text{Volume of diluted sample (ml)}}{\text{weight of sample used (g)}}\right] /1000$$

The results were expressed as mg kg^{-1} or µg kg^{-1} dry weight.

2.9 Cr accumulation in liver and gut tissues

The procedure of AOAC (2002) for metal analysis was followed to determine Cr concentration in liver and gut tissues. Six fish from each treatment were sampled for whole liver and gut chromium content analysis. The samples were dried in an oven at 100 ºC for 24 h until a constant weight was achieved. Dry samples (0.1 g) were then transferred into scintillation vials and digested in 4 ml concentrated nitric acid for 2 h at 120 ºC in a water bath. After cooling, the solutions were made up to 16 ml with distilled water ready for analysis using (ICP-OES) as described previously.

2.10 Plasma glucose level measurement

Plasma glucose concentrations were measured at the end of each experiment after 24 h of last feeding time by the glucose oxidase method (Braham and Trinder, 1972) on six individual fish per treatment. A VersaMax microplate reader (Molecular Devices) Sunnyvale, California, USA was used to read the absorbance at 505 nm. All the chemicals used in plasma glucose determination (as mentioned below in the equations) were provided from Sigma Aldrich Company (Poole, UK).

The enzymatic reactions involved in the assay are as follows:

$$Glucose + H_2O_2 \xrightarrow{Glcose\ oxidase} Gluconic\ acid + H_2O_2$$

$$H_2O_2 + 4 - Aminoantipyrine + p - Hydroxybenzene\ sulfonate \xrightarrow{Peroxidase} Quinineimine\ dye + H_2O_2$$

Whole blood was collected via the caudal vein into 1 ml heparinized syringes. The blood was centrifuged at 4000 xg for 5 minutes and the resultant plasma was collected in Eppendorf tubes, labelled and stored at -20 ºC until analysis.

2.11 Enzyme activity measurements

Six fish per treatment were randomly selected and anaesthetized with buffered MS-222. These fish were dissected to obtain the liver which were immediately removed and stored in cryogenic tubes and frozen in liquid nitrogen, then moved to store at -80 °C until analysis. All the buffers used for liver extraction were prepared according to Enes et al. (2008a). After the homogenization and centrifugation processes for each assay, the resultant supernatant was removed and stored at -80 °C for enzymatic analysis.

All enzyme assays were performed in triplicate monitoring the absorbance at 340 nm using a VersaMax microplate reader (Molecular Devices) Sunnyvale, California, USA. The assay temperature was maintained at 37 ^{o}C. The results from the microplate reader were analysed using SigmaPlot program (version 12) for the replication separately to get the result in µmol min^{-1} ml^{-1} of the liver extract. The concentration of the protein in the same extract was measured for each sample using the Bradford assay (Bradford, 1976) to obtain the final results of enzyme activity as U mg^{-1} protein.

The Bradford protein protocol is a colorimetric assay for measuring total protein concentration in a solution. It involves the binding of the Brilliant Blue dye to protein in an acidic solution and the absorbance was recorded at 595 nm. Bovine Serum Albumin (BSA, Sigma-Aldrich, UK) was used to prepare the protein standard curve.

One unit of enzyme activity is defined as the amount of enzyme that catalyzed the hydrolysis of 1 µmol of substrate per minute at the assay temperature.

2.11.1 Hexokinase (HK 2.7.1.1)

Principle

$$Glucose + ATP \xrightarrow{Hexokinase} Glucose\ 6-Phosphate\ + ADP + H^{+}$$

$$Cresol\ Red\ + H^{+} \rightarrow Reduced\ Cresol\ Red$$

Frozen liver was homogenized (1 g of tissue: 9 ml of buffer) in ice-cold buffer (80 mM Tris buffer, pH 7.6; containing 5 mM EDTA; 2 mM dithiothreitol; 1 mM benzamidine; 1 mM 4-(2-aminoethyl) benzenesulphonyl fluoride). After centrifugation (900 g for 10 min), Hexokinase (HK; EC 2.7.1.1) activity was measured in the extracts essentially according to Bergmeyer et al. (1983) except that the reaction cocktail volume was 250 µl and 10 µl of sample was used.

2.11.2 Glucose-6-phosphate dehydrogenase (1.1.1.49)

Principle

$$D-Glucose\ 6-Phosphate + \beta-NADP \frac{G-6-PDH}{Mg^{2+}} 6-Phospho-D-gluconate + \beta-NADPH$$

G-6-PDH = Glucose-6-phosphate dehydrogenase

β-NADP = β-Nicotinamide Adenine Dinucleotide phosphate, oxidized form

β-NADPH = β-Nicotinamide Adenine Dinucleotide phosphate, reduced form

Frozen liver samples were homogenized (1 g of tissue: 4 ml of buffer) in ice-cold buffer (0.02 M Tris buffer, pH 7.4, containing 0.25 M sucrose; 2 mM EDTA; 0.1 M sodium fluoride; 0.5 mM phenyl methyl sulphonyl fluoride (PMSF); 0.01 M β-mercaptoethanol Homogenates were centrifuged at 30,000 g for 20 min. Glucose-6-phosphate dehydrogenase (G6PD) activity was measured in the liver extracts by following the reduction of $NADP^{+}$ to NADPH according to Barman (1969) with a reduction in the final volume size of the reaction cocktail to 250 µl and with 10 µl of sample.

2.12 Histological examination

For histological assessment, tissues will go through several steps in order to obtain a suitable structural format ready for sectioning and diagnostic purposes (Table 2.2). The livers and the mid-section of the intestine were collected from each fish and stored separately in a pre-labelled glass vial that had been pre-filled with 10% neutral buffered formalin (NBF). The tissues were fixed in NBF for a week and then dehydrated automatically using an automatic tissue processor (Leica Microsystem TP 1020, Germany). In the latter step, small pieces of tissues were passed through a series of water/alcohol solutions to absolute alcohol and then through a HistoClear (xylene). Later, tissues were embedded in melted paraffin to provide a matrix that can support the tissues during sectioning. Finally, blocks cut into 7 µm slices using Leica Microsystem microtome model RM2235 (Germany) and finally stained with Hematoxylin and Eosin (H & E) by Leica Microsystem auto strainer XL, Germany.

Table 2. 2 Tissue processing steps for histological assessment.

Process	**Fixation**	**Dehydration**	**Clearing**	**Infiltration**
Reagents	10% neutral buffered formalin	70% ethanol 90% ethanol 100% absolute	Xylene	Paraffin
Action	Stabilize protein	Removal of water	Removal of alcohol	Interpenetration

Histological examination was performed on samples of two individuals taken from each tank (six fish per treatment) at the end of the experiment. Three small pieces from each liver and mid-gut sample were selected randomly and placed in the same cassette and processed together as described above. Later, these pieces were embedded in the same paraffin block.

Three slides were prepared from each block and the sections were collected from different locations in the block to be representative for the whole tissue. Qualitative assessment for the histological observation was performed to compare the differences between the experimental groups.

2.13 Statistical analysis

All results in figures belong to experiment I and II are expressed as mean values ± standard error (±SE). Statistical analyses were conducted using SPSS statistics version 18 for windows (SPSS Inc., Chicago, IL, USA). Data analysis was performed using one-way ANOVA. Post hoc LSD test was used to identify where significant differences occurred at the 95 % confidence level (associated probability < 0.05). Statistical analysis of the data for experiment III is mentioned in the relevant experimental chapter.

Chapter 3

Experiment I

Dietary chromium requirement of common carp

- Results from this chapter have been published in Journal of Trace Element in Medicine and Biology.

  Arafat R. Ahmed, A. John Moody, Andrew Fisher, Simon J. Davies (2012). Growth performance and starch utilization in common carp (*Cyprinus carpio* L.) in response to dietary chromium chloride supplementation. Trace Element in Medicine and Biology. DOI: 10.1016/j.jtemb.2012.05.006
- The same study has been presented at Alltech's 26th Annual International Symposium, May 2010, USA.
- The same study was presented at the School of Biomedical and Biological Sciences under the seminar programme, May 2011, University of Plymouth, UK.
- Results from this chapter have been presented at the 8th Marine Biological Association Postgraduate Conference (Oral presentation), May 2011, Queen's University, Belfast, Northern Ireland.

Abstract

A nutrition trial was conducted on juvenile common carp (*C. carpio*), initial mean body weight 15 ± 0.4 g under a controlled facility at 25 ± 0.5 ºC. Six diets containing various levels of supplementary Cr (0, 0.2, 0.5, 1.0, 1.5, and 2.0) mg Cr kg^{-1} of diet as Cr chloride hexahydrate were fed to carp for a period of 10 weeks. Lower growth performance was observed in fish fed on the control diet and the diet supplemented with the highest level of Cr (2.0 mg Cr kg^{-1}). Although fish fed 0.5 mg Cr kg^{-1} showed the best growth performance, this was not significantly different ($P > 0.05$) from fish fed 1.0 mg Cr kg^{-1}. The regression of plasma glucose concentration was linear ($R^2 = 0.97$ and P value = 0.001) as the Cr content of the diet increased (up to 1.5 mg Cr kg^{-1}).

Cr carcass content was elevated with an increasing level of dietary Cr supplementation up to 1.5 mg Cr kg^{-1}; but fish fed on the diet supplemented with the highest level of Cr (2.0 mg Cr kg^{-1}) showed a decrease in Cr carcass content.

Histological examination to evaluate the impact of different Cr supplementation on liver and gut tissues showed notable changes. The higher level of Cr (2.0 mg Cr kg^{-1}) in the diet gave rise to elevated hepatocyte vacuolization and changes in gut tissue morphology.

It appeared that Cr chloride significantly improved growth within a defined range (0.2-1.5) mg Cr kg^{-1} without any negative impact, while 2.0 mg Cr kg^{-1} in carp diet seems to be the threshold for the initiation of toxicity.

Chapter 3: Dietary chromium(III) requirement of common carp using the carp practical diet

3.1 Introduction

Using carbohydrates as a source of energy in fish diets could reduce the cost of feed and lower the organic pollution resulting from the catabolism of proteins in intensive aquaculture systems (Moon, 2001). However, fish species differ in their ability to utilize dietary carbohydrate and generally have poor regulation over blood glucose levels (Krogdahl et al., 2005).

As discussed in the "General introduction chapter", numerous studies have attempted to explain the poor utilization of dietary carbohydrate in fish. For example, Hertz et al. (1989) suggested that the sensitivity of the glucose regulatory system to the levels of protein and amino acids is more than to levels of circulating plasma glucose, while Parrizas et al. (1994) reported that insulin receptor levels in fish muscle are relatively low compared to mammals. The poor ability could be due to reduced glucose phosphorylation capacity (Cowey and Walton, 1989). In this context, Wilson (1994) has suggested that the level of hexokinase is low in all fish species, but another possibility is a lack of glucose transporters in fish muscle (Wright et al., 1989). At the level of digestion, the poor utilization of carbohydrate might also be the result of low levels of digestive enzymes in different fish species, e.g. it has been reported that amylase activity in the digestive tract of fish is lower than that in terrestrial animals (Takeuchi et al., 2002).

Trivalent chromium is an essential mineral that plays a key role in modulating carbohydrate, lipid, and protein metabolism in mammals (Anderson, 1998). The nutritional biochemistry of chromium (Cr) has gained considerable interest in recent years for humans (Vincent, 2000b; 2004; Nelson, 2007) and many species of farmed animals (Lee et al., 2000; Shelton et al., 2003; Ahmed et al., 2005a; Eseceli et al., 2010). However, studies with fish are fairly limited

due to the technical difficulties associated with trace element investigations in aquatic environments (Lall, 2002). The mechanisms by which chromium can affect carbohydrate metabolism are not fully understood. However, Pechova and Pavlata (2007) reported that chromium is biologically active as a part of an oligopeptide (chromodulin, also known as low molecular weight chromium binding substance) which has the ability to potentiate the action of insulin and facilitate insulin binding to its receptors at the cell surface. Further, chromium inhibits phosphotyrosine phosphatase enzyme which activates cleavage of phosphate from the insulin receptor, leading to decrease in insulin sensitivity (Cefalu and Hu, 2004). In contrast, chromodulin has been found to activate the insulin receptor kinase activity, the enzyme that stimulates the transfer of a phosphate group to the insulin receptor (Vincent, 2004). As a result, the activation of the insulin receptor kinase and inhibition of the insulin receptor phosphatase would lead to increased phosphorylation of the insulin receptor and thus an increase in insulin sensitivity (Davis et al., 1996; Anderson, 1998).

The supplementation of different chromium compounds in animal diets has produced remarkable benefits. The inorganic form as chromium chloride has been widely used as a source of chromium in animal studies with positive effects on growth performance and nutrient utilization in broiler chickens and on the immune response of quail chicks and calves (Kegley et al., 1996; Ahmed et al., 2005; El-Hommosany, 2008). The organic compounds such as chromium picolinate (CrPic) are suggested to be more bioavailable and relatively well absorbed form of chromium (Anderson et al., 1996). CrPic supplementation increased weight gain and improved carbohydrate metabolism in Goto-Kakizaki (GK) diabetic rats (Kim et al., 2004). Other benefits were reported from inclusion of chromium yeast in the feed of laying hens, including decreased food consumption, increased feed conversion and decreased egg yolk cholesterol (Eseceli et al., 2010).

In fish studies, it has been reported that chromium supplementation has a positive influence on growth performance in carp (Liu et al., 2010) and tilapia (Shiau and Liang, 1995; Shiau and Shy, 1998; Pan et al., 2002), and improves the immune response, decreases serum glucose and cholesterol levels in the blood of rainbow trout (*Oncorhynchus mykiss*) (Gatta et al., 2001; Küçükbay et al., 2006). Surprisingly, the insoluble inorganic form of chromium-chromium oxide-which is used as an indirect marker for measuring nutrients digestibility values, has been found to be a stimulator of glucose utilization and growth improvement in tilapia more so than chromium chloride and sodium dichromate (Shiau and Chen, 1993). In contrast, other studies on rainbow trout (Tacon and Beveridge,1982; Selcuk et al., 2010), and with gilthead sea bream (*Sparus aurata*) (Gatta et al., 2001a) and tilapia (Pan et al., 2003) were unable to determine any significant effects of chromium supplementation on either growth performance or feed utilization.

The scientific literature regarding the functional role of chromium in fish is fraught with conflicting results which merit further studies. It is important to obtain data on the potential benefits of chromium supplementation for carp, since carp have economic value in many countries and also have the distinction of being omnivorous in nature with a high capacity for carbohydrate assimilation (Stone, 2003). As there is little reliable evidence in the literature on the effects of chromium in carp, the aim of this study was to test the following hypothesises:

Hypothesis 1: Cr(III) supplementation affects growth performance in carp at a specific level when Cr acts as an essential trace element.

Hypothesis 2: Cr(III) affects starch utilization by affecting the activity of some key liver enzymes related to carbohydrate metabolism.

Hypothesis 3: Dietary Cr(III) affects the whole body composition (moisture, protein, lipid, and ash content).

3.2 Materials and methods

3.2.1 Growth trial protocol

The aim of this study was to determine chromium requirement of carp using a practical diet.

The table below shows the growth trial experimental protocol.

Table 3. 1 Experimental protocol for growth trial.

Experimental fish	Common carp
Stocking density	15 fish in each tank.
Acclimation period	4 weeks, the fish fed EWOS Sigma diet (please see chapter 2 for the diet ingredients).
Diets	6 diets containing different levels of chromium (0-2.0 mg Cr kg^{-1}).
Chromium source	Inorganic compound (chromium chloride $CrCl_3.6H_2O$).
Duration of study	10 weeks.
Feeding regime	3% of body weight four times a day.
Parameters tested	Growth parameters, initial and final body composition, chromium accumulation, plasma glucose level, key liver enzymes (HK, G6PD, and ALT), gut and liver histology.

3.2.2 Diet ingredients and preparation

Ingredients and proximate composition of experimental diets are presented in Table 3.2. The ingredients sources and the diets preparation procedure were as described in Chapter 2. The basal diets were supplemented with 0.0, 0.2, 0.5, 1.0, 1.5, and 2.0 mg Cr kg^{-1} of diet as chromium chloride hexahydrate ($CrCl_3.6H_2O$). The required concentration of chromium has been dissolved in 500 ml of distilled water and gradually mixed with the other ingredients to produce a uniform mixture.

Table 3. 2 Formulation and chemical composition of experimental diets.

	Diets					
Ingredients (g kg^{-1})	**Control**	**0.2**	**0.5**	**1.0**	**1.5**	**2.0**
Herring meal LT94	250	250	250	250	250	250
Maize starch	385.2	385.2	385.2	385.2	385.2	385.2
Pea protein	164.5	164.5	164.5	164.5	164.5	164.5
Corn gluten	150	150	150	150	150	150
Sunflower oil	33.3	33.3	33.3	33.3	33.3	33.3
Mineral premix	20	20	20	20	20	20
*Cr analyzed (mg kg^{-1})	0.68	0.93	1.10	1.65	2.0	2.49
Proximate composition (g kg^{-1})						
Moisture	43.0±2.0	43.6±1.7	42.8±0.8	42.9±3.2	42.3±2.6	43.5±1.3
Protein	382.1±2.3	383.2±4.5	383.1±2.0	381.9±3.7	382.6±1.4	381.5±3.4
Lipid	73.6±0.8	72.7±1.7	73.2±3.6	73.9±0.5	72.4±2.1	73.5±2.6
Ash	55.0±0.4	55.7±1.0	55.2±2.4	56.2±1.9	56.5±0.7	55.2±1.1

*$CrCl_3$.6H2O: Sigma-Aldrich, UK. Proximate composition values are mean ± SE, n=3

3.2.3 Experimental fish and husbandry

Prior to the feeding trial, fish were acclimated and fed daily as described previously in Chapter 2. At the beginning of the trial, batches of 15 fish (average initial body weight 15 ± 0.4 g fish^{-1}) were distributed into 18 tanks (tanks dimensions and the system maintenance procedure were described in the General materials and methods (Chapter 2).

The fish were fed four times a day to ensure that the diet will completely consumed by the fish in order to avoid any accumulation of feeds in the system.

During the experimental period (10 weeks), the water temperature was set at 25 ± 0.5 °C and there was a regular monitoring of the pH (6.8-7.5), NH_3 (0.01-0.06 mg L^{-1}), NO_2^- (0.006-0.02 mg L^{-1}) and NO_3^- (21.6-71.7 mg L^{-1}); and dissolved oxygen levels ranged between 92.3 and 95.3 %, while the concentration of Cr in the system was 0.05-0.07 µg L^{-1}.

3.2.4 Fish weighing and sampling

Fish weighing, anaesthesia, and sampling procedure were as described in Chapter 2.

3.2.5 Growth performance calculation

To evaluate growth performance of fish at the end of feeding trial, growth performance equations described in Chapter 2 have been applied.

3.2.6 Proximate analysis of the diets and whole fish tissues

Moisture, crude protein, crude lipid, and ash determination were described in Chapter 2.

3.2.7 Cr analysis in the diets and whole fish tissue

The description of Cr analysis in the experimental diets and whole fish tissues were as described in Chapter 2.

3.2.8 Cr accumulation in liver and gut tissues

Tissues digestion and Cr analysis in liver and gut tissues were performed as described in Chapter 2.

3.2.9 Plasma glucose measurement

Number of samples, blood sampling, plasma collection and storage, and plasma glucose measurement were followed as described in Chapter 2.

3.2.10 Enzyme activity measurement

Number of samples, anaesthesia, liver collection and storage, and enzyme measurement technique and analysis conditions were as described in Chapter 2.

3.2.10.1 Hexokinase

Buffer preparation and HK measurement protocol were as described in Chapter 2.

3.2.10.2 Glucose-6-phosphate dehydrogenase

Buffer preparation and G6PD measurement protocol were described in Chapter 2.

3.2.10.3 Alanine aminotransferase (ALT 2.6.1.2)

Principle

$$a - KG + \beta - NADPH + \ NH_4^+ \xrightarrow{L-Glutamic\ Dehydrogenase} \text{L-Glu} + \beta\text{-NADP+}\ H_2O_2$$

Alpha-KG= a- Ketoglutarate

L-Glu= L-Glutamate

Frozen liver tissue was homogenized (1 part of tissue: 9 parts of buffer) in ice-cold buffer (30 mM sodium HEPES buffer, pH 7.4, containing 0.25 mM sucrose, 0.5 mM EDTA, 5 mM K2HPO4, 1 mM dithiothreitol). After centrifugation at 900 g for 10 min, the supernatants were isolated and centrifuged again at 13,000 g for 20 min. Alanine aminotransferase (ALT; EC 2.6.1.2) activity was measured in the supernatants according to Bergmeyer et al. (1983) with the slight modification that there was a reduction in the final volume of the reaction cocktail to 250 µl and 10 µl of sample was used.

3.2.11 Histology

The procedure for histological sample preparation was followed as described in Chapter 2.

3.2.12 Statistical analysis

The statistical analysis was performed as described in Chapter 2.

3.3 Results

3.3.1 Growth performance

The survival rate of the experimental fish was 100% for all the fish fed on different diets. The growth performance parameters of common carp fed diets containing different levels of chromium for 10 weeks are shown in Table 3.3. Weight gain (WG), specific growth rate (%SGR), feed conversion ratio (FCR), and protein efficiency ratio (PER) were significantly improved by Cr supplementation at different levels compared to the control except the highest level of Cr (2.0 mg Cr kg^{-1}) which caused decrease in those values. The highest weight gain was observed in fish fed 0.5 mg Cr kg^{-1} but that was not significantly different ($P > 0.05$) from fish fed 1.0 mg Cr kg^{-1}.

The highest values of SGR (2.14) and PER (3.11) with the lowest FCR value (0.95) recorded in fish fed 0.5 mg Cr kg^{-1}, while the lowest values for the same parameters of SGR (1.61) and PER (1.98) with the highest FCR value (1.46) were recorded in fish fed 2.0 mg Cr kg^{-1}.

Table 3. 3 Growth and feed utilization performance of common carp fed diets containing different levels of chromium chloride over 10 weeks.

	Diets supplemented with different levels of Cr					
Parameter	**Control**	**0.2**	**0.5**	**1.0**	**1.5**	**2.0**
IBW (g)	15.5±0.26	15.4±0.02	15.2±0.22	15.3±0.31	15.5±0.33	15.5±0.33
FBW (g)	48.30±1.48 a	63.29±1.52 b	68.32±1.12 c	65.36±1.81 bc	61.85±1.26 b	49.48±1.62 a
WG %	211.60±9.26a	310.97±9.87 b	349.47±7.38 c	327.17±11.83 bc	299.04±8.12 b	219.04±8.12 a
SGR %	1.63±0.04 a	2.01±0.03b	2.14±0.02 c	2.07±0.04 bc	1.96±0.03b	1.61±0.04 a
PER	1.86±0.08a	2.49±0.09 b	3.11±0.10 c	2.75±0.09 bc	2.73±0.07 b	1.98±0.1 a
FCR	1.48±0.04 a	1.08±0.05 b	0.95±0.03 c	1.09±0.03bc	1.07±0.03 b	1.46±0.05 a

Values are means ± SE (n= 3). In the same line, values with different letters are significantly different ($P < 0.05$).

IBW: initial body weight; FBW: final body weight; WG: weight gain

SGR: specific growth rate; FCR: feed conversion ratio, PER: protein efficiency ratio

The weekly weight gain of common carp (g) as a response to diets with various levels of chromium supplementation is presented in Figure 3.1. It is clear from the figure below that chromium requirement of common carp does not exceed 1.0 mg Cr kg^{-1} and the highest level (2.0 mg kg^{-1}) significantly ($P < 0.05$) reduced the weight gain.

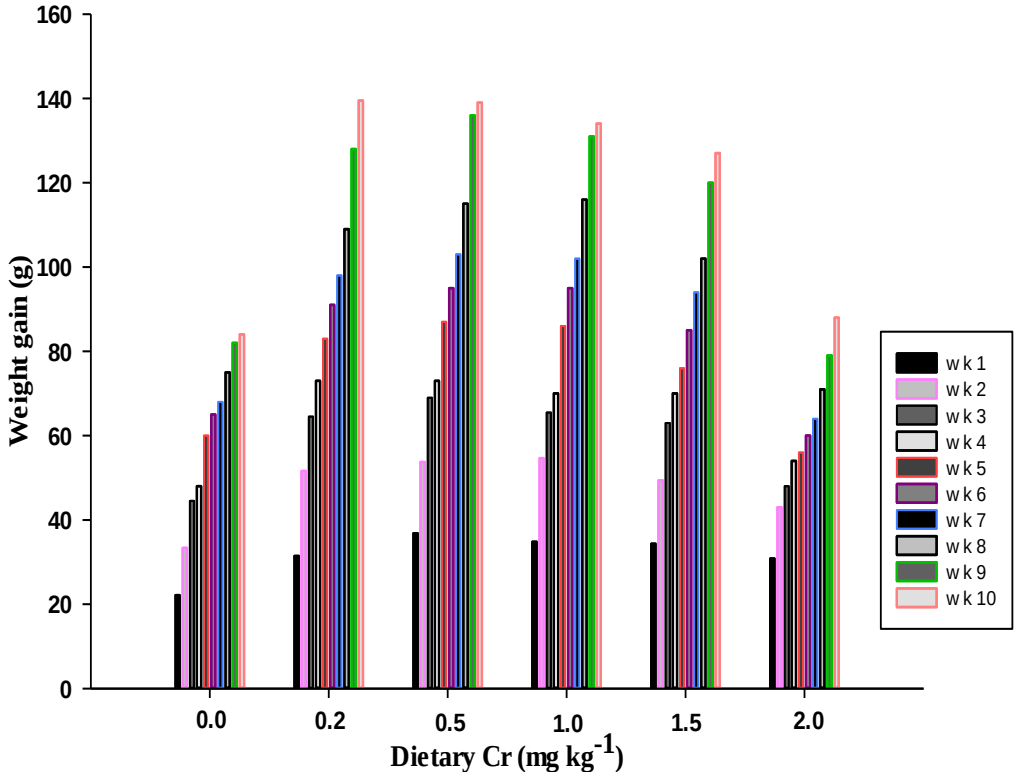


Figure 3. 1 Weekly weight gain of different fish groups fed with diets containing different levels of chromium (as chromium chloride) for 10 weeks.

The response of carp (as mean body weight) to diets with various levels of chromium supplementation is presented in Figure 3.2. Again, the lowest growth rates were observed in fish fed the control diet and fish fed the highest level of chromium (2.0 mg Cr kg^{-1}) compared to all other treatments. Although fish fed 0.5 mg Cr kg^{-1} displayed the best growth, this was not significantly different ($P > 0.05$) from fish fed 1.0 mg Cr kg^{-1}.

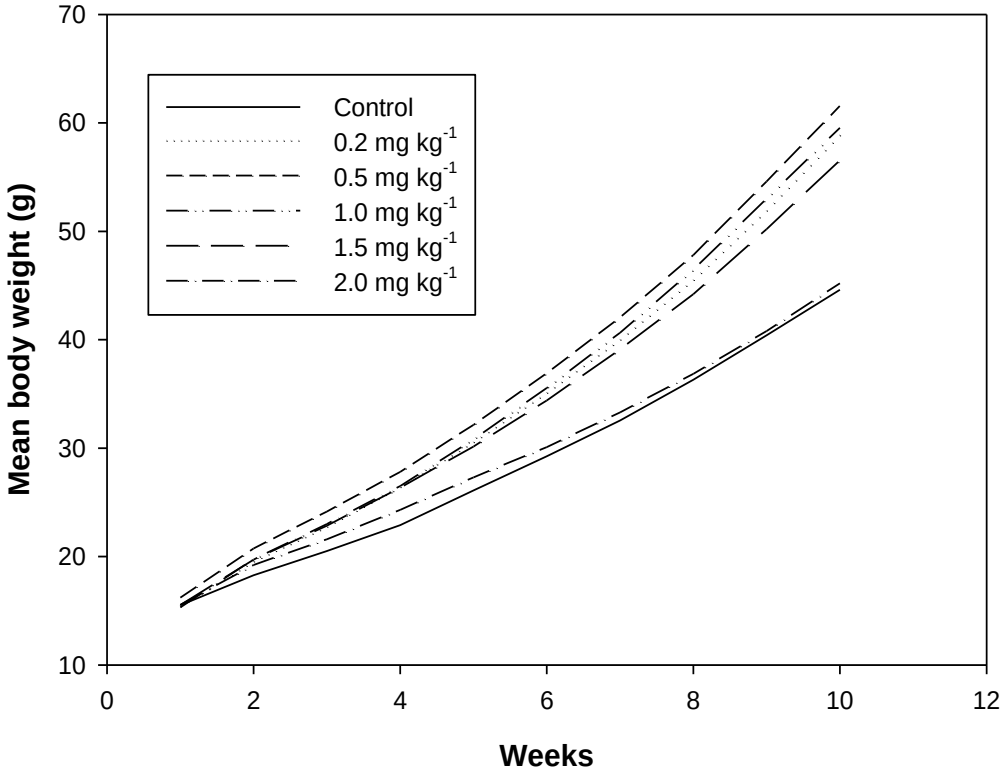


Figure 3. 2 Growth performance of carp fed with respective diets at weekly intervals over 10 weeks.

3.3.2 Proximate composition of whole fish tissue

The whole body proximate composition (moisture, protein, lipid, and ash) at the beginning and after 10 weeks of feeding on the experimental diets is shown in Table 3.4. The results showed that Cr supplementation at different levels had no effect on the final body composition.

Table 3. 4 Initial and final body composition (% wet weight) of common carp fed with different diets over 10 weeks.

		Diets					
Parameter	**Initial**	**Control**	**0.2**	**0.5**	**1.0**	**1.5**	**2.0**
Moisture	70.0 ± 0.1	70.2 ± 0.2	69.3 ± 3.1	70.7 ±0.2	69.6 ± 0.1	69.6 ±0.3	69.6± 0.3
Protein	12.4 ± 0.2	14.4 ± 0.3	14.5 ± 0.3	14.7 ± 0.1	14.4 ± 0.3	14.5 ± 0.1	14.5 ± 0.4
Lipid	10.9 ± 0.3	11.8 ± 0.1	12.0 ± 0.5	11.9 ± 0.1	11.7 ± 0.4	11.9 ± 0.3	11.7 ± 0.2
Ash	2.9 ± 0.1	2.8 ± 0.9	2.6 ± 0.2	2.4 ± 0.1	2.6 ± 0.3	2.6 ± 0.2	2.5 ± 0.2

Values are ±SE, *n*=3 per treatment.

3.3.3 Cr content in liver

The concentrations of chromium in the liver of fish fed different levels of chromium are presented in Table 3.5. No significant differences ($P > 0.05$) were detected between treatments in Cr content of liver and the values ranged from 16.06 µg Cr kg^{-1} in the liver of fish fed on the control diet and 21.14 µg Cr kg^{-1} in the liver of fish fed diet contains 2.0 mg Cr kg^{-1}.

Table 3. 5 Chromium content in the liver of fish fed with diets containing graded levels of chromium at the end of the 10 weeks.

Diets	Control	0.2	0.5	1.0	1.5	2.0
Cr content (µg kg^{-1})	16.06±3.6	16.41±4.21	18.46±2.72	17.47±2.87	17.07±5.37	21.14±6.38

Data are means ± SE (n = 2 per tank, 6 per treatment).

3.3.4 Chromium content in whole fish tissues

Figure 3.3 illustrates the concentration of chromium (mg Cr kg^{-1} dry tissue) detected in the whole fish carcass at the end of the feeding trial. Significant differences ($P < 0.05$) were found in Cr concentration in whole fish tissue. Cr concentration showed increase with an increasing the level of dietary Cr supplementation in the diet up to 1.5 mg Cr kg^{-1}; but fish fed the diet supplemented with the highest level of Cr (2.0 mg Cr kg^{-1}) showed a decrease in Cr carcass content but not significantly different from the levels detected in the whole carcass of fish fed Cr at 0.5-1.5 mg Cr kg^{-1}.

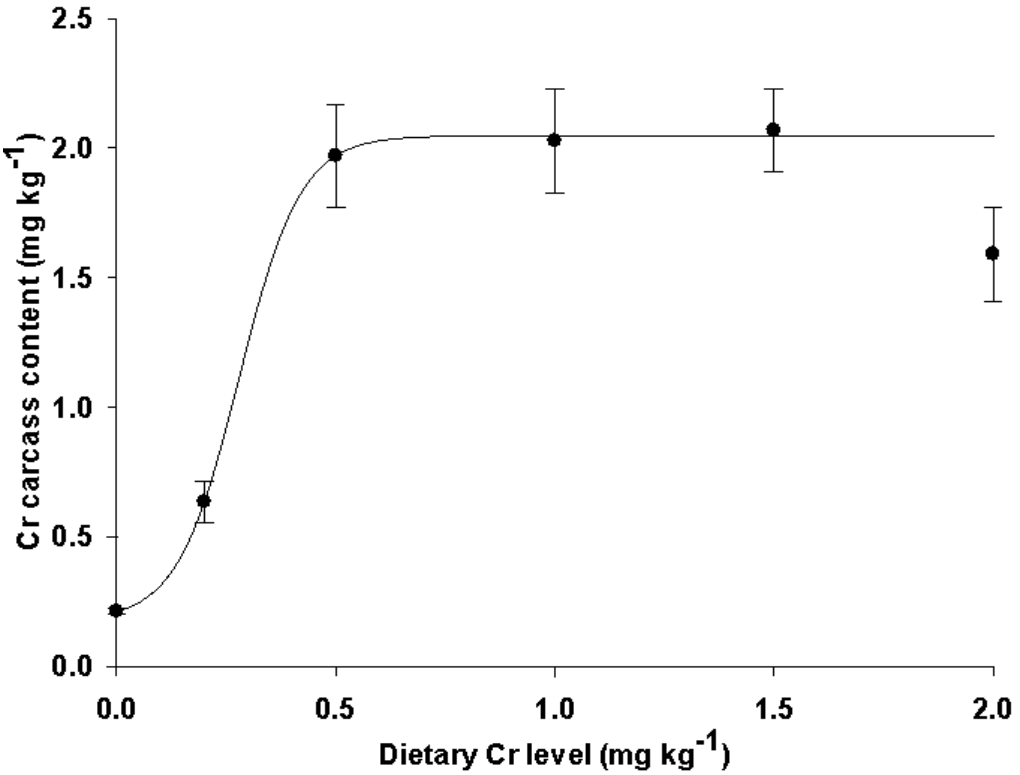


Figure 3. 3 Chromium (Cr) content of whole fish tissue (mg Cr kg^{-1}) dry weight (Data represent mean SE, *n*=6 per treatment).

3.3.5 Plasma glucose concentration

Figure 3.4 shows the levels of plasma glucose of common carp fed different diets at the end of 10 weeks. No significant differences ($P > 0.05$) were detected in plasma glucose concentration between fish groups fed diets supplemented with different levels of chromium. However, the regression of mean values up to 1.5 mg Cr kg^{-1} was linear ($R^2 = 0.97$ and P value = 0.001) showing the effect of Cr on blood glucose clearance.

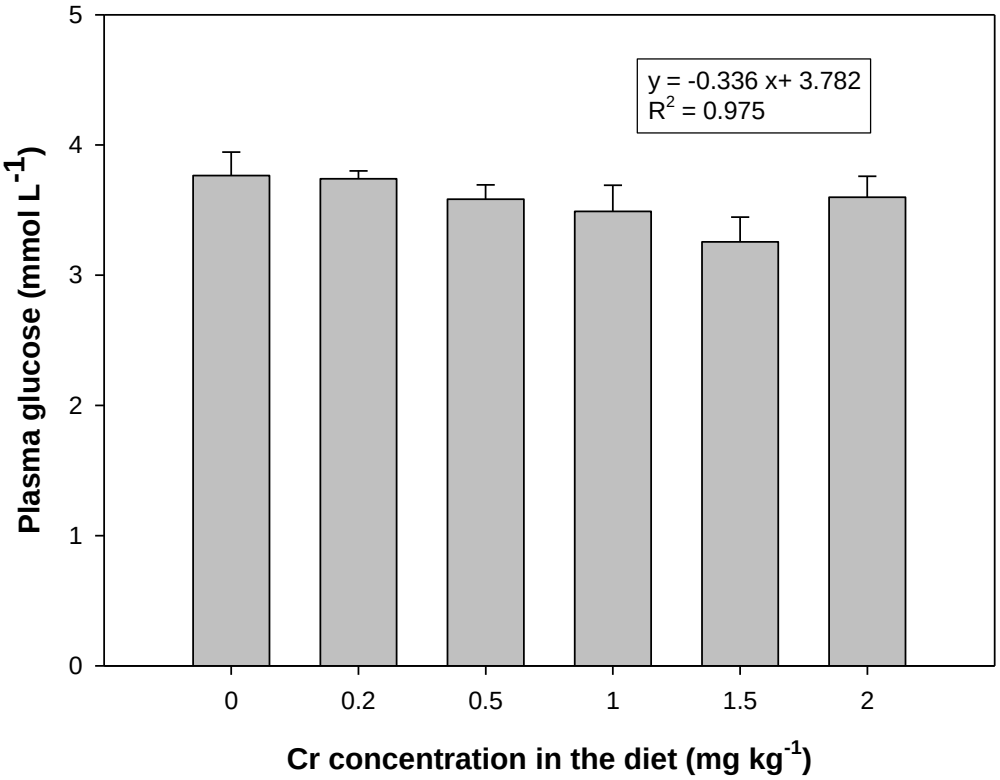


Figure 3. 4 Plasma glucose concentration (mmol l^{-1}) of carp fed each diet at the end of the experiment. (The data represent mean SE, *n*=6 per treatment).

3.3.6 Enzyme activity

The activity of the key liver enzymes (HK, G6PD, and ALT) is presented in Table 3.6. The results suggest that dietary chromium supplementation at different levels had no effect on HK, G6PD and ALT activity in the liver of different experimental groups.

Table 3. 6 Enzyme activity profiles (mU mg^{-1} protein) associated with carbohydrate metabolism for carp fed each diet after 10 weeks.

Dietary Cr supplementation (mg Cr kg^{-1})	HK	G6PD	ALT
0.0	3.03 ±0.31	96.23 ±3.35	1712 ±53.5
0.2	3.20 ±0.23	102.17 ±5.83	1807 ±61.5
0.5	3.84 ±0.36	116.75 ±7.21	1804 ±49.7
1.0	3.62 ±0.31	117.47 ±6.20	1760 ±55.8
1.5	2.87 ±0.26	97.88 ±6.04	1790 ±44.7
2.0	3.50 ±0.31	102.20 ±5.06	1789 ±53.3

Data present mean ± SE (*n*=6 fish per treatment) and *P* values were 0.24, 0.14 and 0.83 for HK, G6PD, and ALT respectively.

3.3.7 Histological examination

3.3.7.1 Liver histology

The histology of liver tissues taken from the fish fed on the experimental diets is presented in Figure 3.5. Histological examination of the liver showed vacuolization in almost all the high level of chromium (2.0 mg Cr kg^{-1}) fed fish, while a normal structure (without clear vacuoles) was found in all other groups.

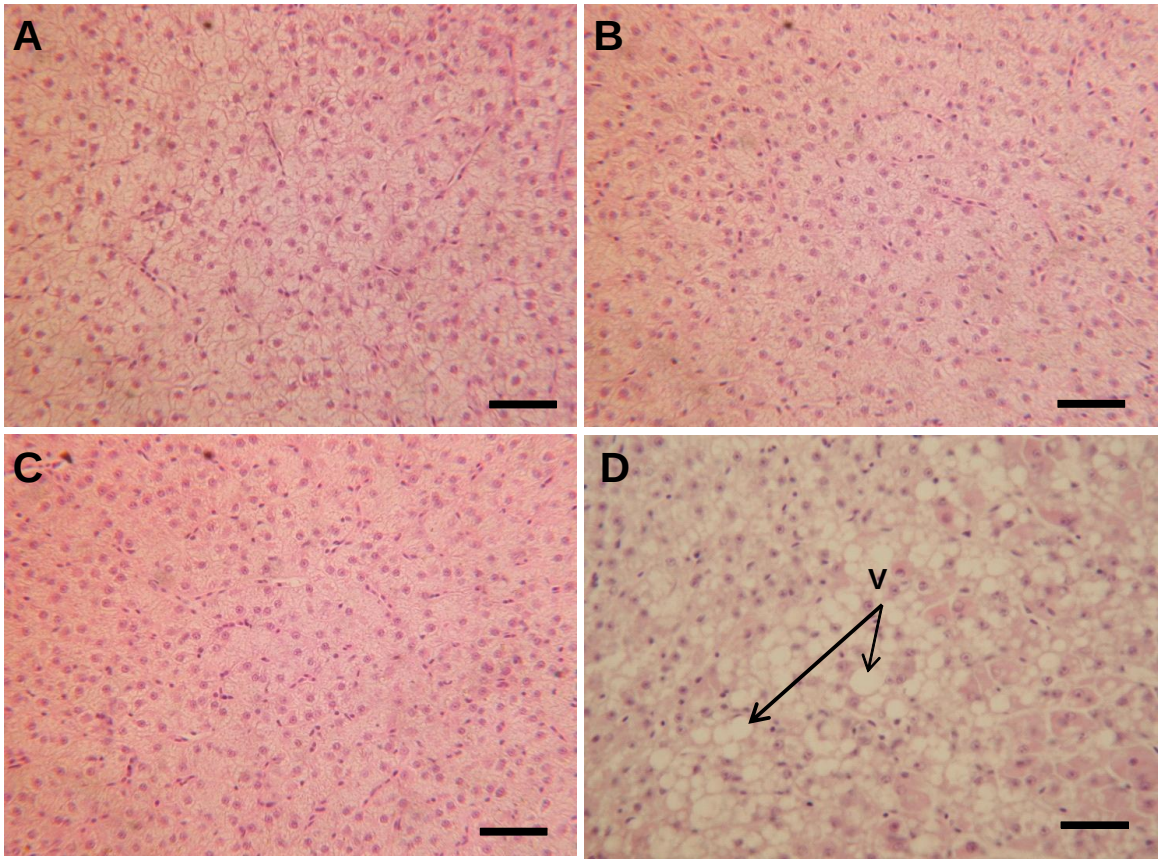


Figure 3. 5 Histology of carp liver tissue samples at the end of the experiment. (A) Liver section from carp fed on the control diet; (B) liver tissue from fish fed on 0.5 mg Cr kg^{-1}; (C) liver tissue from fish fed on 1.0 mg Cr kg^{-1} showing the normal hepatic structure, (D) liver section from fish fed on 2.0 mg Cr kg^{-1} showing liver vacuolization (V). Sections were 7µm thickness and stained with H&E. n= 6 fish per treatment.

*Images for 0.2 and 1.5 mg Cr kg^{-1} treatments are not included

3.3.7.2 Gut histology

Figure 3.6 illustrates the histology of gut sections taken from fish fed the experimental diets. The highest level of Cr (2.0 mg kg^{-1}) in the diet induced a range of different changes in the mid-section of the carp intestine including erosion, necrosis, hyperplasia and tip fusion in three out of six samples (each sample has the same abnormal signs compared to the control); while no histological changes were observed in the mid-section of intestine of fish fed on the other diets.

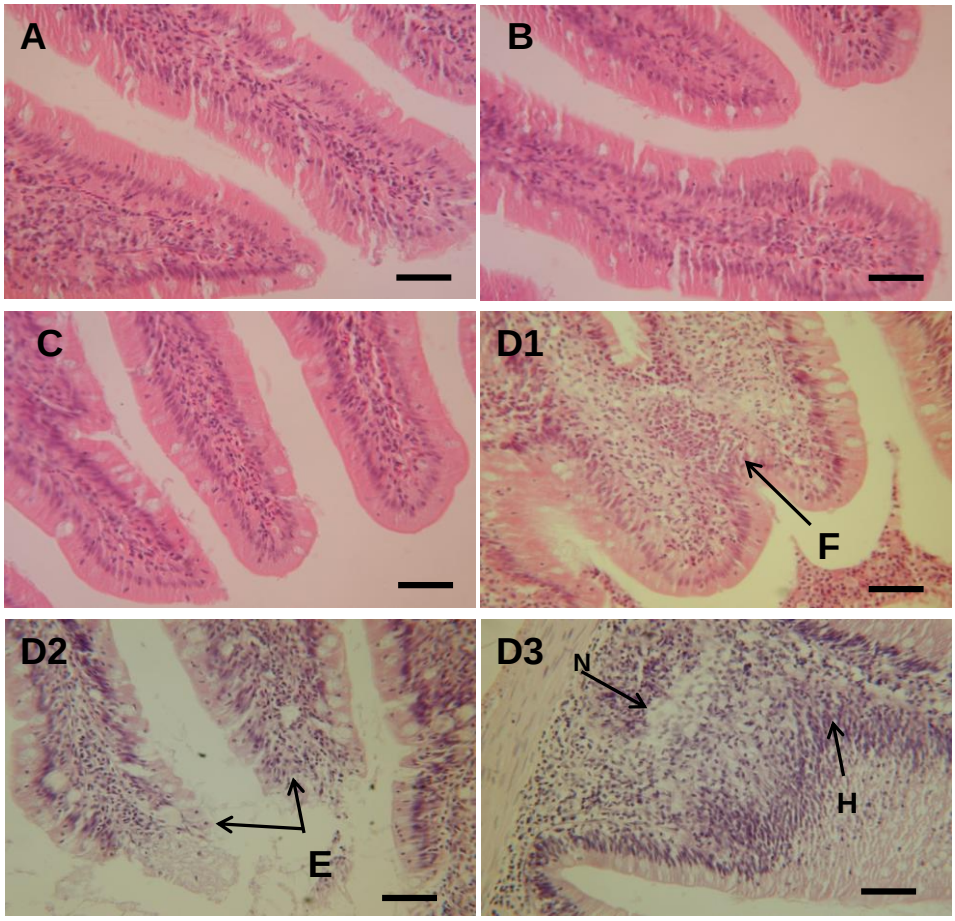


Figure 3. 6 Histology of carp mid intestine section at the end of the experiment (A) Common carp fed on the control diet; (B) fish fed on a diet contains 0.5 mg Cr kg^{-1}; (C) fish fed on a diet contains 1.0 mg Cr kg^{-1} depicting normal intestine features; (D1) Section from fish fed 2.0 mg Cr kg^{-1} showing tip fusion (F); (D2) Intestine from fish fed 2.0 mg Cr kg^{-1} illustrating tip erosion (E); (D3) Intestine from fish fed 2.0 mg Cr kg^{-1} showing necrosis (N) and hyperplasia (H). Sections were 7µm thickness and stained with H&E. n=6 fish per treatment.

*Images for 0.2 and 1.5 mg Cr kg^{-1} treatments are not included

3.4 Discussion

The present investigation with common carp shows the effects of supplementing a practical basal diet formulation (containing a background level of trivalent Cr) with Cr supplementation over a defined range from 0.2-2.0 mg Cr kg^{-1} (providing total Cr dietary levels in the range 0.68-2.5 mg Cr kg^{-1}). The study clearly demonstrated that supplementary Cr over a narrow range (0.2-1.5 mg Cr kg^{-1}) significantly improved weight gain, feed conversion and protein efficiency. Optimum growth was obtained when fish were fed a diet fortified with 0.5 mg Cr kg^{-1} as the trivalent chromium chloride salt. In humans and rats, it has been found that trivalent chromium potentiates the action of insulin via an increase the activity of protein tyrosine kinase, and increased insulin binding, insulin receptor number, insulin internalization and beta cell sensitivity, thereby enhance glucose, amino acids, and fat metabolism (Anderson, 1997; Daveis and Vincent, 1997; Fekete et al., 2001). The other possible explanation for growth improvement in the requirement level range is that chromium may enhance the glucose transporter and subsequently improve carbohydrate assimilation. A relatively lower growth performance was observed in carp fed on the control diet (without Cr supplementation) could be explained by a reduced feed consumption rate and lower feed utilization efficiency. A relatively lower value for specific growth rate was also recorded in fish fed on 2.0 mg Cr kg^{-1}. It is possible that high level of Cr lead to toxicity and growth reduction. These findings are in agreement with Liu et al. (2010) who reported that Cr supplementation at a level 0.8 mg Cr kg^{-1} in the diet of grass carp (*Ctenopharyngodon idellus*) fingerlings resulted in significantly improved weight gain and feed efficiency compared to the un-supplemented control diet. Improved carbohydrate utilization by dietary chromium chloride supplementation has been documented in tilapia (Shiau and Chen, 1993; Shiau and Liang, 1995). Both these species (carp and tilapia) are considered to be omnivorous fish with a preference towards primarily a carbohydrate mode of energy metabolism. Salmonids and many other carnivorous fish generally have lower abilities to

utilize carbohydrates and are more dependent on protein and lipid as principal energy sources (Stone, 2003; Wilson, 1994).

In previous work undertaken by Tacon and Beveridge (1982) with rainbow trout, dietary supplementation with 1.0 mg Cr kg^{-1} produced favourable growth (but not significant) compared to the control diet whilst 6.0 mg Cr kg^{-1} caused a marked decrease in growth performance. In the latter investigation, casein was the protein source employed and corn starch and dextrin were included in their experimental diets contributing up to 25 % of dietary carbohydrate which is considered to be a high level in modern salmonid feeds. The carp diets in our study were based on a practical level of dietary carbohydrate resembling current standard commercial carp diets with mixtures of plant proteins and limited inclusion of fish meal. Chromium apparently was of benefit for carp under the conditions of this experiment with a similar response to trout in a defined range based on trivalent chromium presented as inorganic chromium chloride.

The role of chromium in fish nutrition is unclear with contradictory views presented in the literature (NRC, 1997). Whilst some studies have suggested that dietary chromium supplementation has no effect on growth performance or carbohydrate utilization, others seem to indicate that dietary chromium plays a vital role in fish nutrition through its primary involvement in carbohydrate metabolism as well as other related metabolic processes. Although the situation in fish has so far not been clarified, it seems likely that similar processes occur as in mammals and other vertebrates which are well documented by Vincent (2007).

In the present trial, chromium supplementation had no effect on carcass composition; this result is similar to those of Gatta et al. (2001) on gilthead seabream and Pan et al. (2003) on

tilapia. Likewise Tacon and Beveridge (1982) reported no significant effects on carcass composition in rainbow trout fed 1.0-6.0 mg Cr kg^{-1} of dietary chromium.

In humans, trivalent chromium tends to accumulate in epidermal tissues, bones, liver, kidney, spleen, lungs and large intestine (Pechova and Pavlata, 2007), while chromium chloride supplemented in the diet of broiler chicks had a tendency to accumulate in the heavy muscles of the thigh and breast rather than in the liver (Ahmed et al., 2005). In the current study, no significant differences were detected in chromium concentration in the liver. The possible explanation for this result might be that chromium accumulated in other organs or tissues. Although this was not tested in the current study.

In the present investigation, carp fed 0.5 mg Cr kg^{-1} of supplementary Cr had a final carcass retention of 2.0 mg Cr kg^{-1} (DM) which was not significant to those fish fed higher dietary Cr levels up to 1.5 mg Cr kg^{-1} indicating that whole body Cr saturation had been attained.

Since it has been reported that chromium in active form (GTF) is essential in the regulation of carbohydrate metabolism by improving the uptake of glucose into cells (Mertz, 1974a), it was the aim of the current study to assess the effect of Cr status on a selection of metabolic parameters such as plasma glucose concentration and specific enzymes associated with glucose assimilation. In this trial, plasma glucose concentration showed a significant linear downward trend as the Cr content of the diet increased (up to 1.5 mg Cr kg^{-1}). It has been proposed a potential relationship between LMWCr and insulin signalling which increased glucose assimilation (Vincent, 2000a; Vincent, 2000c). This result is consistent with the study of Küçükbay et al. (2006) where they reported that serum glucose decreased with increasing dietary chromium supplementation in experiments involving rainbow trout fed a series of diets supplemented with chromium picolinate. Values for plasma glucose in carp

were consistent with values reported by Groff and Zinkl (1999) in a comprehensive article on the haematology and clinical chemistry of cyprinid fish.

Key liver enzyme activities of intermediary metabolism could be used as indicators of nutritional status and growth performance of farmed fish (Metón et al., 1999). However, some key carbohydrate metabolism enzymes may not adapt to changes in feeding regimen, e.g. hexokinase, (Tung and Shiau, 1991). A study by Enes et al. (2008a) supported this, showing that HK activity was not influenced by dietary carbohydrate sources or level; this was also found in the current study. G6PD is involved in the first step of the pentose phosphate pathway. Additionally, it has also been reported that chromium increases the rate of lipogenesis by increasing the rate of conversion of glucose to acetyl-CoA (Steel and Rosebrough, 1981). However, G6PD activity was not significantly affected by dietary Cr supplementation. Similarly, Pan et al. (2003) reported that G6PD activity was not affected by dietary Cr supplementation in tilapia fed a diet containing 2.0 mg Cr kg^{-1} as chromium picolinate. ALT plays an important role in amino acid metabolism and gluconeogenesis in higher vertebrates including fish. ALT has also been used as marker of tissue injury or hepatotoxicity in humans and animals and as a bio-marker of adaptive reactions (Lindblom et al., 2007). However, in this study with carp, different Cr supplementation had no effect on ALT activity in the liver suggesting normal hepatic capacity for protein metabolism and the highest level of Cr in the carp diet did not induce liver damage.

Liver histology is widely used as an indicator of the nutritional and physiological status in fish studies due to the vital role of the liver in various metabolic functions and in detoxification ((linhua et al., 2009). Loss of lipid in the liver may happen as a result of intoxication, starvation, stress, or certain diseases. In contrast, accumulations of fat in the liver may occur as a result of toxic exposure (Wolf and Wolfe, 2005). Histological examination of liver removed at the end of the experiment from carp fed the diet

supplemented with 2.0 mg Cr kg^{-1} showed elevated hepatocyte lipid vacuoles. This result might indicate an imbalance between the rate of synthesis and the rate of release of stored lipid reserves in the liver due to chemical intoxication (Gingerich, 1982). To our knowledge, dietary trivalent toxicity in common carp has not been fully investigated compared to aqueous toxicity of water borne Cr in the hexavalent state for fish in general. Mishra and Mohanty (2008) reported different changes in liver hepatocytes in *channa punctatus* (Bloch) included vacuolization and necrosis as a result to hexavalent chromium exposure.

The histological examination provided clear evidence that highest level of chromium (2.0 mg Cr kg^{-1}) induced fusion of intestinal mucosal folds with associated necrosis and hyperplasia of the enterocytes and evidence of tip erosion. It has been reported that the direct contact between the metal or its salts and the tissue surface may induce local irritative effects in the stomach and intestine (Schroeder, 1976). This result could add further interpretation for the poor growth rate in fish fed on a diet containing high level of Cr. It is likely that tip erosion reduced digestion and nutrient absorption (Berntssen et al., 1999).

In conclusion, this investigation on carp suggests an optimum chromium supplementation of at least 0.5 mg Cr kg^{-1} as desirable to enhance growth and feed performance of fish fed practical feeds, thereby to achieve a total dietary Cr of 1.01 mg Cr kg^{-1} dry matter (DM). A level of 2.0 mg Cr kg^{-1} DM supplementation (2.5 mg Cr kg^{-1} DM of total Cr) seems to be the threshold for the initiation of toxicity leading to reduced growth.

It is likely that in practice carp receive sufficient Cr from diets containing appreciable levels of animal based ingredients, but the bioavailability of Cr is highly variable in different plant by products following processing and feed production. Since legislation in Europe prevents additional Cr supplementation in trace element premixes for aquafeeds deficiencies are likely to occur in practical diets and optimum performance is therefore not always guaranteed.

Future investigations must be directed towards validating the bioavailability of dietary chromium from different sources. There is growing research interest in evaluating mineral speciation for application in animal nutrition and especially in aquafeeds. The potential for using such forms as chromium picolinate and natural chromium yeast complexes remain to be tested for carp and other fish species as safer and more effective additives.

A summary of the experimental results:

- Cr chloride supplementation increased growth performance in common carp at different levels (0.2-1.5 mg Cr kg^{-1}) compared to the control diet fed group.
- Cr carcass content increased with increasing dietary Cr supplementation.
- Different levels of Cr had no effect on the final body composition or key liver enzymes activity.
- High level of Cr (2.0 mg Cr kg^{-1}) in the carp diet resulted in liver vacuolization and different changes in the gut tissue.

Chapter 4

Experiment II

Chromium bioavailability

- This chapter has been published in Biological Trace Element Research:

  Arafat R. Ahmed, Awadhesh N. Jha, Simon J. Davies (2012). The Efficacy of Chromium as a Growth Enhancer for Mirror carp (*Cyprinus carpio* L): An Integrated Study Using Biochemical, Genetic and Histological Responses. Biological Trace Element Research. DOI: 10.1007/s12011-012-9354-4.

- The same study has been presented at Alltech's 26th Annual International Symposium, May 2010, USA.
- The same study was presented at the school of Biomedical and Biological Sciences (Oral presentation), 15th May 2011, Plymouth University, UK.
- Results from this chapter have been presented at the 8th Marine Biological Association Postgraduate Conference (Oral presentation), 17th May 2011, Queen's University Belfast, Northern Ireland.

Abstract

A growth trial was conducted on juvenile mirror carp (*Cyprinus carpio* L.) for eight weeks to compare the efficacy of three chromium compounds (Cr chloride, Cr picolinate, and Cr yeast) at a level 0.5 mg kg^{-1} as a potential growth enhancer. In addition, a high level of Cr (2.0 mg kg^{-1}) as Cr chloride has also been added in parallel for comparison. All Cr fortified diets at a level 0.5 mg kg^{-1} produced superior growth for carp compared to the control group and the group fed the high level of Cr chloride (2.0 mg kg^{-1}). Metabolic indicators measured included two of the key liver enzymes (HK and glucose-6-G6PD) activity. The results validated the positive effect of Cr at a level 0.5 mg kg^{-1} on enzyme activity and carbohydrate utilization producing significantly better growth performance for mirror carp. The study also included measurement of DNA strand breaks in the erythrocytes using the comet assay which revealed significantly ($P < 0.05$) increased DNA damage in fish fed on high level of Cr chloride (2.0 mg kg^{-1}) but the other treatments were not significantly different ($P > 0.05$) from the control groups. The concentration of Cr in the liver, gut, and whole fish tissues increased with increasing dietary Cr supplementation. Overall, Cr supplementation at a level 0.5 mg Cr kg^{-1} from different sources may affect growth performance in carp by activation some key liver enzymes (HK and G6PD).

Chapter 4: Chromium bioavailability

4.1 Introduction

Chromium has been classified as an essential micronutrient for maintenance of proper carbohydrate and lipid metabolism. Different chromium compounds (organic and inorganic) have showed remarkable advantages in animal nutrition studies (NRC, 1997). In this regard, inorganic compound (Cr chloride) supplementation has showed to promote growth and nutrient utilization in black Bengal bucks Capra hircus (Paul et al., 2005) and in broiler chickens (Ahmed et al., 2005). Similarly, different advantages have been detected from dietary organic Cr supplementation as Cr picolinate (CrPic) including positive influences on egg production of laying quails (Yildiz et al., 2004) and improve glucose metabolism in yearling horses (Ott and Kivipelto, 1999). More recently, dietary CrPic has been found to increase fertility in beef cows (Stahlhut et al., 2006). Another source of organic chromium, Cr yeast, improved glucose tolerance in pigs (Guan et al., 2000) and decreased the mortality rate in broiler chickens (Debski et al., 2004).

Investigations of the influence of chromium in fish nutrition have been limited; although more attention has been focused on this area recently with conflicting results which may be due to the difference in chromium source, chromium concentration and fish species used for these studies. The results of the previous experiment (Experiment I, Chapter 3) have indicated that inorganic chromium, Cr chloride, had a positive effect on glucose utilization and growth performance in common carp at different levels (0.2-1.5) mg Cr kg^{-1} of diet without negative side effects; while 2.0 mg Cr kg^{-1} in the diets significantly impaired the general health of fish and reduced weight gain. Gatta et al., (2001a) reported that organic Cr as Cr yeast had the ability to modulate the immune response in rainbow trout at different levels (1.5, 2.3 and 4.1 mg Cr kg^{-1}) but the same compound at two concentrations (i.e. 0.8 and 5.3 mg Cr kg^{-1}) had no effect on growth performance in gilthead sea bream (Gatta et al.,

2001b). More recently, the study of Liu et al. (2010) suggested that CrPic significantly improved growth and carbohydrate metabolism in grass carp fingerlings at a level 0.8 mg Cr kg-1; while conversely no beneficial effect was detected for tilapia (Pan et al., 2003) or rainbow trout (Selcuk et al., 2010) from inclusion of dietary CrPic in their diets at a level 2.0 and 1.6 mg Cr kg^{-1} respectively. Based on human or mammalian studies, it is likely that the response to trivalent chromium supplementation is dependent on the amount and form of supplemental Cr (Anderson, 1998).

It is known that the hexavalent state, Cr(VI), represents the genotoxic form of chromium due to its solubility and bioavailability (De Flora et al., 1990). The genetoxicity of the Cr(VI) is resulting from the reduction of the latter form to trivalent state, Cr(III), which is effective in generating the reactive oxygen species (ROS) that can induce oxidative stress and DNA damage (Shi and Dalal, 1990; Gaddameedi et al., 2011). Growth impairment and survival reduction have been recorded in Chinook salmon (*Oncorhynchus tshawytsch*) exposed to aqueous chromium concentrations (0-266 μl^{-1}) (Farag et al., 2006). Another study recorded DNA damage as a result of Cr(VI) exposure in Prussian carp (*Carassius auratus gibelio*) (Al-Sabti et al., 1994). However, the safety of Cr(III) has not been proved because the biochemistry and bioavailability of Cr(III) is changeable due to its ability to form coordination ligands (Stearns, 2007). It has been suggested that some coordinated complexes are able to migrate from the cytoplasm to the nucleus and attack the DNA structure (NRC, 1997). In addition, the more stable state of chromium, Cr(III), is considered the ultimate form that interacts with the DNA molecule (O'Brien et al., 2003; Zhitkovich, 2005; Gaddameedi et al., 2011). A number of experiments have demonstrated that the reactive intermediates Cr(III), can produce harmful reactive oxygen species through the Fenton and Haber-Weiss reactions (Poljsak et al., 2010). The Fenton reaction is a step in the lipid peroxidation pathway where metals, such as Cr, reduce hydrogen peroxide to hydroxyl radicals leading to

oxidative DNA lesions and lipid peroxidation of poly unsaturated fatty acids in cells membranes (Farag et al., 2006).

The application of comet assay or single cell gel electrophoresis has been established as a vital means for detecting strand breaks in the DNA of single cells from blood and different tissues. The protocol has been described as a precise method for investigating the genotoxicity of nutrients and micronutrients at the level of DNA in humans (Collins, 2004) and is considered to be a sensitive tool to evaluate the genotoxicity in aquatic animals (Mitchelmore and Chipman, 1998; Lee and Steinert, 2003; Jha, 2004).

So far, there is no comparative study in the literature has investigated the effect of different sources of Cr(III) on growth, carbohydrate utilization in carp and no data are available pertaining to the effects of dietary Cr(III) on DNA structure in fish. Therefore, this experiment was designed to assess the relative effect of Cr chloride, CrPic and Cr yeast on growth performance, carbohydrate utilization, DNA structure and activities of some key liver enzymes that associated with carbohydrate assimilation

The specific hypotheses tested in this investigation are:

Hypothesis 1: Growth performance is affected by the form and level of Cr.

Hypothesis 2: Different forms of Cr (organic and inorganic) affect the activity of the key liver enzymes.

4.2 Materials and methods

4.2.1 Growth trial protocol

The aim of this trial was to investigate the bioavailability of chromium from different sources. The design of the growth trial is presented in Table 4.1.

Table 4. 1 Growth trial protocol for chromium bioavailability experiment.

Fish	Mirror carp
Stocking density	15 fish in each tank
Acclimation period	Due to the small size of the stock fish when obtained from the farm, the acclimation period has been extended for 6 weeks to allow the fish growing to the suitable size (10-15) g.
Diets	5 diets containing different levels and sources of chromium
Chromium source	Inorganic form (Cr chloride) and two sources of organic chromium (Cr picolinate and Cr yeast).
Duration of study	8 weeks
Feeding regime	3% of body weight four times a day.
Parameters tested	Growth parameters, initial and final body composition, Cr accumulation in liver, gut and whole fish tissue, plasma glucose level, key liver enzymes (HK and G6PD) in liver, comet assay, gut and liver histology.

4.2.2 Diets ingredients and preparation

Formulation of the experimental diets and chemical composition are presented in Table 4.2. Three experimental diets were supplemented with 0.5 mg Cr kg^{-1} from different sources; Cr chloride hexahydrate ($CrCl_3.6H_2O$), CrPic, and Cr yeast. The fourth diet was supplemented with 2.0 mg Cr kg^{-1} as chromium chloride; while the basal diet (without Cr supplementation) represented the control diet. CrPic is slightly soluble in cold water whilst Cr yeast is insoluble; therefore, they were mixed with the other ingredients as a powder. The defined amount of Cr chloride was dissolved in 500 ml distilled water and added gradually to the

mixture. The sources of the main ingredients and diet preparation protocol were as mentioned in Chapter 2.

Table 4. 2 Formulation and chemical composition of experimental diets.

Ingredients (g kg^{-1})	**Diets**				
Formulation	**Control**	**0.5 $CrCl_3$**	**0.5 CrPic**	**0.5 Cr yeast**	**2.0 $CrCl_3$**
Herring Meal LT 94	250	250	250	250	250
Maize starch	382.2	382.2	382.2	382.2	382.2
Pea protein concentrate	164.5	164.5	164.5	164.5	164.5
Maize gluten	150	150	150	150	150
Sunflower oil	33.3	33.3	33.3	33.3	33.3
Mineral Premix	20	20	20	20	20
Cr added (mg kg^{-1})	0.00	0.50	0.50	0.50	2.00
Cr analyzed (mg kg^{-1})	0.69	1.24	1.22	1.21	2.46
Proximate analysis (g kg^{-1}dry matter)					
Moisture	36.0±0.6	37.0±1.1	37.5±0.8	36.8±2.1	37.7±0.4
Protein	390.4±2.9	388.9±1.7	390.5±1.2	389.6±1.5	389.8±2.0
Lipid	73.0±1.5	74.0±1.8	74.0±0.9	73 0±2.1	74.5±0.7
Ash	53.3±0.5	53.8±0.9	53.4±1.6	52.9±0.2	52.0±0.5

$CrCl_3.6H_2O$ (Sigma-Aldrich, UK); CrPic (TCI, Europe, Belgium); Cr yeast (Biochrome™) (Alltech Company, USA). Proximate analysis values are ± SE, n=3

Note: the chemical analysis of Cr yeast product using the ICP found that the product contains 996±11 mg Cr kg^{-1}.

4.2.3 Experimental fish and husbandry

Prior to the nutrition trial, the fish were acclimated and fed daily as described in Chapter 2. At the beginning of the nutrition trial, batches of 15 fish (initial weight 10 ± 0.1g) were randomly distributed in 18 fibreglass tanks (three replicate per treatment). During the trial period, water quality parameters were maintained by daily monitoring the levels of pH which ranged between 6.75 and 7.5, dissolved oxygen level (94.5 and 97.6) % and temperature (25 ºC ± 0.5). Weekly monitoring the levels of Ammonia (0.00-0.199 mg l^{-1}), Nitrite (0.00-0.01 mg l^{-1}), Nitrate (8.70-65.45 mg l^{-1}) and Cr concentration in the system (0.06-0.09) µg l^{-1} were also carried out. In addition, the concentration of Cr in the system ranged between 0.03 and

0.09 µg l^{-1}. The devices used for water chemistry measurements were described previously in Chapter 2.

4.2.4 Fish weighing and sampling

Fish weighing, anaesthesia, and sampling procedure were as described in Chapter 2.

4.2.5 Growth performance calculation

To evaluate growth performance of fish at the end of feeding trial, growth performance equations described in Chapter 2 have been applied.

4.2.6 Proximate composition of the diets and whole fish tissues

Moisture, crude protein, crude lipids, and ash determination were described in details in Chapter 2.

4.2.7 Cr analysis in the experimental diets and whole fish tissue

The description of Cr analysis in the experimental diets and whole fish tissues were as described in Chapter 2.

4.2.8 Cr analysis of Cr yeast product

The concentration of Cr in Cr yeast product has been determined by the same protocol used to determine Cr concentration in the diet and whole fish tissue that described previously in Chapter 2.

4.2.9 Cr accumulation in liver and gut tissues

Tissues digestion and Cr analysis in liver and gut tissues were performed as described in Chapter 2.

4.2.10 Plasma glucose measurement

Number of samples, blood sampling, plasma collection and storage, and plasma glucose measurement protocol were as described in Chapter 2.

4.2.11 Enzyme activity measurement

Number of samples, liver collection and storage, and enzyme measurement technique were as described in Chapter 2.

4.2.11.1 Hexokinase

Buffer preparation and HK measurement protocol were described in Chapter 2.

4.2.11.2 Glucose 6 phosphate dehydrogenase

Buffer preparation and G6PD measurement protocol were described in Chapter 2.

4.2.12 Comet assay or single cell gel electrophoresis (SCGE)

Principle

The single cell gel electrophoresis or comet assay is a sensitive means to evaluate the DNA damage/repair, biomonitoring and genotoxicity in individual cultured cells or cells isolated from animals in (*in vitro* and *in vivo*) (Mitchelmore and Chipman, 1998; Collins, 2004). Different modifications have been made on the original protocol that allows the evaluation of different DNA lesions but the basic procedure involves the following steps (Figure 4.1) as described in detail by Tice et al. (2000).

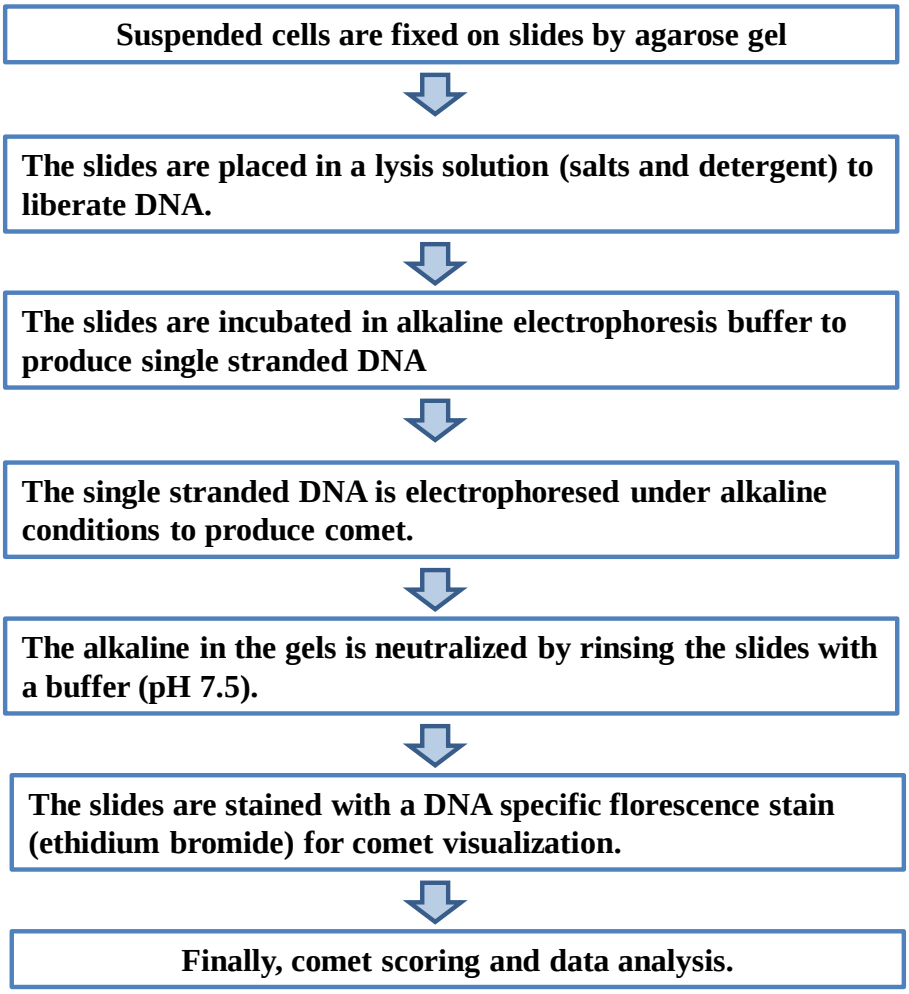


Figure 4. 1 Principle steps of the single cell gel electrophoresis (SCGE) protocol.

4.2.12.1 Methodology

4.2.12.1.1 Blood samples and cell viability measurement

Two fish per tank (six per treatment) were randomly selected and anaesthetized with buffered MS-222 and whole blood was collected via the caudal vein into 1 ml heparinised syringes. Before applying the comet assay protocol, trypan blue exclusion test was used to determine the viable cells present in a cell suspension. The principle of this test is that the viable cell membrane is able to exclude certain dyes (e.g. Trypan blue or eosin), whereas nonviable cells do not possess this ability and a certain dye is taken up by the cell membrane; therefore, nonviable cells stain with blue colour in case of using trypan blue dye. Briefly, 500 µl of 0.4% of trypan blue was added to 500 µl of blood and mixed carefully. The mixture was

incubated for 2-3 minutes at room temperature. Using a haemocytometer, the unstained (viable) and stained (unviable) cells were counted separately. Percentage viable cells was calculated as living cells/ total cells counted (cells should be counted within 5 minutes of mixing with trypan blue). Viable cells should be more than 90% in any sample to be suitable for comet assay protocol.

4.2.12.1.2 Samples preparation

The procedure used as described by Singh et al., (1989) and adapted for the erythrocytes of common carp by Mustafa et al. (2011). Blood samples were diluted 1:5000 (to provide a cell count of 2.5×10^5 cells ml^{-1}) using physiological saline solution (PBS) (Gibco, Invetrogen, UK). The diluted samples were centrifuged at 2,500 xg for 2 min. The resulting pellet was resuspended in 170µl low melting agarose (LMP). Two replicate per sample were prepared by adding 85 µl from the later suspension on the CometSlide™ slides (Trevigen, Gaithersburg, MD). Cover slips were placed over each sample and were incubated at 4°C for 5-10 min.

4.2.12.1.3 Lysis

Subsequently, coverslips were removed and slides were placed in a lysis buffer for 1 h at 4 °C to remove cellular proteins. The lysis buffer used consists of 2.5 mM NaCl, 100 mM Na2EDTA, 10 mM tris-BASE, 1% n-lauroylsarcosinate, 1% Triton X-100, 10% DMSO, pH 10.

4.2.12.1.4 Alkali (pH<13) unwinding

DNA was left to unwind in the electrophoresis chamber (Pharmacia Biotech GNA200) at 4°C in alkaline electrophoresis buffer [1 mM Na2EDTA, 300 Mm NaOH, pH 12.3].

4.2.12.1.5 Electrophoresis

Electrophoresis was performed at 25 V, 300 mA at 4°C in the dark for 20 min.

4.2.12.1.6 Neutralisation and staining

Slides were immersed in a neutralization buffer [0.4 M Tris-BASE, pH 7.5] for 5 minutes and this step was repeated three times. Later, slides were rinsed with distilled water and allowed to dry at the room temperature for 24 h. Finally, to visualise Comets, 40 µl (2 µg ml^{-1} stock) of thidium bromide stain was applied to each gel and coverslips added.

4.2.12.1.7 Scoring method

The percentage Tail length was measured as a reliable parameter (Kumarravel and Jha, 2006) using a camera attached to a fluorescence microscope (Leica, DMR, Germany). A komet 5.0 image analysis software (Kinetic Image Ltd., Liverpool, UK) was used to score 100 cells for each slide (50 cells per gel from each exposed individual fish). Comet image analysis software transfers a picture from the field selected to the computer monitor. Scoring cells from the field under analysis is performed by using the computer mouse to select a single cell. The software will directly calculate all measurement parameters (e.g. tail length and olive tail moment) and then add the data for the cell to list of results. The process is repeated by selecting the next cell to be scored. Once all the cells within the current field of view have been scored, the microscope's stage controls along with the picture on the monitor were used to find the next set of cells to be scored. The movement of the microscope stage should present number 2 in its digital form to prevent scoring the same cells twice.

4.2.13 Histological examination

All procedure for histological sample preparation was followed as described in Chapter 2.

4.2.14 Statistical analysis

The statistical analysis was performed as described in Chapter 2.

4.3 Results

4.3.1 Growth performance

The survival rate of the experimental fish was 100% for all the fish fed on different diets. Wet weight gain (%WG), specific growth rate (% SGR), feed conversion ratio (FCR), and protein efficiency ratio (PER) of mirror carp fed diets supplemented with different levels and sources of chromium are presented in Table 4.3.

All chromium fortified diets at a level 0.5 mg Cr kg^{-1} produced superior % WG, SGR, FCR and PER for carp and were significantly different ($P < 0.05$) from the un-supplemented control group and the group fed with the highest level of chromium (2.0 mg Cr kg^{-1}). Cr yeast displayed the highest weight gain performance but the other growth parameters were not significantly different ($P > 0.05$) from fish fed with 0.5 mg Cr kg^{-1}as chromium chloride and chromium picolinate. All growth parameters (WG, SGR, FCR and PER) in fish fed 2.0 mg Cr kg^{-1} were lower and significantly different ($P < 0.05$) compared to the other treatments.

Table 4. 3 Growth and feed utilization performance of mirror carp fed diets containing different levels and sources of chromium over 8 week.

	Experimental diets				
Parameters	**Control**	**0.5 $CrCl_3$**	**0.5 CrPic**	**0.5 Cr yeast**	**2.0 $CrCl_3$**
Initial mean weight (g)	9.80 ± 0.14	10.03 ± 0.04	9.83 ±0.04	9.95 ±0.07	10.05 ± 0.07
Final mean weight (g)	26.30 ± 0.14 a	29.73 ± 0.10 b	29.93 ± 0.20 b	31.75 ± 0.33 c	24.18 ± 0.31 d
Weight gain %	168.38 ± 1.72 a	204.85 ± 8.32 b	196.51 ± 1.48 b	211.61 ± 1.95 b	140.56 ± 3.40 c
SGR	1.79 ± 0.03 a	1.98 ± 0.01 b	2.05± 0.06 b	2.08 ± 0.06 b	1.58 ± 0.04 c
FCR	1.39 ± 0.01 a	1.19 ± 0.03 b	1.17 ± 0.01 b	1.14 ± 0.00 b	1.46 ± 0.05 c
PER	1.90 ± 0.00 a	2.22 ± 0.01 b	2.25 ± 0.03 b	2.31 ± 0.04 b	1.76 ± 0.02 c

Values are means ± SE (n= 3). In the same line, values with different letters are significantly different ($P< 0.05$).

The response of carp to diets of various chromium sources supplementation (as mean body weight) is presented in Figure 4.2. Again, the lower growth presented as mean body weight (g) was observed in fish fed on the control diet and a reduced growth rate was recorded in fish fed diet supplemented on the highest level of chromium (2.0 mg Cr kg^{-1}) and significantly different from the other treatments ($P < 0.05$).

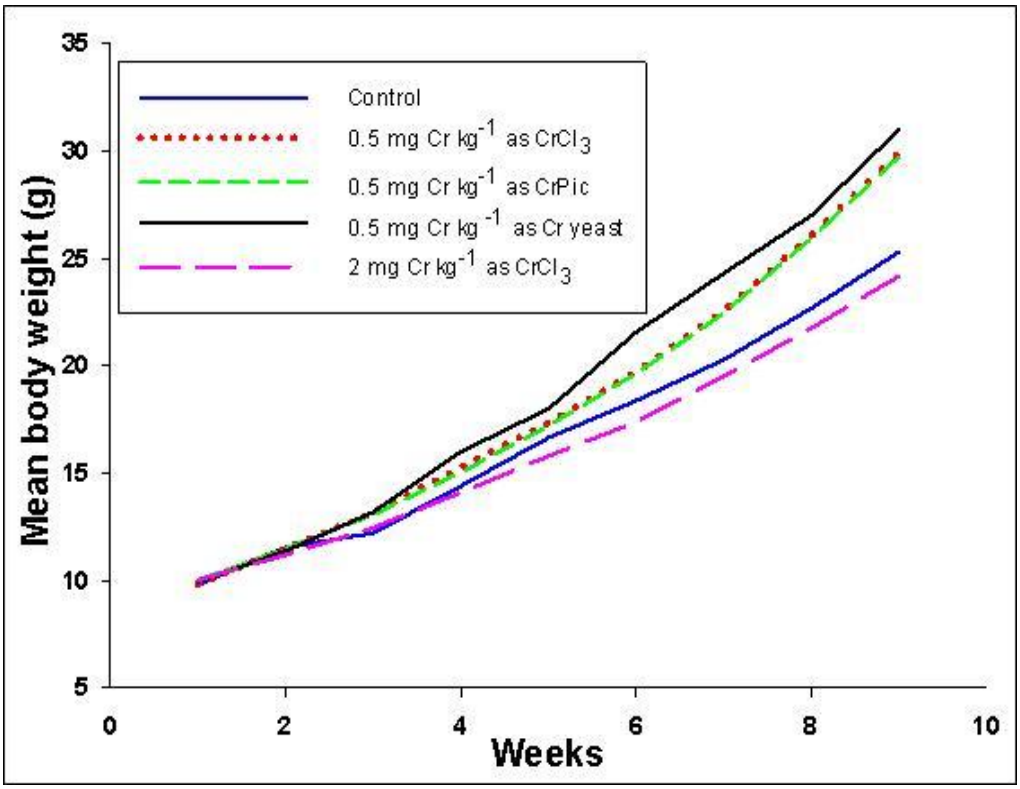


Figure 4. 2 Growth performance of mirror carp fed with respective diets at weekly intervals of 8 weeks.

4.3.2 Proximate composition of whole fish tissue

The whole body composition at the beginning and after 8 weeks of feeding trial is presented in Table 4.4. The chemical analysis demonstrated that crude lipid was high in fish fed different levels and sources of chromium and significantly different (P =0.001) compared to the control diet fed group.

Table 4. 4 The proximate nutrient composition of whole body (% wet weight) of mirror carp before the experimental and at the end of 8 weeks.

		Diets				
Parameters	**Initial fish**	**Control**	**0.5 $CrCl_3$**	**0.5 CrPic**	**0.5 Cr yeast**	**2.0 $CrCl_3$**
Moisture	70.7 ± 0.7	71.0 ± 0.2	71.7 ± 0.1	71.4 ± 0.3	71.9 ± 0.6	71.6 ± 0.8
Crude protein	12.6 ± 0.5	13.4 ± 0.5	14.1 ± 0.5	13.0 ± 0.4	14.3 ± 0.4	13.8 ± 0.8
Crude lipid	9.2 ± 0.3	9.42 ± 0.1a	10.23 0.3 b	10.6 ± 0.6 bc	11.06±0.4 c	10.08 ± 0.2b
Ash	4.8 ± 0.2	4.6 ± 0.2	4.4 ± 0.2	4.2 ± 0.2	4.3 ± 0.2	4.4 ± 0.2

(Values are ±SE, *n*=3 per treatment).

4.3.3 Cr content in whole fish, liver and gut tissues

The concentration of Cr in whole fish tissue, liver and gut samples are presented in Table 4.5. At the end of feeding trial, significant differences ($P < 0.05$) were detected in Cr content in whole fish tissue, the lowest value (0.29 mg Cr kg^{-1}) was recorded in fish fed the control diet and the highest value (0.58 mg Cr kg^{-1}) in fish fed diet containing high level of Cr (2.0 mg Cr kg^{-1}). Despite the low Cr content in the liver, significant differences ($P < 0.05$) have been detected in Cr concentration in the liver of fish fed different diets. The lowest value (13.43µg Cr kg^{-1}) was observed in the control diet fed fish and the highest value (17.04 µg Cr kg^{-1}) in fish fed 0.5 mg Cr kg^{-1} as Cr yeast. From the data in Table 19, it is clear that the concentration of Cr in the gut was higher than that in the liver. Similarly, significant differences ($P < 0.05$) have been found in the gut of fish fed different diets. The lowest value (32.35 µg kg^{-1}) was detected in the gut of fish fed the control diet and the highest value (49.27µg kg^{-1}) in the gut of fish fed on a diet containing 0.5 mg Cr kg^{-1} of Cr yeast.

Table 4. 5 Cr content of whole fish tissue (mg kg^{-1}), liver and gut (µg g^{-1}) dry weight of mirror carp after 8 weeks.

Diets	Cr added (mg kg^{-1})	Cr analyzed in whole fish tissue (mg kg^{-1})	Cr analyzed in liver (µg kg^{-1})	Cr analysed in the gut (µg kg^{-1})
Control	0.0	0.29 ± 0.02 a	13.45 ± 0.19 a	32.35 ± 2.08 a
0.5 $CrCl_3$	0.5	0.52 ± 0.02 b	14.25 ± 0.45 a	34.45 ± 2.44 a
0.5 CrPic	0.5	0.39 ± 0.05 c	16.45 ± 0.24 b	37.28 ± 2.73 a
0.5 Cr yeast	0.5	0.43 ± 0.03 c	16.47 ± 0.23 b	47.97 ± 1.73 b
2.0 $CrCl_3$	2.0	0.58 ± 0.09 b	16.74 ± 0.16 b	49.27 ± 3.46 b

Values are presented as mean ± standard error (SE), n = 6 per treatment and $P < 0.05$.

4.3.4 Plasma glucose concentration

The values of plasma glucose concentration of carp at the end of the feeding trial are presented in Table 4.6. No significant differences ($P > 0.05$) were detected in terms of plasma glucose concentration between the experimental treatments. The maximum value (3.26 mmol l^{-1}) was recorded in fish fed with the control diet and the minimum value (2.86 mmol l^{-1}) was recorded in fish fed diet containing 0.5 mg Cr kg^{-1} Cr yeast.

Table 4. 6 Plasma glucose concentrations of carp fed diets containing different levels and sources of chromium at the end of the feeding trial.

	Diets				
Parameters	**Control**	**0.5 $CrCl_3$**	**CrPic**	**Cr yeast**	**2.0 $CrCl_3$**
Glucose (mmol/l)	3.26±0.36	3.21±0.38	3.05±0.38	2.84±0.33	3.13±0.26

Values are presented as ± SE (N= 6 per treatment).

4.3.5 Enzyme activity

4.3.5.1 Hexokinase

From the data presented in Figure 4.3, it is apparent that fish fed on diets supplemented with 0.5 mg Cr kg^{-1} as organic Cr showed the higher HK activity (5.50 and 6.56 mU mg^{-1} protein) for CrPic and Cr yeast respectively. The Cr yeast group displayed superior HK activity and significantly different ($P < 0.05$) compared to the other treatments including CrPic fed group.

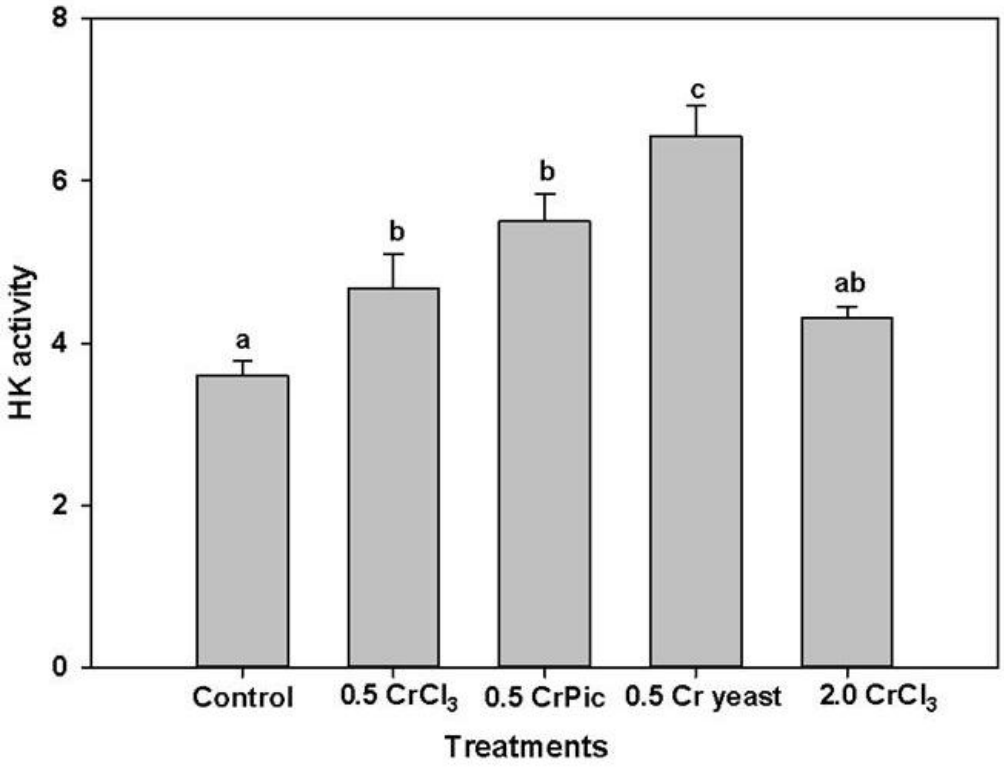


Figure 4. 3 Hexokinase activity profile in response to different levels and sources of chromium at the end of feeding trial. (Values are ±SE, *P* value= 0.000, n=6 per treatment).

4.3.5.2 Glucose 6 phosphate dehydrogenase (G6PD)

Figure 4.4 illustrates that the lowest activity for G6PD (71.60 mU mg^{-1} protein) was in fish fed the control diet and significantly different ($P < 0.05$) from fish fed on diets supplemented with different sources and levels of chromium which also showed differences in the activity depending on the source and level of chromium supplementation.

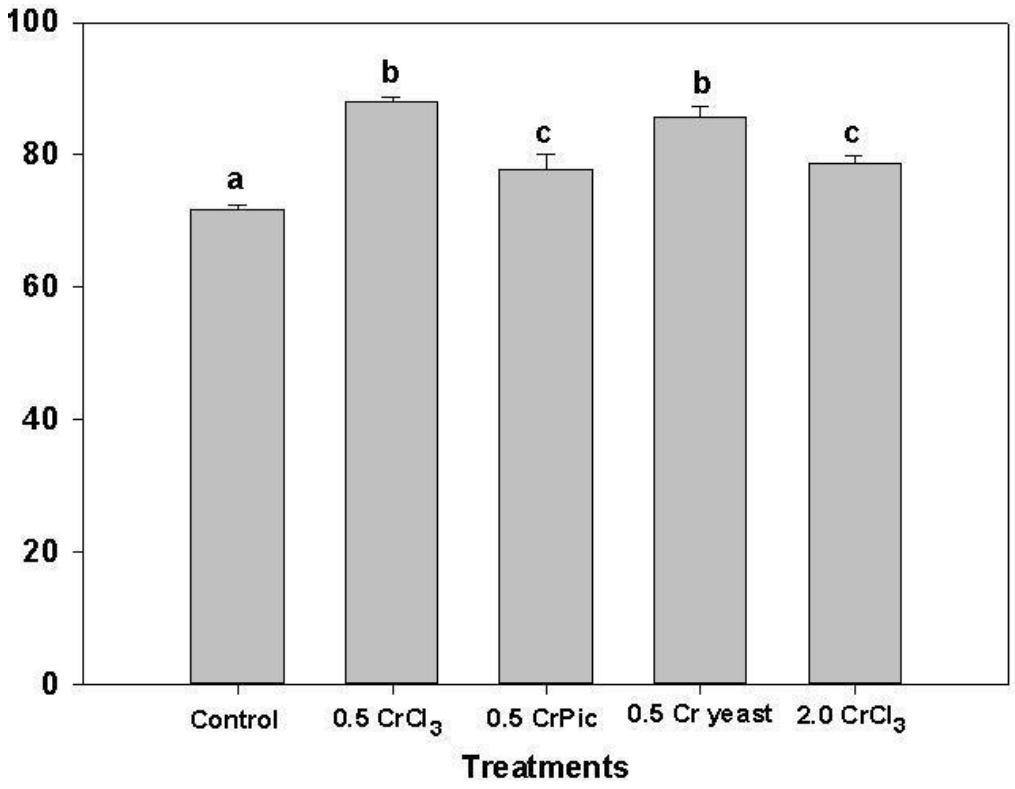


Figure 4. 4 G6PD activity profile in response to different levels and sources of chromium at the end of feeding trial (Values are ±SE, *n*=6 per treatment and *P* value=0.000).

4.3.6 Comet assay

The comet assay results presented as percentage Tail DNA damage showed significant differences ($P < 0.05$) between fish fed the diet supplemented with 2.0 mg Cr kg^{-1} compared with the other treatments (Figure 4.5). The percentage value for the highest level group was 32.33 and ranged between 16.74 and 18.96 for the other groups.

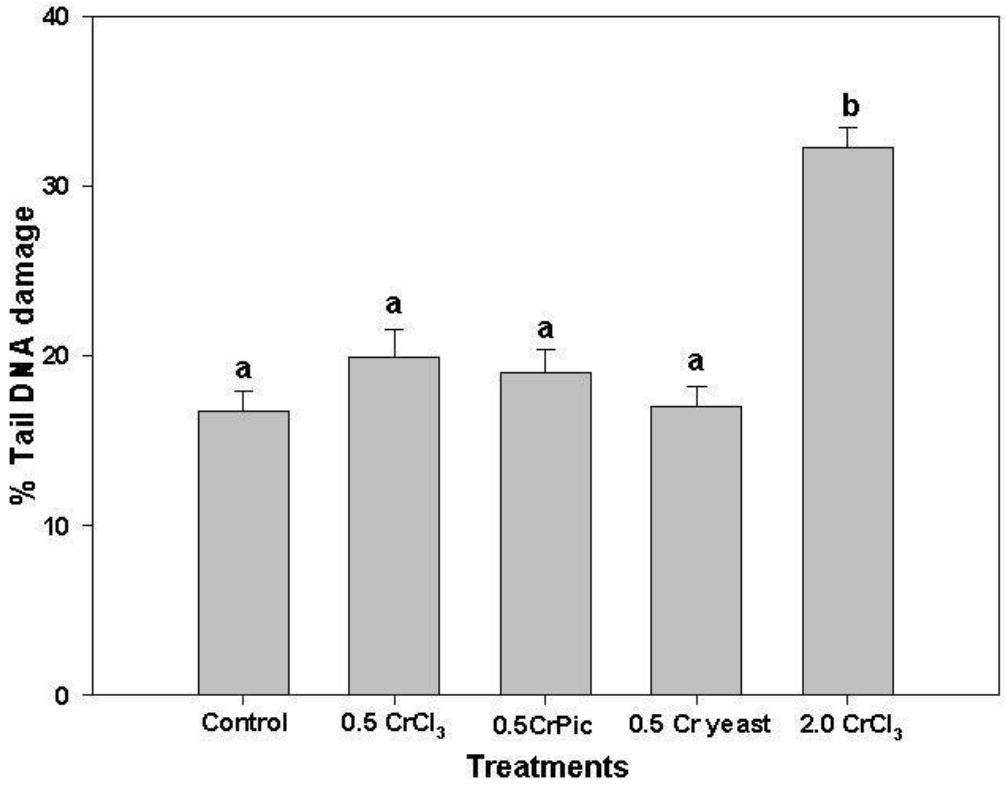


Figure 4. 5 Percentage Tail DNA damage in blood cells of mirror carp fed diets containing different levels and sources of chromium after 8 weeks. Each point represents the mean value and standard error of six fish per treatment. Different letters indicate significantly different from the control ($P = 0.00$).

Comet assay images of DNA damage in blood cells of mirror carp fed on different diets for 8 weeks are presented in Figure 4.6.

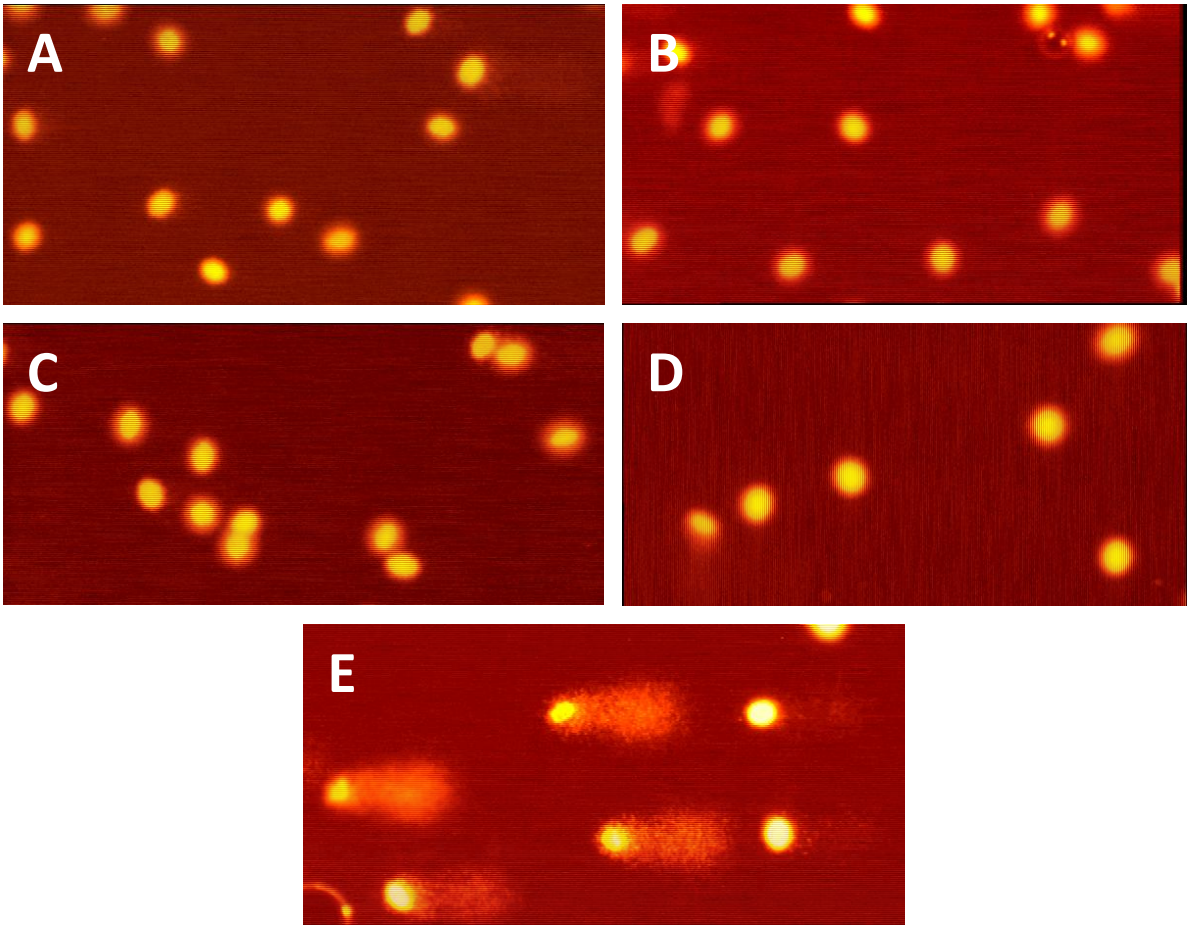


Figure 4. 6 DNA damage profile in blood cells of mirror carp fed with diets containing different levels and sources of chromium after 8 weeks. (A) Sample from fish fed the control diet, (B) sample from fish fed Cr chloride diet, (C) sample from fish fed Cr Pic diet, (D) sample from fish fed Cr yeast diet and (E) sample from fish fed 2.0 mg Cr kg^{-1} Cr chloride.

4.3.7 Histology

4.3.7.1 Liver histology

The histological examination of the liver tissue showed lipid vacuoles in four out of six fish fed on 2.0 mg Cr kg^{-1}; while a normal structure (without clear lipid vacuolization) was observed in the other groups including the control (Figure 4.7).

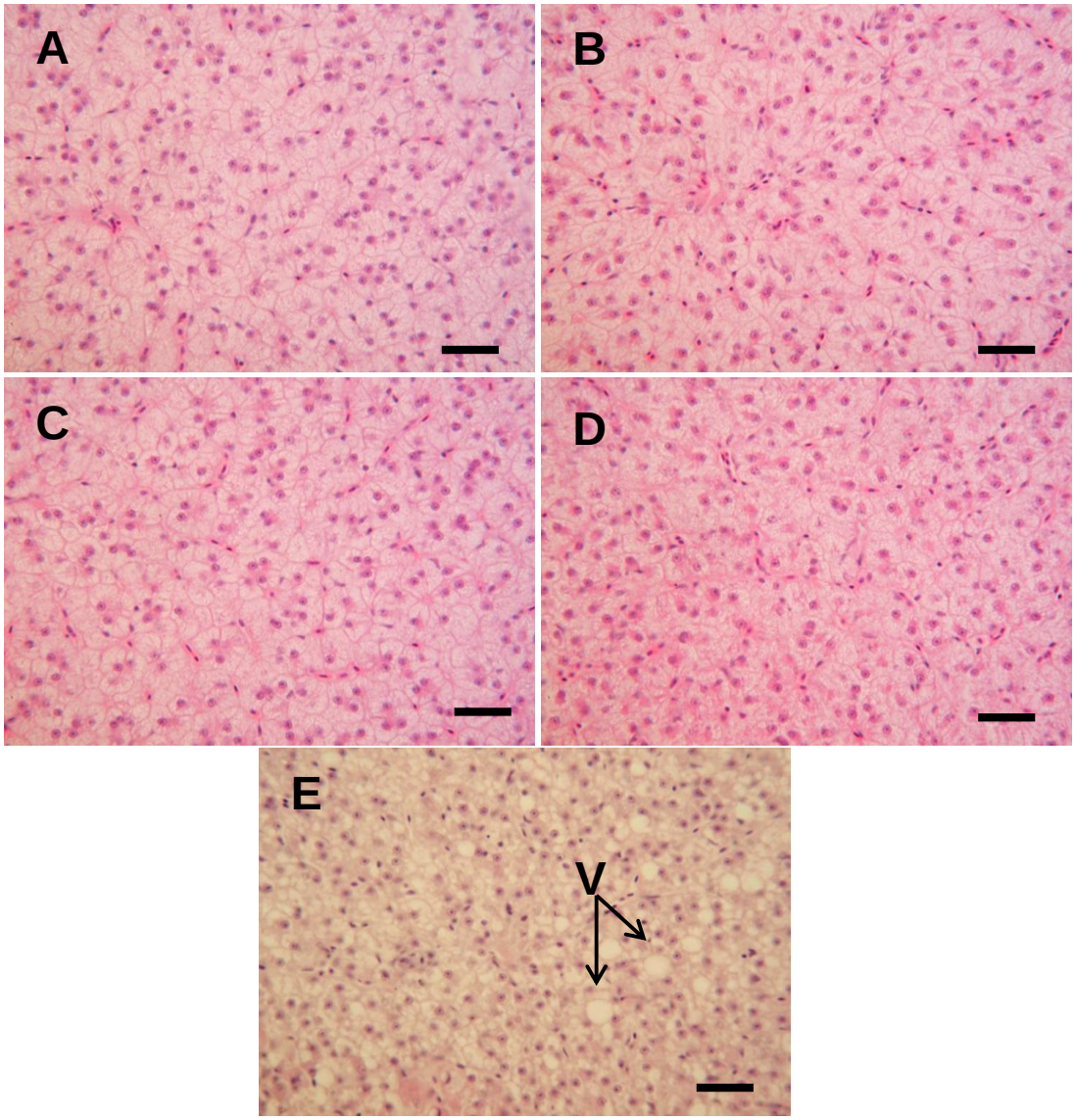


Figure 4. 7 Liver histology of fish fed experimental diets at the end of the experiment. (A) is a sample from fish fed on the control diet; (B, C, and D) are samples from fish fed on diets containing 0.5 mg Cr kg^{-1} as $CrCl_3$, CrPic, and Cr yeast respectively showing normal structure; while the image (E) is sample from fish fed on 2.0 mg Cr kg^{-1} showing liver vacuoles (V).

4.3.7.2 Gut histology

High level of Cr (2.0 mg Cr kg^{-1}) in fish diet induced different changes in the mid-section of the intestine of fish such as necrosis and fusion in mucosal folds in four out of six samples taken at the end of the nutrition trial, while the normal structure of intestine has been found in the mid-section of fish fed the other diets including the control (Figure 4.8).

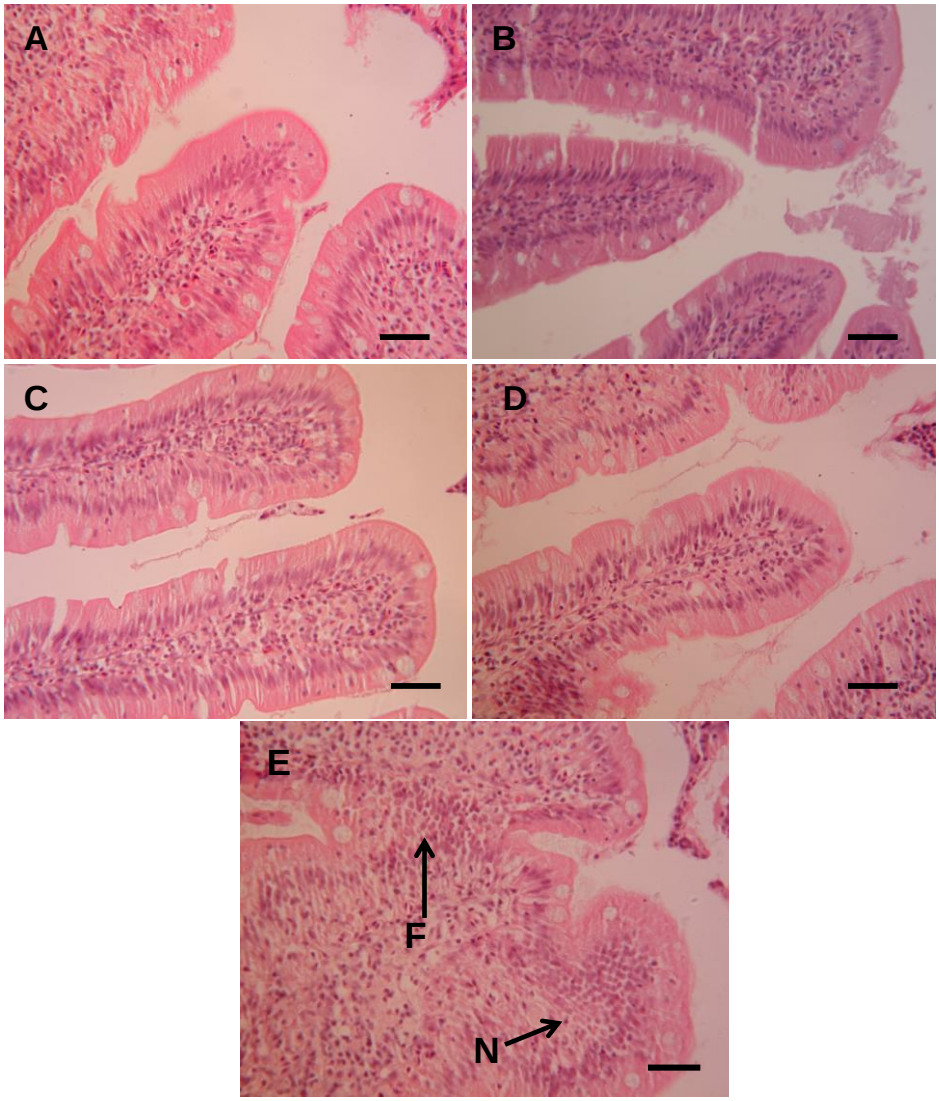


Figure 4. 8 Microscopic examination of mid intestine section of mirror carp at the end of the experiment (A) Fish fed on the control diet depicting normal intestine features; (B, C, and D) are samples from fish fed on diets containing 0.5 mg Cr kg^{-1} as $CrCl_3$, CrPic, and Cr yeast respectively showing normal structure; (E) Intestine section from fish fed 2.0 mg Cr kg^{-1} showing necrosis (N) and fusion (F) in mucosal folds. Scale bar: 50 µm, sections were 7µm thickness and stained with H&E.

The same samples of fish fed on the high level of chromium (2.0 mg Cr kg^{-1}) showed different degree of tissue fusion in the mucosal folds as presented in Figure 4.9.

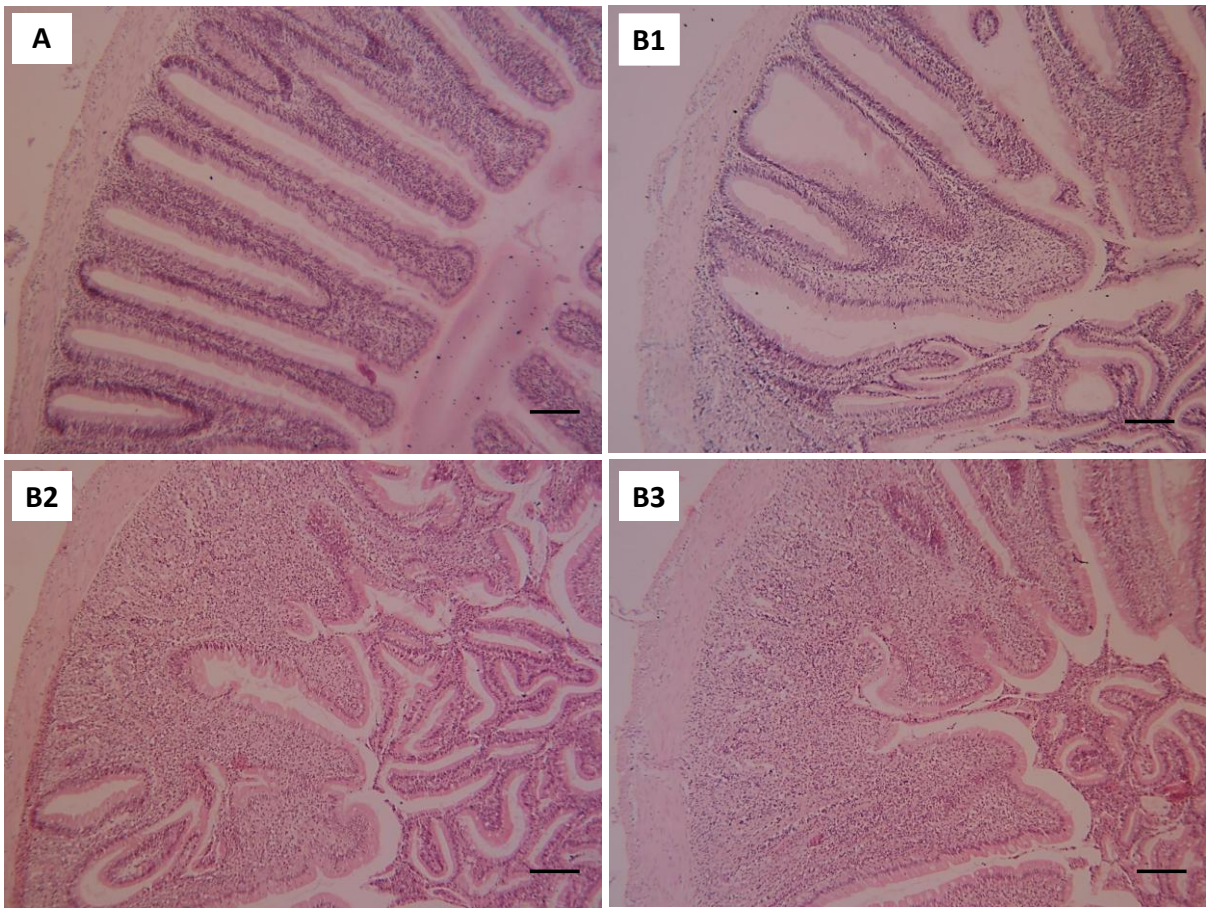


Figure 4. 9 Histology of mirror carp mid intestine section at the end of the experiment (A) Mirror carp fed on the control diet depicting normal mucosal folds. B1, B2, and B3 sections from fish fed on 2.0 mg Cr kg^{-1} illustrating tissue shrinkage in mucosal folds and different degrees of tissue disruption. Scale bar: 200 µm and sections were 7µm thickness and stained with H&E.

4.4 Discussion

The benefits of dietary Cr(III) supplementation of farmed animals (i.e. growth promotion, increasing fecundity, decreasing mortality and immunity system improvement) have been well documented in the scientific literature (NRC, 1997). In the present study, dietary Cr supplementation at a level 0.5 mg Cr kg^{-1} from different sources significantly improved WG, SGR, FCR, and PER of mirror carp compared to fish fed on the control diet. At the molecular level, it has been reported that chromium may acts as a part of the oligopeptide low molecular weight chromium binding substances (LMW-Cr) or chromodulin (Pechova and Pavlata, 2007). This compound has been found to play a vital role in an auto amplification mechanism in insulin signalling via activating the receptors tyrosin kinase and subsequently affects carbohydrate and lipid metabolism (Anderson, 1981; Vincent, 2000b). In addition, the Cr yeast fed carp gave the highest growth performance (although not significant) compared to the other groups indicating that Cr yeast is the more bioavailable form of Cr in fish diet. It has been reported that the bioavailability of the minerals from the chelated compounds is higher compared with the other chemical forms because the element from the chelated compound will be released in ionic form which make the element ready for the absorption process or will be absorbed as intact chelate (Scott et al., 1982). In addition, the minerals from organic compounds and chelated minerals are less sensitive to the inhibitors and have more bioavailability in practical diets (Davis and Gatlin, 1996). Since 2.0 mg Cr kg^{-1} in fish feeds is far from the optimal level required for the best growth of carp (0.5 mg Cr kg^{-1}) (the results of Cr requirement in Chapter 3), it is noticeable that fish fed at 2.0 mg Cr kg^{-1} had showed a reduction in growth performance compared to the other Cr levels from each source due to the toxic effects associated with the high level of Cr supplementation. This finding is in accordance with the results obtained from the first experiment (Chapter 3).

The results of the final body composition of the current trial demonstrated that dietary Cr supplementation at different levels and sources had no effect on the final body moisture, crude protein, and ash. These results support the finding of the first experiment (Chapter 3) and in line with a previous investigation by Gatta et al. (2001) on rainbow trout. However, the same treatments were effective in increasing the final body lipid content compared to the fish fed on the control diet. It has been suggested that chromium elevated the lipid synthesis by increasing the rate of the lipogenesis pathway in a study carried out on turkey poults (Steel and Rosebrough, 1981). The same results have been reported recently by Magzoub et al. (2010) when tilapia fed different sources of chromium (Cr oxide and Cr yeast).

Although the trivalent form is poorly absorbed, many studies have reported Cr accumulation in animal tissues after a period of diet administration (Tacon and Beveridge, 1982; Debaski, 2004; Ahmed et al., 2005; Zha et al 2007). In this experiment, Cr carcass content increased with increasing dietary Cr concentration and the same increasing trend has been observed in rats (Zha et al., 2007) and fish (Jain et al., 1994) experiments. In the current investigation, the concentration Cr of in liver and gut were very low compared to the total Cr accumulated in whole fish carcass. It has been suggested that the increased in insulin secretion rate and insulin action due to Cr supplementation caused withdrawal of Cr from the liver to be exist in the insulin sensitive tissues (Ahmed et al., 2005).

It has been suggested that carp have a great ability to maintain glucose haemostasis via the adaptation to experimental diets formulated to contain various levels of carbohydrate (Capilla, 2004) which may explain the similarity in glucose levels of the experimental fish despite the significant differences in growth performance between different treatments. In another study, no differences have been detected in blood glucose levels of tilapia fed diets containing Cr yeast or Cr oxide despite that blood samples have been taken at different intervals from the last feeding time (Magzoub et al., 2010).

Different mechanisms may cause DNA single and double strand breaks in living cells such as ionising radiation, ultra violate light, reactive oxygen and reactive intermediates (Lindahl and Ljungquist, 1975). The exposure of cells to genotoxic chemicals may result in DNA modification; however, the chemical exposure could be an indirect reason for strand breakage process (Mitchelmore and Chipman, 1998). The results of the comet assay in the present investigation demonstrated that % Tail DNA damage of fish fed with diet containing 2.0 mg Cr kg^{-1} was significantly higher ($P < 0.05$) compared to all other treatments including the control. It has been reported that Cr(III) directly reacts with DNA molecule producing DNA-mono adducts, DNA-DNA as well as DNA-protein cross-link (Gaddameedi et al., 2011). This result might indicate that dietary inorganic Cr(III) at high levels is genotoxic to mirror carp under the current experimental conditions. The comet assay results could explain the reduced growth rate in this group compared to the lower levels of Cr supplementation from each Cr source. This supports an earlier study in our laboratory conditions where hypoxia and hyperoxia induced DNA damage has been linked with specific growth rate (SGR) in carp (Mustafa et al., 2011). In this regard, it has also been reported that DNA damage in aquatic animals collected from contaminated areas was associated with negative effects on growth, reproduction and population dynamics (Lee and Steinert, 2003). Likewise, reduced growth and increased DNA damage have been reported in controlled studies with mussels (*Mytilus edulis*) when subjected to poly aromatic hydrocarbons and UV exposure (Steinert et al., 1998). To our knowledge the genotoxicity of dietary Cr(III) in fish has not been investigated both in a nutritional and environmental context.

Little is known about the effect of Cr on the key liver metabolic enzymes; however, the glycolytic enzyme -HK- which is associated in the first step of the glycolysis pathway and the lipogenic enzyme -G6PD- which is involved in the initial step of lipogenesis pathway are widely identified in fish nutrition studies which aim to elucidate the mechanisms controlling

carbohydrate utilization (Carvalho and Fernandes, 2008; Enes et al., 2008b; Enes et al., 2010). Since organic Cr has more bioavailability and is well absorbed compared to inorganic compounds (Pechova and Pavlata, 2007), it was not surprising that fish fed with 0.5 mg Cr kg^{-1} of diet as organic Cr (CrPic and Cr yeast) showed significantly higher HK activity compared to the other treatments. It has been reported that chromium oxide supplementation facilitates the glycolysis pathway leading to glucose utilization improvement (Shiau and Liang, 1995), although Cr oxide is recognized to be an inert dietary marker used for digestibility studies (Davies and Gouveia, 2010).

In this study, all groups fed diets supplemented with Cr at different levels and sources showed significant differences in the activity of G6PD. It has been reported that Cr chloride improves the lipogenesis pathway (Steel and Rosebrough, 1981).

In fish nutrition studies, liver histology is considered to be a proper tool to evaluate the general fish health due to the important role of the liver in various metabolic processes (Mela et al., 2007; Nogales Mérida et al., 2010; Ferri et al., 2011). In this study, the liver of fish fed with 2.0 mg Cr kg^{-1} showed fatty changes and vacuoles which can be explained by the poor regulation of lipid stores due to the toxicity effects. To our knowledge, the effect of dietary chromium (III) at high levels on liver histology has not been investigated. However, liver vacuolization has been observed in the liver of goldfish exposed to Cr(VI) (Velma et al., 2010). The results of the current experiment showed that dietary Cr supplementation at a level 2.0 mg Cr kg^{-1} caused necrosis and fusion in the mucosal folds of the intestine and different levels of tissue fusion of mirror carp fed high Cr level for 8 weeks. The necrosis of different tissues (liver and kidney) of goldfish exposed to high levels of Cr (VI) in a study by Velma et al., (2010) was attributed to the accumulation of inflammatory cells.

In conclusion the benefits of feeding dietary Cr at 0.5 mg Cr kg^{-1} supplementation from different sources are enhanced carbohydrate utilization and growth performance in carp and Cr may affect growth rate by activation of some key liver enzymes (HK and G6PD).

A summary of the experimental results:

• Fish fed on different levels of Cr gave the same growth performance.

• The current study on mirror carp showed that different sources of Cr affected the final lipid content of fish carcass compared to the fish fed the control diet. This result is inconsistent with the result of the previous investigation that curried out on common carp indicating that different strains may have different responses to the dietary Cr supplementation.

• Cr carcass increased with increasing dietary Cr supplementation.

• High level of Cr (2.0 mg kg^{-1}) induced DNA damage in erythrocytes cells.

• Different sources of Cr significantly induced HK and G6PD activity in the liver compared to the control diet fed group.

Chapter 5

Experiment III

The effect of different levels of chromium yeast on the utilization of starch and dextrin in Common carp

Results from this chapter have been published in Journal of Biological Trace Element Research:

Arafat R. Ahmed, Awadhesh N. Jha and Simon J. Davies. The Effect of Dietary Organic Chromium on Specific Growth Rate, Tissue Chromium Concentrations, Enzyme Activities and Histology in Common carp, *Cyprinus carpio* L. Journal of Biological Trace Element Research. DOI: 10.1007/s12011-012-9436-3.

Abstract

A 63-day feeding trial was carried out to investigate the effect of three levels of Cr yeast (0.5, 1.0, and 2.0 mg Cr kg^{-1}) on the utilization of diets containing 38.5 % of maize starch or dextrin in common carp, *C. carpio* L. (initial mean body mass 14 ± 0.3 g) in auto circulator system at 25 ± 0.5 ºC. A two-way analysis of variance (ANOVA) showed that the final body weight, weight gain, specific growth rate, and feed conversion ratio were significantly (P < 0.05) affected by the two sources of variation (carbohydrate source and Cr level). In general, fish fed on a diet containing starch and fortified with 0.5 mg Cr kg^{-1} performed significantly higher FBW (47.23 g), % WG (225.11), SGR (1.91), and lower value of FCR (1.24) compared to fish fed on the other diets. Carp fed on 2.0 mg Cr kg^{-1} with maze starch ad 1.0 0mg Cr kg^{-1} with dextrin based diet showed a significant reduction (P < 0.05) in whole body lipid content as confirmed by a two-way ANOVA. Fish fed on maize starch-based diet and supplemented with 0.5 and 1.0 mg Cr kg^{-1} recorded the highest activities for hexokinase (HK) enzyme (although not significant). Glucose-6-phosphate dehydrogenase (G6PD) activity was neither affected by Cr concentration nor by dietary carbohydrate source. Fish fed dextrin-based diets accumulated higher Cr in the whole tissue compared to fish fed on starch-based diets. Normal histological structures in the liver and gut tissues were observed in all groups. The present data clearly showed that dietary Cr yeast was safe in the fish diet at the levels tested.

Chapter 5: The effect of different levels of chromium yeast on the utilization of starch and dextrin in Common carp

5.1 Introduction

Regardless of species, fish do not have specific requirements for dietary carbohydrate per se and they grow normally when fed on a diet free from carbohydrate (NRC, 2011). The reason for this is due to their gluconeogenesis capacity whereby glucose is synthesized from non-glucose precursors such as amino acids (Berg et al., 2007). However; from the economic and an environmental perspective, carbohydrate is considered to be a regular source of nutrients in fish diets with little negative impact on the ecosystem (Moon, 2001; Enes et al., 2010). Fish have a limited capacity for dietary carbohydrate digestion and the efficiency of carbohydrate utilization by fish depends on the molecular complexity of carbohydrate (Stone, 2003; Enes et al., 2009). It has been reported that most fish species use complex carbohydrate for growth better than the more simple forms such as glucose (Tung and Shiau, 1991; Lee et al., 2003; Tan et al., 2006). On the other hand, other studies reported the opposite observation (Tian et al., 2004; Enes et al., 2008). It seems that the variation in carbohydrate utilization by fish can be affected by different factors such as the differences in the digestive and physiology metabolic system of each species (Walton and Cowey, 1982), carbohydrate source (Tan et al., 2006), and dietary carbohydrate level inclusion (Peres et al., 2002).

With the rapid expansion in aquaculture industries, carbohydrate utilization improvement in farmed fish is one of the major challenges. Hence, different studies have been carried out in order to reduce cost and enhance carbohydrate utilization in different fish species. Creating transgenic fish is one of the possible strategies that can be used to achieve this goal (Ense et al., 2009). As an attempt to improve the efficiency of carbohydrate metabolism in salmonid fish, human glucose transporter type I (hGluT1) and rat hexokinase type II cDNAs were cloned with viral (CMV) and piscine promoters and microinjected into rainbow trout and arctic charr (*Salvelinus alpinus* L.) eggs (Pitkanen et al.,1999). The latter study expected to

obtain practical results and conclusions in the second generation of fish. However, it seems that the application of this kind of studies in aquaculture is limited because the improvement of growth performance by transferring the growth hormone gene (as an example) have been shown to affect other characters (could be negative or positive) such as body shape and composition, feed conversion efficiency and disease resistance (Pillay and Kutty, 2005). Therefore, the enhancement of dietary carbohydrate utilization in fish by diet modification could be economic and more acceptable. In addition, consumer acceptance as well as potential negative views concerning the use of the genetically modified organisms in aquaculture.

Chromium has been distinguished as a vital element involved in glucose metabolism and subsequently growth improvement for different domesticated and farmed animals (NRC, 1997). Several studies have provided a clear evidence of the positive effect of Cr (organic and inorganic compounds) as a growth inducer in carp (Experiments I and II) and tilapia (Shiau and Lin, 1993; Pan et al., 2002). Despite that, the mechanism by which Cr affects or modulates carbohydrate utilization is still unknown. As previously mentioned, the poor utilization of dietary carbohydrate in fish was partly attributed to the low level of hexokinase enzyme which is responsible for providing the intermediate metabolite molecule (glucose-6-phosphate) for the glycogenesis and pentose-phosphate pathway. The most interesting result of the second experiment (Chapter 4) demonstrated that the different sources of Cr (Cr chloride, Cr picolinate and Cr yeast) at a level 0.5 mg Cr kg^{-1} significantly improved growth and carbohydrate utilization in mirror carp compared with fish fed on the control diet and Cr yeast showed the highest weight gain concurrent with the highest HK activity. The findings were attributed partly to the activation of liver HK. Therefore, this experiment was designed to evaluate the effect of different levels of organic Cr (Cr yeast) on the utilization of two

sources of carbohydrate (starch and dextrin) and impact of Cr and starch or dextrin diet on liver HK at the enzymatic and molecular level in common carp.

The specific hypotheses tested in this experiment were as follows:

Hypothesis 1: Growth performance is affected by the source of carbohydrate and the level of Cr.

Hypothesis 2: Different sources of carbohydrate and various levels of Cr affect enzymes activity, gene expression, and liver and gut histopathology.

Hypothesis 3: Different treatments affect the accumulation of Cr in whole fish tissue.

5.2 Materials and methods

5.2.1 Growth trial protocol

The aim of the experiment is to investigate the effect of different levels of chromium yeast on the utilization of two sources of carbohydrate (maize starch and dextrin). The growth trial protocol is presented in Table 5.1.

Table 5. 1 Experimental protocol for growth trial.

Fish	Common carp
Stocking density	15 fish in each tank.
Acclimation period	4 weeks fed EWOS Sigma diet (please see Chapter 2 for the ingredients).
Diets	6 diets contain three levels of chromium (0.5, 1.0, and 2.0) mg Cr $kg^{-1}$and two sources of carbohydrates (starch and dextrin).
Chromium source	Organic compound (chromium yeast).
Duration of study	9 weeks.
Feeding regime	3% of body weight four times a day.
Parameters tested	Growth parameters, initial and final body composition, Cr accumulation in liver, gut and whole fish tissue, plasma glucose level, key liver enzymes (HK and G-6-PD), HK gene expression, and gut and liver histology.

5.2.2 Diets ingredients and preparation

Ingredients and proximate composition of the experimental diets are presented in Table 5.2. Two sources of carbohydrate (starch and dextrin) were used to formulate the experimental diets. Three diets were formulated for each carbohydrate source to contain 0.5, 1.0 and 2.0 mg Cr kg^{-1} as Cr yeast and labelled as follows: 0.5 M, 1.0 M, 2.0 M, 0.5 D, 1.0 D, and 2.0 D. As Cr yeast is insoluble in water, the defined amount has been mixed with the other

ingredients as powder. The sources of the main ingredients and diet preparation protocol were described in Chapter 2.

Table 5. 2 Formulation and chemical composition of experimental diets.

	Diets					
Ingredients (g kg^{-1})	**0.5 M**	**1.0 M**	**2.0 M**	**0.5 D**	**1.0 D**	**2.0 D**
Herring Meal LT94	250	250	250	250	250	250
Maize starch or dextrin	382.2	382.2	382.2	382.2	382.2	382.2
Pea protein concentrate	164.5	164.5	164.5	164.5	164.5	164.5
Corn gluten concentrate	150	150	150	150	150	150
Sunflower oil	33.3	33.3	33.3	33.3	33.3	33.3
Mineral Premix	20	20	20	20	20	20
*Cr added (mg kg^{-1})	0.5	1.0	2.0	0.5	1.0	2.0
Cr analysed (mg kg^{-1})	0.67	0.97	1.60	0.69	0.99	1.59
Proximate composition (g kg^{-1}dry matter)						
Moisture	52.7±1.3	53.1±1.1	53.2±2.8	54.2±1.5	53.6±0.7	53.5±2.5
Protein	382.1±2.8	384.7±3.1	383.6±1.3	383.5±0.9	383.3±4.1	382.3±2.6
Lipid	63.4±2.2	64.5±1.6	64.5±3.1	63.6±2.7	64.5±0.8	63.7±1.1
Ash	52.3±0.2	52.2±0.8	51.9±1.3	51.8±0.4	52.4±0.9	52.7±0.3

Proximate composition data are mean ± SD

*Cr yeast source (BiochromeTM): Alltech, USA; maize starch and dextrin: Roquette Company, Frêres, France.

5.2.3 Growth trial

Prior to the growth trial, the fish were acclimated and fed daily as mentioned in Chapter 2. At the beginning of the nutrition trial, batches of 15 fish (initial mean body weight 14 ±0. 3g) were distributed in 18 tanks (three replicate per treatment). The system design was described in Chapter 2.

5.2.4 Water quality

During the trial, water temperature was adjusted to 25 ± 0.5°C. The following pH (6.48 - 7.03), dissolved oxygen (92-97 %), Ammonia (0.000-0.509 mg l^{-1}), Nitrite (0.000-0.009) mg l^{-1} and Nitrate (11.22-36.92 mg l^{-1}) ranges were recorded. The range of Cr concentration in the system was 0.02-0.07 µg l^{-1}.

5.2.5 Fish weighing and sampling

Fish weighing, anaesthesia, and sampling procedure were as described in Chapter 2.

5.2.6 Growth performance calculation

To evaluate growth performance of fish at the end of feeding trial, growth performance equations described in Chapter 2 have been applied.

5.2.7 Proximate analysis of the diets and whole fish tissues

Moisture, crude protein, crude lipids, and ash determination were described in details in Chapter 2.

5.2.8 Cr analysis in Cr yeast product, diets and whole fish tissue

The description of Cr analysis in the experimental diets and whole fish tissues were as described in Chapter 2.

5.2.9 Cr accumulation in liver and gut tissues

Tissues digestion and Cr analysis in liver and gut tissues were performed as described in Chapter 2.

5.2.10 Plasma glucose measurement

Number of samples, blood sampling, plasma collection and storage, and plasma glucose measurement protocol were followed as described in Chapter 2.

5.2.11 Enzyme activity measurement

Liver collection, storage, and enzyme measurement protocols were as described in Chapter 2.

5.2.11.1 Hexokinase

Buffer preparation and HK measurement protocol were described in Chapter 2. It is important to mention that each liver sample has been divided into two pieces (one piece for HK enzyme assay and the other for the gene expression assay) and both samples were stored at -80ºC until analysis. Enzyme assay was performed on 2 fish per tank (6 fish per treatment).

5.2.11.2 Glucose 6 phosphate dehydrogenase

Buffer preparation and G6PD measurement protocol were described in Chapter 2. Enzyme assay was performed on 2 fish per tank (6 per treatment).

5.2.12 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is an *in vitro* technique established in 1983 in molecular biology laboratories and rapidly became one of the most reliable and sensitive tool used in different medical and biological studies. Figure 5.1 presents the major steps of the PCR assay.

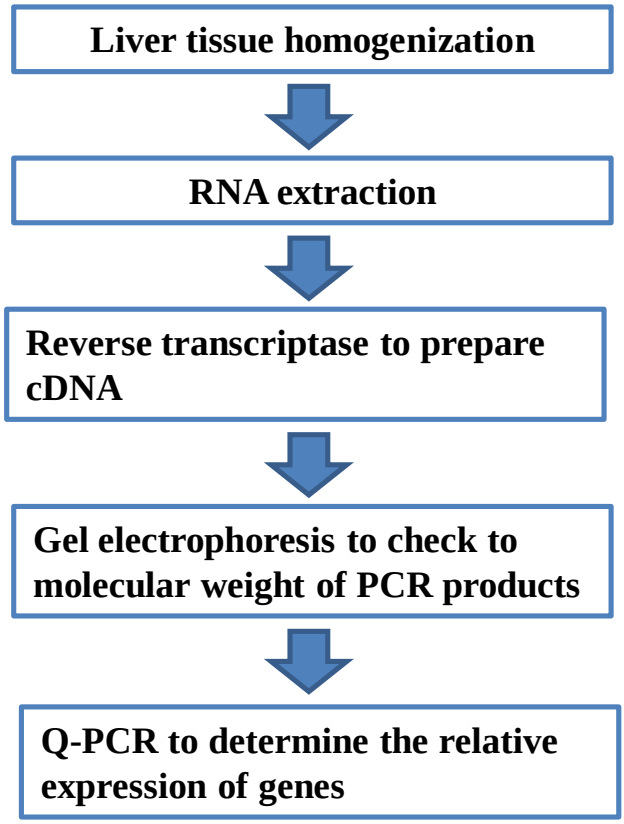


Figure 5. 1 Major steps of the polymerase chain reaction assay.

5.2.12.1 RNA extraction

Total RNA was extracted from ~ 20 mg of frozen liver tissue using a Sigma GenElute mammalian total RNA extraction kit (RTN70, Sigma, Poole, UK) following the manufacturer's procedure. The weighed sample was placed in a plastic micro-centrifuge tube

and 500µl of lysis buffer and 5 µl of mercapto-ethanol were added. Later, the tube is embedded in small ice box and the tissue homogenized using a Microsone Ultrasonic Cell Disruptor, USA for a few seconds. After tissue homogenization, the manufacturer's protocol for RNA extraction was applied and the extracted RNA was collected in pre-labelled tubes. RNA concentration and quality was evaluated using a NanoDrop spectrophotometer at A260:280. (RNA samples with low purity have been ignored and the extraction has been repeated). The RNA samples were stored at -80 ºC until analysis. The traditional RNA isolation steps are presented in Figure 5.2.

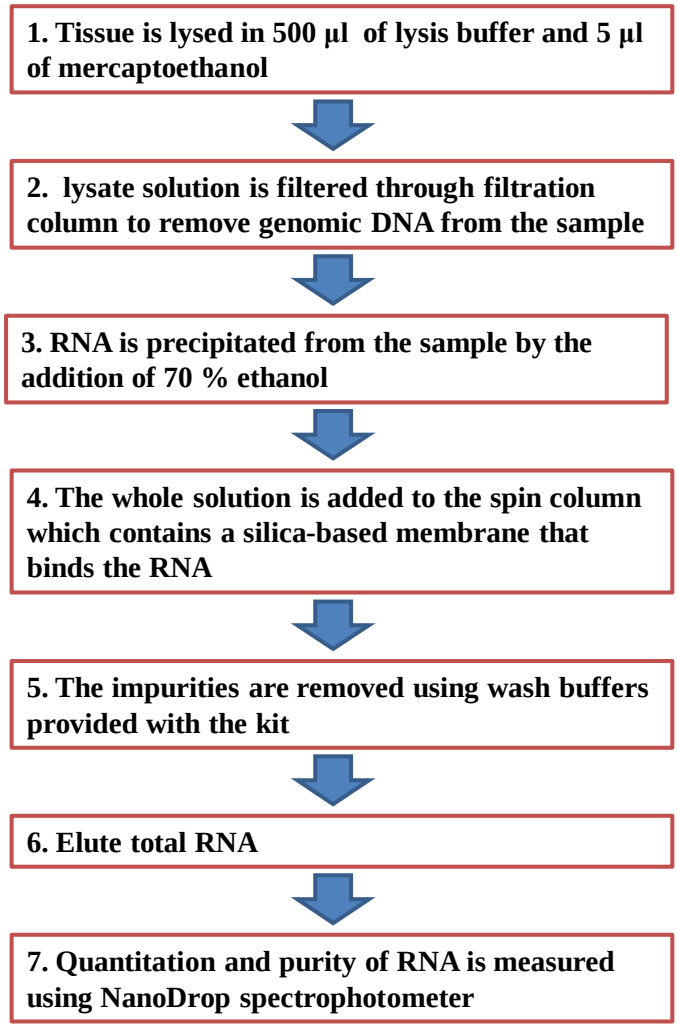


Figure 5. 2 Principle steps involved in RNA extraction using Sigma a GenElute mammalian total RNA extraction kit.

Step no. 5 in Figure 5.2 above involves two washes of the column filter using wash solution I and another two washes using wash solution II (500 µl was used in each step). After the first

wash, 10 µl of DNase and 70 µl of digestion buffer were added on the top of each filter and the columns were incubated for 15 minutes at room temperature. The purpose of this step is to remove any DNA contamination to produce a high quality RNA sample.

5.2.12.2 M-MLV Reverse Transcriptase procedure for the preparation of cDNA

The manufacture's procedure (Sigma, Poole, UK) was followed to convert the total RNA to the cDNA samples using 1 µl of total RNA template. The components and volumes required for each 1 µl of RNA are as listed below:

Table 5. 3 Master mix number 1.

1 µl Dioxynucleotide triphosphate sodium
1 µl Specific primer, random nonamers
1 µl RNA template
(?) µl Nuclease free water
10 µl Total volume

These components were mixed gently and were incubated at 70 °C for 10 minutes in StepOne PCR Biosystem, UK. Later, the tubes were removed and placed on ice for 5 minutes. During the incubation period mentioned above, the master mix no.2 was prepared (Table 5.4).

Table 5. 4 Master mix number 2.

2 µl Reverse Transcriptase buffer
1 µl Reverse Transcriptase enzyme
0.5 µl RNase inhibitor
6.5 µl Nuclease free water
10 µl Total volume

The total volume of the mixture no. 2 (10µl) was added to the total volume of the mixture no.1 to produce 20 µl as a final volume which was centrifuged for 5 seconds and incubated at room temperature for 10 minutes prior to the 37 °C incubation for 50 minutes. The reaction conditions were 21 °C for 10 minutes, 37 °C for 20 minutes, 94 °C for 5 minutes. The produced cDNA samples were stored at -20 °C until further analysis.

5.2.12.3 Primers design

Using the housekeeping genes in PCR assay relies on the assumption that their levels of expression remain constant in different cells, samples, and treatments. Therefore they have been used to normalize the data. Using ß-actin as a housekeeping gene is common in genomic studies carried out on fish (Maples and Bain, 2004; Hook et al., 2006; Soengas, et al., 2006).

ß-actin as a housekeeping gene for *Cyprinus carpio* (accession number: M24113.1) and HK-I for the same species (accession number: 119837) were designed using the National Centre for Biotechnology Information database (NCBI) http://www.ncbi.nlm.nih.gov/ and the primers characteristics were assessed using Sigma DNA calculator website http://www.sigma-genosys.com/calc/DNACalc.asp.

HK and beta actin specific primers used in the PCR procedure are presented in Table 5.5.

Table 5. 5 Hexokinase and ß-actin primers (forward and reverse) used in the experiment.

Gene	5´-3´ Forward primer	3´-5´ Reverse primer
β-actin	TCGCTTAGGCCTTGCTCTTCAAACA	GGCTGTCGCGTGCACATTGC
HK-I	CGCCTCCGCACGATAGTGGG	GACTCCGGCACCAGACGACG

The product size of these primers was 92 and 101 pb for β-actin and HK-I respectively.

5.2.12.4 Polymerase chain reaction procedure

Principle

The principle of the PCR is to generate a huge number of copies from one single copy of the target gene. The amplification process of the PCR can selectively amplified a single copy of a desired sequence that exists in a complex mixture containing optimized concentrations of the DNA template, Taq polymerase enzyme, primers and deoxynucleotides (dNTPs). DNA polymerase will add complimentary deoxynucleotides (dNTPs) to the DNA template producing a new DNA molecule. The primers (small pieces of DNA consist of 15-30

nucleotides long) are used as a starting point in the polymerase process. Heating and cooling the reaction mixture is an essential factor controlling the whole procedure as described in Figure 5.3.

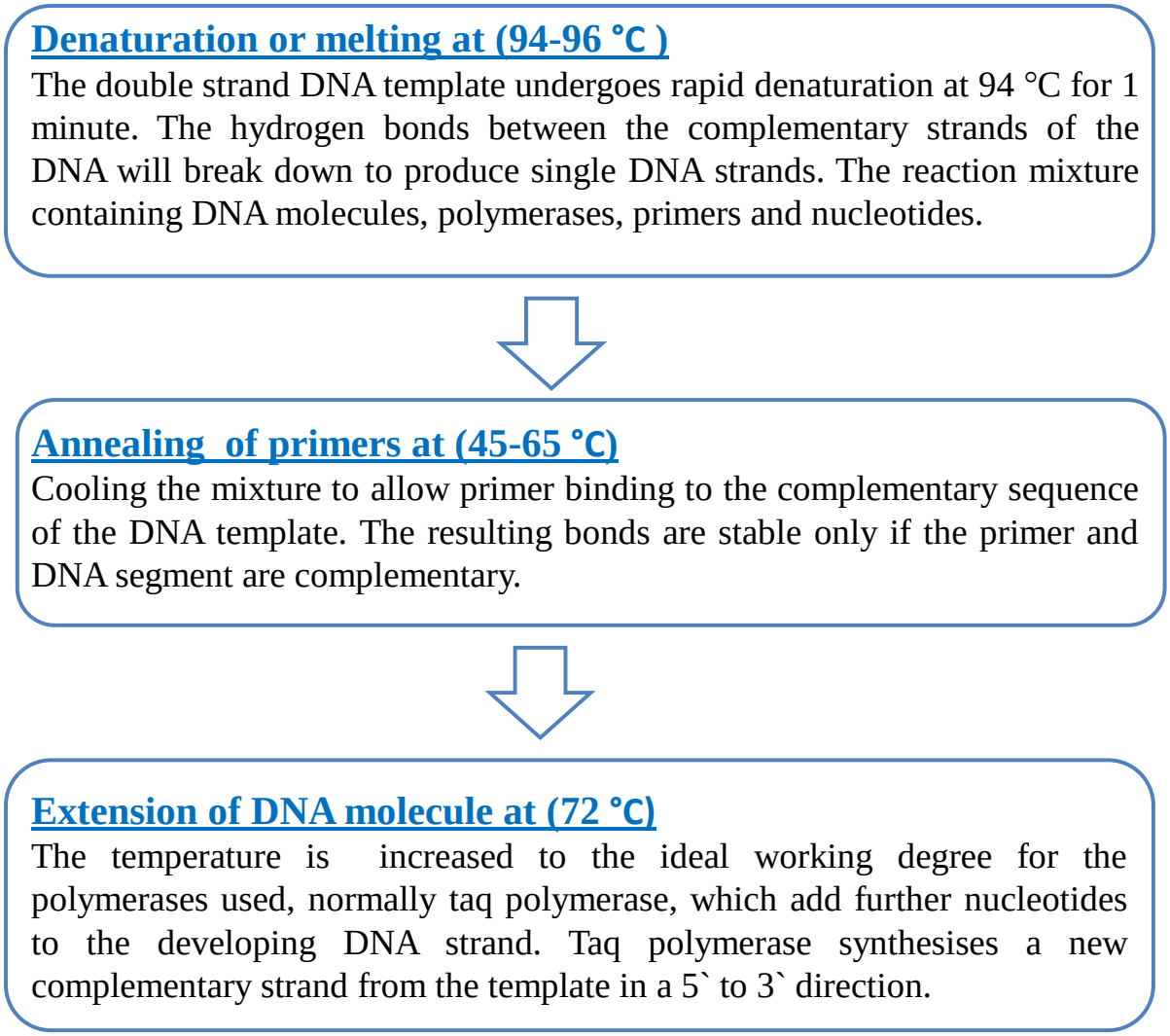


Figure 5. 3 The principle steps of the polymerase chain reaction (PCR) protocol.

The number of DNA strand copied doubles with each cycle (30-40 cycle) as presented in Figure 5.4. It is worth mentioning that the amplification process is limited due to two factors. Firstly, the amount and the activity of enzyme will decrease after 25-30 cycles of PCR. Secondly, the efficiency of reannealing of target strands will decrease with increasing the concentration (Newton and Graham, 1995).

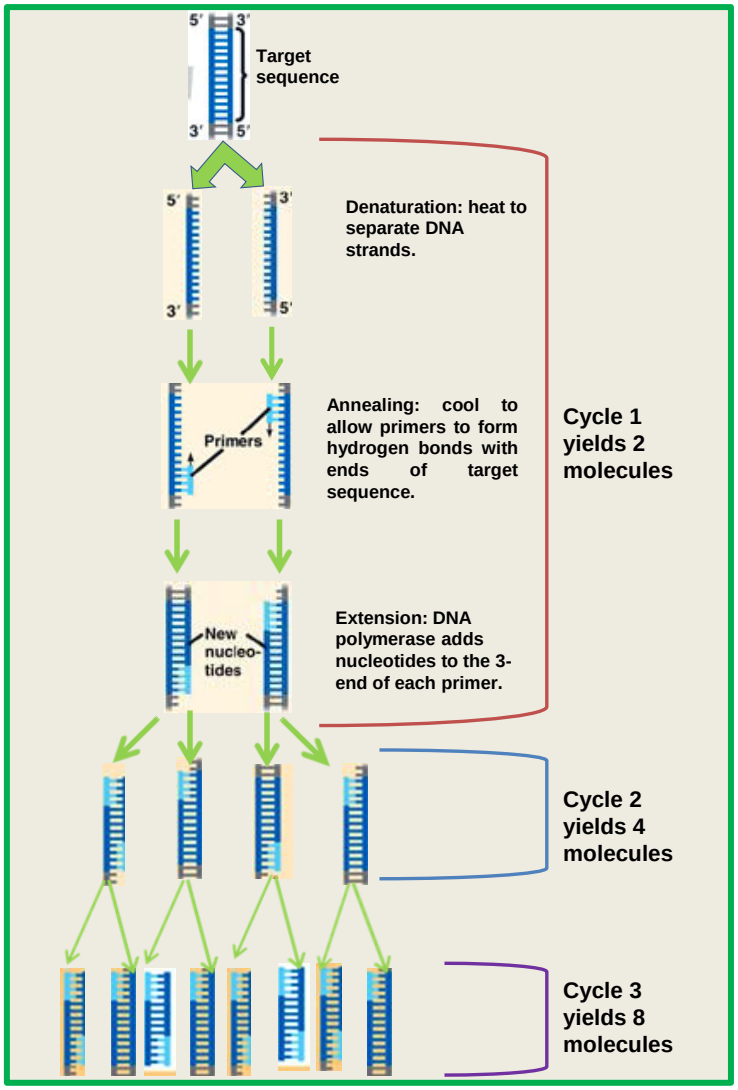


Figure 5. 4 Schematic representation of polymerase chain reaction.

5.2.12.5 Relative quantification

Quantitative polymerase chain reaction was applied on six samples per treatment using the Syber Green method. Negative controls along with samples of target and housekeeping gene were performed in triplicate. PCR reaction conditions were 35 cycle of denaturation at 94 °C for 1 minute, annealing at 60 °C for 40 second, and extension at 72 °C for 1 minute. The SyberGreen master mix components and their volumes are presented in Table 5.6.

Table 5. 6 Preparation of SyberGreen master mix for the PCR reaction.

12.5 µl SYBER Green jumpStar
0.25 µl Reference dye
0.5 µl Forward primer
0.5 µl Reverse primer
2.0 µl cDNA template
9.5 µl Molecular water

Later, Gel electrophoresis is used in order to determine the presence or absence of PCR products and quantify the length of the DNA molecule. In this technique, the DNA molecule is separated by its movement through an agarose gel under the influence of an electric field. An agarose gel is prepared by suspending 1.4 g of agarose in a buffer solution (70 ml 1x Tris Acitate EDTA or TAE), boiling in a microwave until the solution becomes clear, and then pouring it into a tray provided for this purpose and allowing it to cool. During electrophoresis, the gel is submersed in a chamber containing a buffer solution (1x TAE) and provided with a positive and negative electrode. The DNA samples were mixed with 2.5 µl of a suitable DNA dye (orange dye) and 25 µl of each sample was loaded into the wells of the gel along with a ladder sample. Under an electrical field, DNA will move to the positive electrode (red side) and away from the negative electrode (black side). DNA movement rate depends on the size of the DNA molecules, strength of the electrical field, and the concentration of agarose used in gel preparation. The size of PCR products is confirmed by comparison with the known size of DNA fragments (a ladder) loaded along with DNA samples. A DNA ladder is a solution of DNA molecule of different lengths used as a reference to estimate the size of unknown DNA molecule.

Calculations

The data were calculated as follows:-

(ΔCt) = Ct of target gene – Ct of endogenous control (actin)

ΔΔCt is the difference between the sample ΔCt and that of the internal control.

Data are expressed as relative quantity (RQ).

Relative quantity = 2 – (Δ CT sample – Δ Ct control)

Where CT is the cycle threshold

5.2.12.6 Histological examination

Liver and gut tissue collection, processing, sectioning and staining were as described in Chapter 2.

5.2.13 Statistical analysis

Data were analysed using Statgraphics 5.1 (StatPoint Technologies Inc, USA). The General linear model was used to compare means of the main effects and to detect the interaction between the variables followed by LSD to detect the significant differences between the means. The *P* value was fixed at 0.05. All data are presented as mean ± standard deviation (SD).

5.3 Results

5.3.1 Growth performance

The survival rate of the experimental fish was 100% for all the fish fed on different diets.

Growth performance of common carp throughout 5.5 weeks of feeding is presented in Figure 36. It is apparent from the mean body weight that fish fed on starch-base diet with 0.5 mg Cr kg^{-1} performed significantly ($P < 0.05$) the best growth performance compared with the other experimental groups.

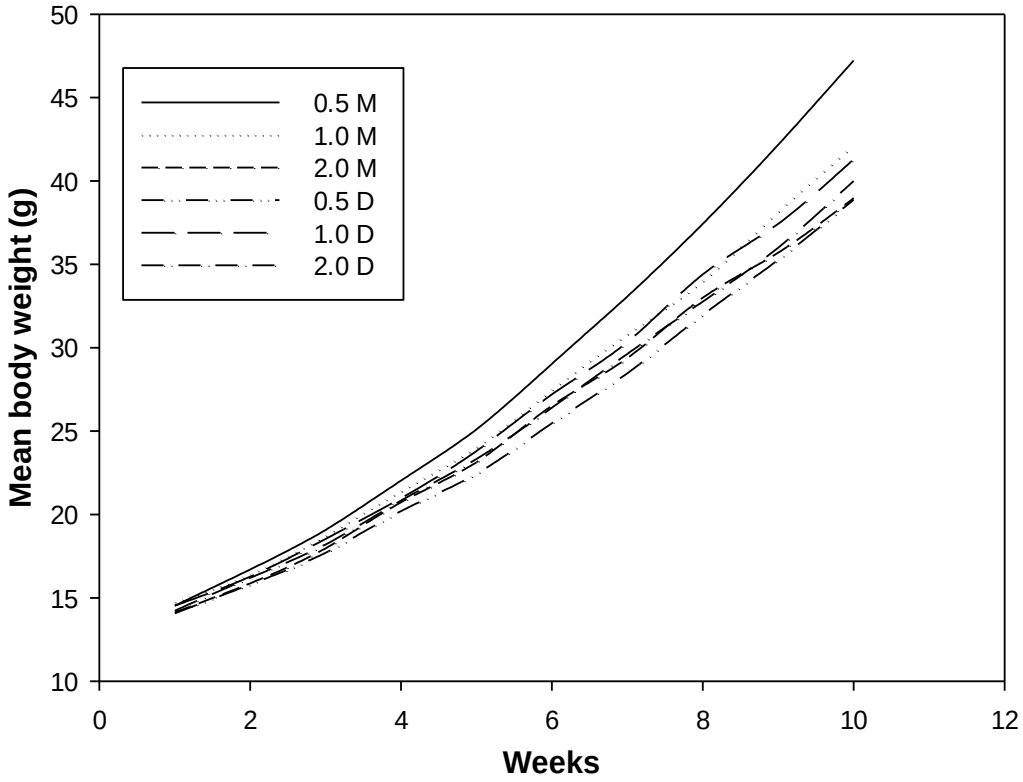


Figure 5. 5 Growth performance of common carp fed with respective diets at weekly intervals of 9 weeks.

In general, fish fed with 0.5 M diet showed significantly ($P < 0.05$) higher response in terms of final body weight (47.23), % weight gain (225.11), specific growth rate (1.91) and feed conversion ratio (1.24), and protein efficiency ratio (2.02) than those fed on the other experimental diets. The General linear model revealed a significant interaction ($P < 0.05$) between these two variables (carbohydrate source and Cr level) for all growth parameters except the protein efficiency ratio (PER). Although fish fed 0.5 M showed significantly ($P< 0.05$) higher protein utilization compared to the other groups. The growth parameters recorded in the current study are presented in Table 5.7.

Table 5. 7 Growth performance and feed utilization of common carp fed diets containing different levels and sources of chromium over 9 weeks.

	Experimental diets						Interaction
Parameters	**0.5 M**	**1.0 M**	**2.0 M**	**0.5 D**	**1.0 D**	**2.0 D**	**(*P value*)**
Initial mean weight (g)	14.53±0.18	14.47±0.09	14.13±0.28	14.07±0.47	14.53±0.18	14.23±0.14	-
Final mean weight (g)	47.23±1.74a	42.13±0.09b	38.97±0.04c	38.87±1.22c	41.30±1.83bc	40.00±0.23bc	0.001
Weight gain %	225.11±16.22a	191.29±5.04b	175.37±4.01b	176.34±10.57b	184.12±3.43b	189.25±4.53b	0.006
SGR	1.91±0.08a	1.72±0.02b	1.64±0.03b	1.71±0.03b	1.68±0.06b	1.72±0.04b	0.007
FCR	1.24±0.05a	1.38±0.01b	1.47±0.04c	1.44±0.06bc	1.41±0.02b	1.38±0.01bc	0.004
PER	2.02±0.08a	1.81±0.02b	1.70±0.095 b	1.73±0.07b	1.77±0.03b	1.81±0.01b	ns

Values are mean ±SD and *n*= 3.

5.3.2 Proximate body composition

The proximate composition of whole fish body (final and initial) is presented in Table 5.8. The results of proximate analysis of whole fish carcass showed that moisture, protein, and ash content were similar in different groups regardless the source of carbohydrate or the level of Cr in the diets, while lipid were significantly affected ($P < 0.05$) by the two variables (carbohydrate source and chromium level). The lowest values of lipid (9.4 and 9.6 g kg^{-1}) were recorded in fish fed on 2.0 M and 1.0 D respectively.

Table 5. 8 Proximate nutrient composition (percentage wet weight) of common carp before the trial (initial) and at the end of 9 weeks.

		Diets						
Parameters	**Initial fish**	**0.5 M**	**1.0 M**	**2.0 M**	**0.5 D**	**1.0 D**	**2.0 D**	**Interaction**
Moisture	72.6±0.3	72.2±0.3	72.3 ±0.6	72.4±0.4	72.6±0.9	72.6±0.4	72.2±0.3	ns
Protein	14.5±0.2	14.4±0.9	14.2±0.4	14.4±0.3	14.6 ±0.3	14.1±0.6	14.5±0.3	ns
Lipid	11.1±0.2	11.0±0.7 a	12.1±0.3 b	9.4±0.1 c	10.3±0.2 d	9.6±0.6 c	10.9±0.1 ad	0.000
Ash	2.5±0.3	2.6±0.10	2.6±0.6	2.5±1.0	2.6±0.8	2.7±1.8	2.5±1.19	ns

Values are mean ±SD and *n*= 3.

5.3.3 Plasma glucose concentrations

Plasma glucose data at the end of the experiment are presented in Table 5.9. The different diets had no significant affect ($P > 0.05$) on the plasma glucose concentration. The highest value of plasma glucose (2.92 mmol l^{-1}) was recorded in fish on 2.0 D diet and the lowest (2.56 mmol l^{-1}) was in fish fed on 1.0 D.

Table 5. 9 Plasma glucose concentrations (mmol l^{-1}) of carp fed diets containing different levels and sources of chromium and the end of the trial.

	Diets						
Parameters	**0.5 M**	**1.0 M**	**2.0 M**	**0.5 D**	**1.0 D**	**2.0 D**	**Interaction**
Glucose (mmol l^{-1})	2.84±0.46	3.15±0.32	2.81±0.40	2.73±0.53	2.65±0.38	2.92±0.43	ns

Data are presented as means ±SD (*n*=6 fish per treatment).

5.3.4 Cr in liver, gut, and whole fish tissue

Cr accumulation data are presented in Table 5.10. No significant differences were detected in Cr content in the liver of fish fed different levels of Cr and two sources of carbohydrate and the values ranged from (12.88-17.71) µg Cr kg^{-1} in fish fed 0.5 M and 2.0 D respectively. Similarly, the concentrations of chromium in the gut tissues taken from fish fed on different diets were similar at the end of the experiment despite an increasing trend reflecting increased dietary chromium levels. On the other hand, the accumulation of Cr in whole fish tissue increased with increasing dietary Cr supplementation. In general, carp fed dextrin based diet showed higher Cr accumulation compared with fish fed on starch based diets. Significant differences ($P < 0.05$) were observed in Cr carcass content between the different treatments. The lowest value (0.44 mg Cr kg^{-1}) was recorded in fish fed with 0.5 M and the highest value (0.97 mg Cr kg^{-1}) in fish fed on 2.0 D.

Table 5. 10 Chromium carcass content (mg Cr kg^{-1}), liver and gut Cr content (µg Cr kg^{-1}) of fish fed different diets for 9 weeks.

Treatment	0.5 M	1.0 M	2.0 M	0.5 D	1.0 D	2.0 D	Interaction
Liver Cr content	12.88±2.30	14.88±2.90	15.48±2.09	14.91±1.93	15.55±1.86	17.71±2.26	ns
Gut Cr content	42.17±4.3	46.19±2.01	48.88±6.80	45.52±4.62	47.04±2.14	48.14±6.25	ns
Carcass Cr content	0.44±0.12 a	0.55±0.14 a	0.75±0.12 b	0.55±0.12 a	0.85±0.11 bc	0.97±0.10 c	ns

Values are presented as means ±SD, *n*= 2 fish per tank, 6 per treatment. Significant differences are indicated by different letters.

5.3.5 Enzyme activity

5.3.5.1 Hexokinase

The activity of HK in the liver of fish fed on the experimental diets is presented in Table 30. The statistical analysis (general linear model) on HK activity data did not show significant differences ($P > 0.05$) between the groups fed on different diets. However; fish fed 0.5 M and 1.0 M diets recorded the highest HK activity (6.36 and 6.06 mU mg protein^{-1}) respectively.

5.3.5.2 Glucose 6 phosphate dehydrogenase

G6PD activity was neither affected by Cr concentration nor dietary carbohydrate source and there were no significant differences ($P > 0.05$) between the different experimental fish fed on different diets. The activity of G6PD data are presented in Table 5.11.

Table 5. 11 Enzyme activity profile (mU mg protein^{-1}) in response to different treatments.

Enzyme	Diets						Interaction
	0.5 M	1.0 M	2.0 M	0.5 D	1.0 D	2.0 D	
HK	6.36±1.90	6.06±1.50	5.14±2.02	4.65±1.38	4.86±1.02	4.40±0.92	ns
G6PD	87.50±9.34	82.37±9.34	75.49±7.97	81.83±7.15	73.68±14.08	83.79±4.32	ns

Values are means standard deviation (±SD), n=6 per treatment.

5.3.6 Hexokinase gene expression

Figure 5.6 illustrates the confirmation of PCR products size using agarose gel electrophoresis. The results suggested that dietary Cr supplementation had no significant effect ($P > 0.05$) on the expression of HK in the liver of fish fed on different diets. However, HK gene expression was higher in fish fed on 0.5 M and 1.0 M diets compared to the others treatments. The relative HK gene expression values are presented in Table 5. 12.

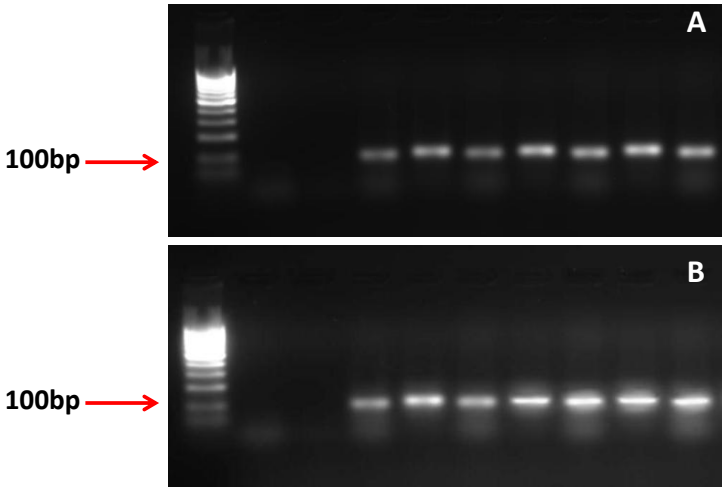


Figure 5. 6 Confirmation the size of PCR products.

1.4 % agarose gels confirming q-PCR products, (A) ß-actin (92bp) and (B) hexokinase (101bp). The samples were loaded in the following order: *Control, 0.5 M, 1.0 M, 2.0 M, 0.5 D, 1.0 D, and 2.0 D.

*The design of this experiment did not include a control treatment for two reasons. Firstly, the positive effect of Cr supplementation on growth has been proved compared to fish fed on the control (un-supplemented diet) in experiment I and II. Secondly, the aim of this experiment was to compare the effect of chromium on the utilization of two sources of carbohydrate. Therefore, the control sample of the second experiment has been used for the PCR run.

Table 5. 12 HK gene expression profile in response to different treatments after 9 weeks of feeding trial.

	Diets						**Interaction**
	0.5 M	**1.0 M**	**2.0 M**	**0.5 D**	**1.0 D**	**2.0 D**	
Relative HK gene expression	8.54±1.52	8.04±2.49	6.53±1.49	6.14±1.77	6.67±1.96	5.59±1.16	ns

Values are means ± SD (*n*=6 per treatment).

5.3.7 Histological examination

5.3.7.1 Liver histology

From the light microscopic examination, no histological changes were observed in the liver of fish irrespective of the experimental diets. Figure 5.7 illustrates the normal structure of liver sections taken from fish fed on starch diets and supplemented with 0.5 or 2.0 mg Cr kg^{-1} and from fish fed on dextrin diets and supplemented with 0.5 or 2.0 mg Cr kg^{-1} at the end of nutrition trial.

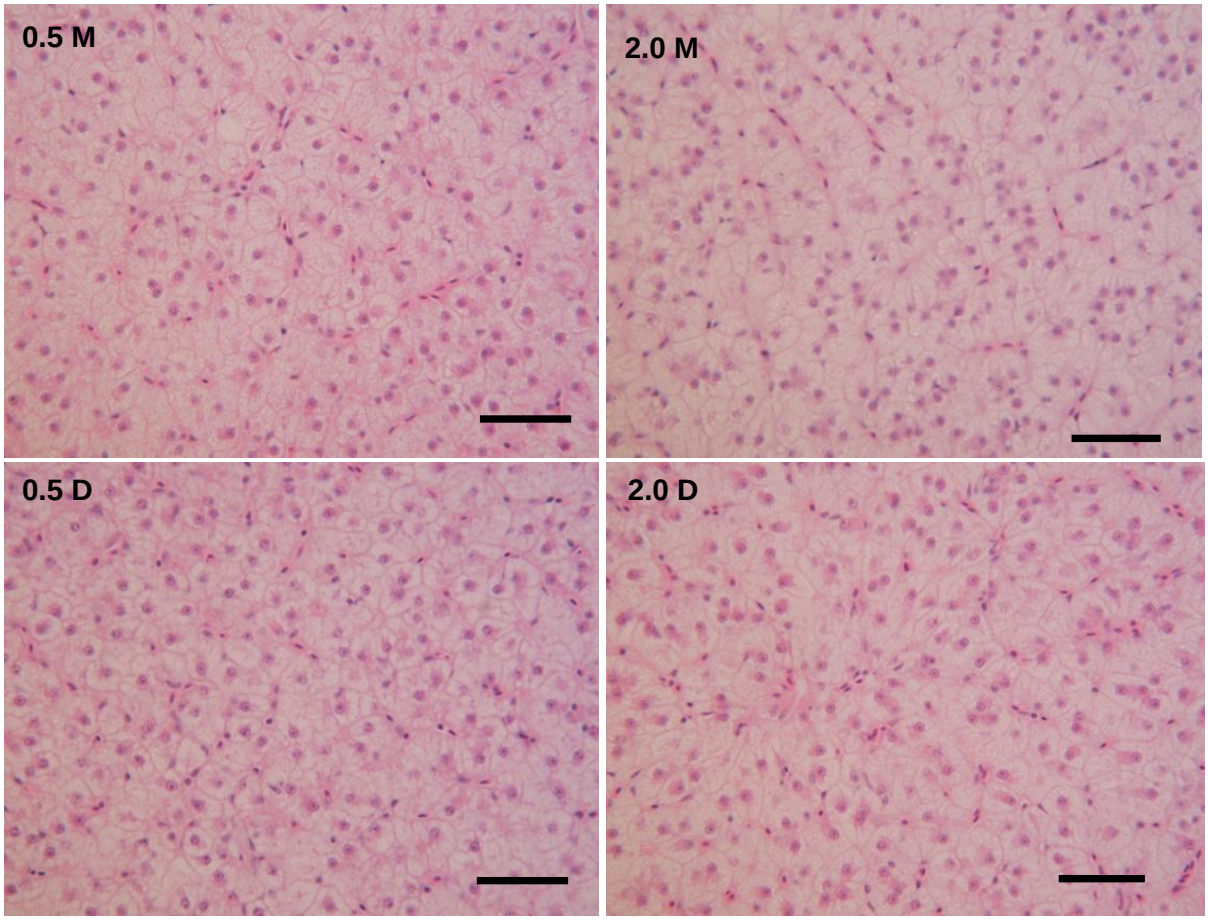


Figure 5. 7 Liver histology of carp fed with maize starch (M) or dextrin (D) diets supplemented with low or high levels of Cr yeast (0.5 and 2.0) mg Cr kg^{-1} showing normal live structure.

*Images for 1.0 M and 1.0 D treatments are not included.

5.3.7.2 Gut histology

From the light microscopic examination, no histological changes were observed in the mid-section of the intestine tissue irrespective of the experimental diets. Figure 5.8 illustrates the normal structure of liver sections taken from fish fed on starch or dextrin diets and supplemented with 0.5 or 2.0 mg Cr kg^{-1} at the end of the nutrition trial.

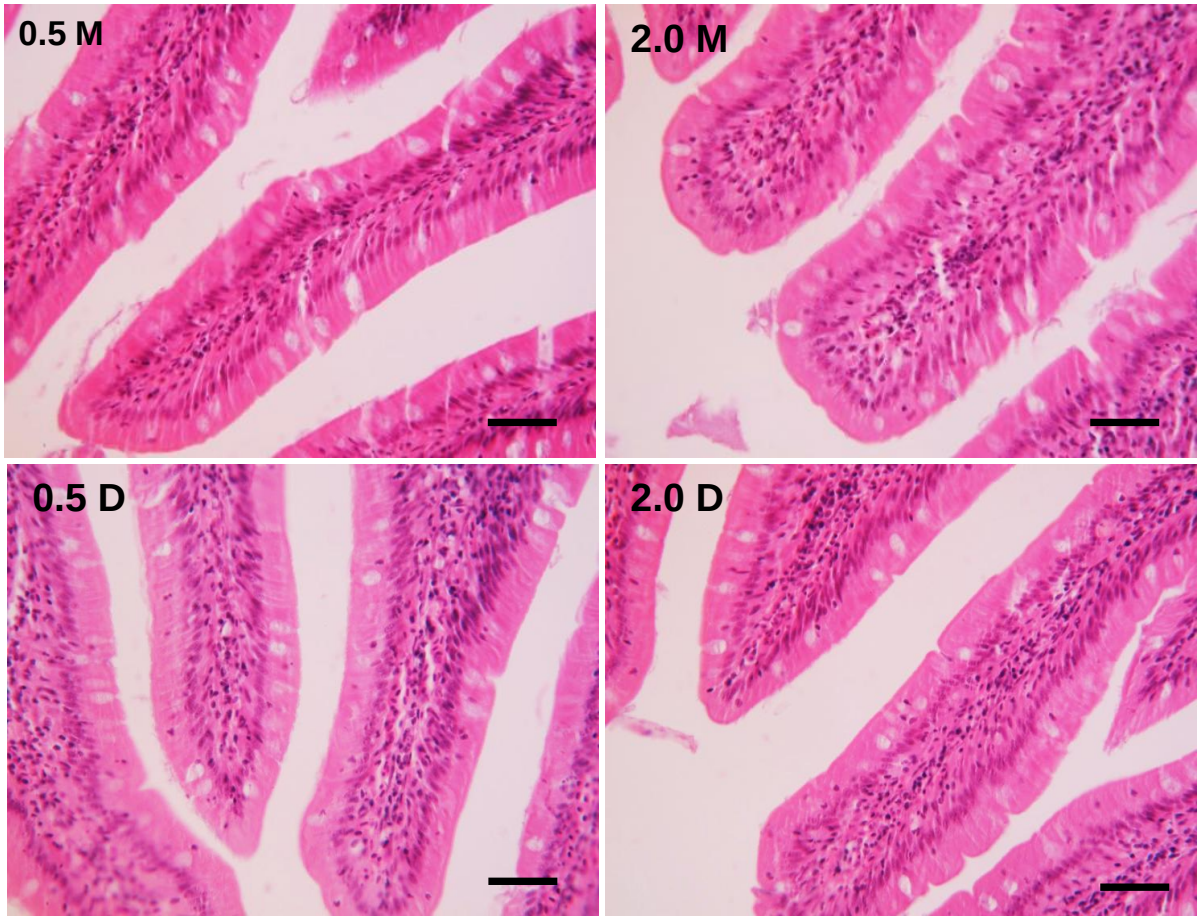


Figure 5. 8 Liver histology of carp fed with maize starch (M) or dextrin (D) diets supplemented with low and high levels of Cr yeast (0.5 and 2.0) mg Cr kg^{-1} depicting normal gut structure.

*Images for 1.0 M and 1.0 D treatments are not included.

5.4 Discussion

Trivalent chromium is often claimed to affect carbohydrate metabolism pathway (Vincent and Bennett, 2007) and the source of carbohydrate could alter the metabolism of chromium (Seaborn and Stoecker, 1989). In this study, growth parameters (except the PER) were significantly affected by the source of carbohydrate and dietary Cr concentration. Fish fed the starch diet supplemented with 0.5 mg Cr kg^{-1} achieved higher weight gain and specific growth rate with the best feed conversion ratio and protein efficiency value. The possible explanation for this result is that complex carbohydrates need more time for digestion and absorption whereas simple sugars are readily absorbed soon after administration which can lead to hyperglycemia and relatively growth reduction (De Silva and Anderson, 1995). The other possible explanation is that fish do not possess the adequate capacity to deal with the high level of absorbed glucose (Panserat et al., 2001). Therefore, the excess amount of the absorbed glucose may be excreted from the blood before the utilization process occurs in the cells (Peres et al., 2002). Similarly, common carp fed on a diet containing 42% maize starch grew better than those fed a diet containing the same level of dextrin (Furuichi and Yone, 1982c). This observation is consistent with the finding reported for catfish (*Mystus nemurus*), an omnivorous species (Hamid et al, 2011). Since that 0.5 mg Cr kg^{-1} meet the requirement of carp (Experiment I), it was not surprising that this level produced the best growth performance (weight gain, SGR, FCR, and PER) in the current study.

In this trial, different treatment did not change the moisture, protein and ash content of whole fish body, and this results support previous investigations carried out on carp (the results of experiment I of this thesis; Liu et al., 2010). Dietary Cr supplementation was able to decreased carcass fat in swine (NRC, 1998) and Japanese quails (Uyanik et al., 2005). This decrease may be due to inhibition of the lipogenesis path way (Gang et al., 2001). The proximate composition analysis of the current investigation demonstrated that both Cr

concentration and carbohydrate sources significantly affected the final lipid content of the whole fish tissue. Two out of six experimental groups showed the lowest lipid content (9.49 and 9.44) % for 2.0 M and 1.0 D diet respectively. It is noteworthy that these groups recorded the lowest G6PD activity (75.49 and 73.68 mU mg protein^{-1}). This finding is in line with Liu et al. (2010) who reported that grass carp fingerlings fed high level of Cr picolinate (1.6 and 3.2 mg Cr kg^{-1}) had significantly lower whole-body lipid content. In addition, a previous study reported that different sources of carbohydrate in the European eel (*Anguilla Anguilla* L.) diet significantly affected the lipid deposition in whole fish tissue (Degani et al., 1986)

In the current trial, the concentrations of Cr were measured in two organs (liver and gut) for specific reasons. The liver considered to be the most important store of the biologically active form of Cr (glucose tolerance factor: GTF) (Outridge et al., 1993), while the intestinal tract is the most effective part of the dietary Cr absorption and excretion. However, the concentration of chromium in the liver was very low and identical in all the experimental groups. Similarly, no differences have been detected in chromium content of the gut between the experimental groups fed on different diets despite the increasing trend reflecting dietary chromium concentration. These results support the previous findings of experiments I and II suggesting that Cr tends to accumulate in other tissues as reported by Beverage and Tacon (1982) in rainbow trout. In contrast, Cr carcass content increased with increasing dietary Cr supplementation in case of starch and dextrin-based diets. Interestingly, fish fed dextrin diets were able to accumulate higher Cr in whole carcass than fish fed on a starch-based diet. The possible explanation for this result is that dextrin has a relatively higher bioavailability and higher absorption rate from the gut compared to starch and this may increase chromium absorption and accumulation. It has been suggested that the source of carbohydrate may alter chromium absorption and retention in mice fed chromium chloride with different sources of carbohydrate included starch, sucrose, fructose, and glucose (Seaborn and Stoecker, 1989). It

is worth mentioning that the latter study demonstrated that mice fed on starch diet accumulated more chromium in their bodies. It is likely that the absorption and retention processes in fish are different from that of mice. In general, the efficiency of mineral absorption by the animal is affected by different factors such as sex, genetic variables, and general health in addition to the nutritional status (Spallholz et al., 1999).

At the end of the growth trial after 1 day of fasting, the plasma glucose concentration was identical for all the experimental groups. The same result was previously observed in the first and second experiments (Chapter 3 and 4). The possible explanation for this finding is that the peak phase of plasma glucose concentration was missed. In addition, it is likely that carp (as an omnivorous fish) have no problem in glucose regulation when fed with different carbohydrate sources as reported in gibel carp (Tan et al., 2006). The result of the current experiment is in agreement with the finding of Liu et al. (2010) who did not observe any significant differences in glucose concentrations of fish fed different levels of Cr-Pic ranged between 0 and 3.2 mg Cr kg^{-1} despite the significant difference in growth performance of grass carp fed different diets.

A previous investigation has indicated that the poor ability in rainbow trout to regulate the blood glucose concentration could be due to the lack of any betterment in glucose phosphorylation capacity in the liver which refers to the stability of HK level that involved in glucose phosphorylation step while increasing the glucose uptake by the fish (Cowey et al., 1977). In this experiment, no significant differences were recorded in HK activity between the different groups. However; the starch-based diet which has supplemented with 0.5 and 1.0 mg Cr kg^{-1} recorded the highest HK activity compared to the other treatments.

It has been suggested that the application of genomic analysis in nutrition studies offered strong outcomes and assessment of nutrients mode of action and the mechanism of effect

(Panserat et al., 2000b; Soengas et al., 2006). In this experiment, no significant differences were detected in HK gene expression in the liver of fish fed on different treatments. The possible explanation for this result is that HK may not adapt to changes in nutritional status or feeding regimen (Tang and Shiau, 1991) or may be the peak of the gene expression was missed.

G6PD catalysis the conversion of glucose-6-phosphate to 6-phosphogluconate in the presence of NADP+ in the first step of the pentose phosphate metabolic pathway (Ciftci et al., 2007). In the present study, the activity of G6PD was not affected by the two variables and there were no significant differences ($P > 0.05$) between the experimental groups fed starch or dextrin with different levels Cr yeast. However, fish fed (1.0 M and 2.0 D) recorded the lowest activity of G6PD which may indicate that chromium at a certain level was able to inhibit the lipogenesis pathway (NRC, 1998).

High level of Cr supplementation (2.0 mg Cr kg^{-1}) as inorganic Cr (Cr chloride) in the carp diet had a negative impact at the structural level of liver and mid-section of intestine which subsequently affected fish health and growth performance (Experiment I and II). On the contrary, the same level of Cr supplementation as Cr yeast (organic Cr) did not show any signs of abnormalities in these organs.

In conclusion, dietary chromium yeast supplementation has showed beneficial effects on growth performance without any side impacts on the parameters tested under the conditions of the current experiment.

A summary of the experimental results:

• Starch-based diet supplemented with 0.5 mg Cr kg^{-1} significantly improved growth performance in common carp.

• Final body weight, weight gain, specific growth rate, feed conversion ratio and whole body lipid content were significantly ($P < 0.05$) affected by the two sources of variation (carbohydrate source and Cr concentration).

• Hexokinase (HK) and Glucose-6-phosphate dehydrogenase (G6PD) activity in the liver tissue were neither affected by Cr concentration nor the dietary carbohydrate source. These results are consistent with the finding of experiment I (Chapter 3) on common carp but inconsistent with the experiment II (Chapter 4) that curried on mirror carp where the mentioned enzymes significantly affected by dietary chromium supplementation.

• Different treatments did not change the normal structure of liver and gut tissues.

Chapter 6

General Discussion

Chapter 6: General Discussion

6.1 General view

The vitamin and mineral premix produced by the leading European animal feed companies including the United Kingdom are mandatory additives in fish diets. Micronutrients such as vitamins and minerals are well known to be essential for the normal life processes at the levels required by each species. Vitamin/mineral premixes are usually complete and include all the principle minerals meeting both macro and trace element requirements. However, the inclusion of Cr (which is considered to be a modifier for protein, lipid, and carbohydrate metabolism) to standard premixes is not allowed because little is known about the requirement level and the risk of the toxicological effects resulting from chromium supplementation (organic and inorganic compounds) to both fish and humans. Therefore, the aim of this thesis was to obtain a holistic view concerning the role of chromium in common and mirror carp nutrition since these are important commercial fish species. Different parameters have been included such as growth performance data, feed utilization, and proximate body composition. Specific key liver enzyme activities (ALT, HK, and G6PD), chromium accumulation in liver, gut, and whole fish tissue, comet assay, liver and gut histology, and gene expression were determined. The main aims were to evaluate the effect of different levels and sources of chromium on growth and the utilization of two sources of carbohydrate (maize starch and dextrin) in common and mirror carp as well as the impact of high levels of chromium on general fish health.

6.2 Cr requirement

As mentioned in the introduction of this thesis (Chapter 1) concerning the previously available studies that elucidated the role of Cr in fish nutrition, the conflicting results that have been obtained in the specific literature may be due to the random selection of Cr concentrations used in different experiments by several workers. Therefore, the first experiment of this thesis (Chapter 3) focused on determination of Cr requirement and

assessment of the potential advantages of dietary inorganic Cr as a growth inducer in common carp. The results of the Cr requirement experiment (Chapter 3) suggested that dietary Cr supplementation at 0.5 and 1.0 mg Cr kg^{-1} as Cr chloride produced the optimal levels of growth parameters (weight gain, specific growth rate, feed conversion efficiency, and protein efficiency ratio). The same results have been found previously for grass carp where 0.8 mg Cr kg^{-1} produced the best growth performance (Liu et al., 2010). It is likely that the level of Cr required by other species is different. For example, Cr supplementation at a level 0.5 mg Cr kg^{-1} did not change the growth performance of rainbow trout (Bureau et al., 1995) and 0.8 mg Cr kg^{-1} in the gilthead sea bream diet did not produce any change in the final body weight (Gatta et al., 2001). These results may reflect the variation in fish capacity to digest dietary carbohydrate. On the other hand, dietary Cr chloride at 2.0 mg Cr kg^{-1} presented as an inorganic compound caused growth reduction which may be attributed to liver and gut tissues impairment as discussed in proceeding chapters, while the same level as organic compound (chromium yeast) did not show any negative effects or toxicological signs such as growth reduction or structural changes in the liver and gut tissues indicating the safety of the organic form of Cr as a supplement in fish diet.

6.3 Whole body proximate composition

It seems that the response of animals including fish to dietary chromium supplementation is not consistent and affected by the physiological status of the animal tested (Anderson, 1998). Chromium showed conflicting effects on the lipid metabolism in the different experiments that were involved in this thesis. In the first experiment, chromium showed no effect on the final lipid content of the whole fish tissue at the various levels tested. This result is in line with the findings of studies carried out on gilthead sea bream (Gatta et al., 2001) and tilapia (Pan et al., 2003). The second experiment suggested that chromium was able to increase lipid synthesis in carp fed on diets containing different sources and levels of chromium compared

with the control groups and the results confirmed the finding of an earlier study carried out on turkey poults which suggested that chromium-supplemented to poults feed increased rates of fatty acid and glycerol synthesis from the glucose substrate and the effect was attributed to the activation of the lipogenesis pathway by dietary chromium supplementation (Steel and Rosebrough, 1981). The same observation has been found recently in tilapia fed chromium yeast or chromium oxide (Magzoub et al, 2010). Surprisingly, the third experiment of this thesis suggested another phase for chromium impact on lipid metabolism when chromium yeast at two levels with different sources of carbohydrate was effective to reduce the lipid content in whole fish carcass. This may indicate that chromium could act as an inhibitor for the lipogenesis pathway at certain levels. Similarly, different sources of chromium were able to decrease fat mass and body fat proportion of rats fed on dietary chromium at a level 300 µg Cr kg^{-1} for 6 weeks (Zha et al, 2007). It has been reported that Cr supplements may become an economical means to decrease fat tissue in pigs (Mertz, 1993). Despite the different effects discussed above, the main conclusion derived from the results of the three experiments of this thesis suggested that the optimal requirement level (0.5 mg Cr kg^{-1}) had no effect on the final body composition of carp.

6.4 Enzyme activity

The activity of enzymes in the liver of fish is affected by different variables (e.g. the nutritional status, diet ingredients, fish species, and the response of an enzyme itself to these variables (Shiau and Liang, 1995; Shikata and Shimeno, 1998; Soengas etal., 2006). Therefore, it is difficult to predict the peak phase of the activity for any liver enzyme under a certain condition. In this regard, the activity of hexokinase was measured in the liver of common carp 6 and 24 hours after being fed on a dietary carbohydrate. The results suggested that the HK activity did not show a significant difference between the two sampling points. The interpretation of these results as suggested by the workers is that the HK activity in the

liver of carp is not highly induced by dietary carbohydrate and there is another factor may regulate the carbohydrate metabolism (Panserat et al., 2008). In another trial, the activity of HK and G6PD were measured in the liver of gilthead sea bream juvenile after 6 hours from being fed on glucose or starch diets. However, no significant differences were detected in the activity of the measured enzymes (Enes et al., 2008).

In the current study, the activity of key liver enzymes (HK and G6PD) was measured after 24 hours from the last feeding time. Interestingly, the two experiments that curried out on the common carp did not show any significant differences between the experimental groups that fed on diets containing 38.5% starch and supplemented with chromium in terms of the enzyme activity, whereas mirror carp showed high significant differences in the activity of the same enzymes. It is noteworthy that common and mirror carp are belong to the same species and they are similar genetically, however, these results may indicate a potential differences in respect of their adaptation to the same diet ingredients. In the third experiment (Chapter 5), fish fed on 0.5 D treatment showed the best growth performance which was significantly different from fish fed the other diets. However, no significant differences were detected in respect of HK activity and expression. The possible explanation for these results is that the only one sampling point is insufficient to establish a definitive relationship between chromium supplementation and hexokinase action or maybe there is another factor affected growth performance in addition to the enzymes action.

Under *in vivo* condition, the synthesis of G6PD is regulated by dietary composition and hormones and the regulation may happen as a direct response of the liver to the dietary composition or indirectly by stimulating a specific endocrine response (Manos et al., 1991). The effect of chromium on the lipogenesis pathway has been discussed previously in a connection with the effect of dietary chromium on lipid content of whole fish tissue.

6.5 Cr bioavailability

Diet preparation in Cr experiments seems to be a critical step highly affects the result of growth performance. Due to the low level of Cr required by fish, diet processing demands more care and a long time for mixing the metal with the other ingredients to produce a uniform mixture especially when Cr compounds used is insoluble in water such as Cr yeast and Cr-Pic.

Chapter 4 investigated the bioavailability of Cr from different sources (Cr chloride, Cr Pic, and Cr yeast). The organic form of Cr, Cr yeast, showed non-significantly higher bioavailability than Cr chloride and Cr-Pic based on the result of the final body weight. This finding may indicate that Cr yeast is the most active form of Cr in the fish diet. Interestingly, the superior growth rate of Cr yeast fed group was concurrent with a higher HK activity which may indicate that the capacity of carbohydrate utilization in fish is affected by the phosphorylation step in glycolysis pathway. Since mirror carp was the experimental animal in the second experiment (Chapter 4), the high level of Cr (2.0 mg Cr kg^{-1}) as Cr chloride has been included again for results confirmation.

It is noteworthy that application of the comet assay proved to be an effective tool to validate the safety of Cr supplementation to the fish diet at the requirement level (0.5 mg Cr kg^{-1}). It has been suggested that the accumulation of Cr(III) in cells may induce DNA damage under certain conditions (Stearns et al, 1995). Increased DNA damage in erythrocytes cells of fish fed with high level of Cr chloride (2.0 mg Cr kg^{-1}) along with the histological examination explained the poor growth rate in fish fed 2.0 mg Crkg^{-1} which seems to be the threshold of toxicity zone.

Since Cr yeast presented the more bioavailable form of Cr at a level 0.5 mg Cr kg^{-1} compared to Cr chloride and Cr picolinate, it was worth testing the effect of Cr yeast at different levels on growth performance and the other parameters. Experiment III (Chapter 5) aimed to

elucidate the interactive effects of the dietary carbohydrate source and chromium yeast level. Common carp were fed a starch or dextrin-based diet and supplemented with three levels of Cr (0.5, 1.0, and 2.0) mg Cr kg^{-1}. The two-way analysis of variance demonstrated a significant interaction between the experimental variables (carbohydrate sources and Cr concentration) which together affected the final body weight, SGR, and FCR. Again, the result confirmed the low dietary requirement level of carp (0.5 mg Cr kg^{-1}). In addition, the best growth rate was observed in fish fed with starch-based diet indicating that fish have sufficient ability to deal with the complex carbohydrate that are digested and absorbed relatively slowly compared to simple sugars (Wilson, 1994).

6.6 Cr accumulation

Most of chromium accumulation studies have been performed on rats while the conditions that lead to Cr(III) accumulation in fish are still unknown. Despite that Cr(III) is poorly absorbed via the intestinal tract, dietary Cr(III) accumulated in different organs or tissues tested in chickens fed on diets supplemented with inorganic chromium as chromium chloride (Ahmed et al., 2005) or an organic form as chromium yeast (Debski et al, 2004). In this study on carp, the concentration of chromium has been analysed in two organs (liver and gut). Comparing the low concentrations of chromium in these two parts (especially in the liver) with the total chromium accumulated in whole fish tissue, it seems that these two organs are not the target for chromium accumulation. It has been suggested that the increased in insulin secretion rate and insulin action due to chromium supplementation caused withdrawal of chromium from the liver to the insulin sensitive tissues (Ahmed et al., 2005).

It is generally accepted that organic chromium compounds are well absorbed compared with inorganic forms (Pechova and Pavlata, 2007) and increased absorption of Cr(III) due to the dietary supplementation may lead to accumulation of Cr in tissues because Cr(III) is slowly excreted from cells (Stearns et al., 1995a). However, the results obtained from this study

demonstrated that the accumulation of inorganic form (Cr chloride) was much higher than that of the organic form (Cr yeast). In a comparative study on rats, it has been found that the absorption efficiency of inorganic chromium ($CrCl_3$) is low compared with the organic chromium (CrPic that used in the rat experiment) but the rapid exchange pool (for the inorganic form) is much smaller, resulting in higher Cr accumulation in muscle and fat tissues (Kottwitz et al., 2009). It is possible that Cr yeast behaves in the same manner as Cr Pic as both of these forms are organic compounds. In general, chromium whole body content increased with increasing dietary chromium level. The same result has been found in fish (Jain et al., 1994) and in rats (Zha et al., 2007).

6.7 Plasma glucose concentration

Plasma glucose measurement was conducted in each experiment of this thesis. However, no significant differences were detected between the groups fed different diets at the end of each experiment. It is likely that one sampling time after 24 h of feed deprivation is insufficient to detect any changes resulting from different treatments (repeated glucose measurement has been avoided in this study to prevent any stress could affect the general fish health or future measurement). Another possible explanation for the result is that carp are able to deal with high level of dietary carbohydrate due to the high affinity of insulin receptors (Parrizas et al., 1994). Furthermore, in a comparative study which included carp, red sea beam and yellowtail, insulin level was measured over time after feeding fish with a diet containing 40% dextrin. The results showed that the insulin level was the highest in carp followed by sea bream and yellow tail (Furruchi and Yone, 1981). These findings may suggest that carp have a greater ability to regulate blood glucose levels compared with carnivorous and herbivorous fish. Similarly, Capilla et al., (2004) observed no changes in plasma glucose levels 6 h after receiving carp carbohydrate rich diets and the author suggested that the glucose homeostasis was maintained due to the adaptation of the carp to the experimental diets. In other species,

Magzoub et al., (2010) reported no significant changes in plasma glucose level of tilapia fed two sources of chromium (chromium oxide and chromium yeast) at two levels (1.0 and 2.0 mg Cr kg^{-1}) despite the plasma glucose level has been measured after 1, 2, 3, 4, 6, 8, and 24 h from the last feeding time. Because glucose concentration in fish blood is highly affected by stress conditions resulting from handling and sampling methods, it appears that plasma glucose concentration is not always the suitable parameter to test the efficacy of certain variables or their action.

6.8 Liver and gut histology

Microscopic examination of liver and gut tissues was an effective tool to detect the toxicological signs which was represented by increased liver vacuoles in fish fed the high level of Cr chloride (Experiment I and II). These results were consistent with the hypothesis that elevated levels of inorganic Cr (2.0 mg kg^{-1}) impaired the normal structure of the liver. It has been reported that some trace elements and their salts induce metabolic toxicity affecting the regulation of different processes (Schroeder, 1976). Similarly, carp exposed to a lethal dose of Cr (VI) for 96 h showed different changed in the liver structure described as fatty changes and necrosis (Parvathi et al., 2011). On the other hand, fish fed high level of the organic form of Cr (Cr yeast) did not show any changes in the liver structure. This observation may indicate that the metabolism of chromium in living cells is affected by its form in addition to the dose administered.

High dietary inorganic Cr inclusions in the carp diet induced severe lesions in gut tissues including tip erosion, fusion, and necrosis. It is known that Cr chloride is poorly absorbed from the gastrointestinal tract and it has an ability to form insoluble complexes. Therefore, the direct contact between these complexes and the tissue surface may result in local tissue irritation. On the other hand, 2.0 mg Cr kg^{-1} as chromium yeast in fish diet did not induce any

signs of toxicity in the mentioned organs. Again, the high absorption efficiency of Cr yeast compared to Cr chloride may explain the safety of the former at the levels tested.

6.9 Conclusions

The results derived from this study provided a holistic view about the potential advantages of dietary Cr supplementation in the nutrition and health of cultivated carp.

- Despite that the concentration of Cr used in the experiments of this thesis acceded 2.5 mg Cr kg^{-1} (in total), the survival rate of fish was 100 % in all dietary groups for all the experiments undertaken.
- An optimum supplemental level of chromium (0.5 mg kg^{-1}) to the carp practical diet has been confirmed in the three experiments reported in this thesis.
- The organic form (Cr yeast) was found to be efficacious in modulating carbohydrate assimilation and improving growth performance in carp and less toxic compared with the inorganic form (chromium chloride).
- Chromium supplementation at (0.5 mg kg^{-1}) to the carp practical diet did not affect the final body composition.
- The parameters used in these experiments (enzyme activity, comet assay, and the histological examination of the liver and gut tissues) have been showed to be sensitive and reliable markers reflect the health condition of the experimental fish.
- Formulation of practical diets with the natural forms of Cr such as Cr yeast may produce significant profits and enhance the aquaculture industry as well as the environmental sustainability.

6.10 Future perspectives

Since integrated studies addressing the potential advantages of chromium as a modifier for carbohydrate metabolism in fish are limited, the influence of dietary chromium for other fish species remains to be elucidated.

Despite the main question of this thesis concerning the growth enhancement of carp by dietary chromium being answered; different areas still need further investigations which can be summarized by the following points:-

• In this study, we used a practical diet containing a background level of chromium (0.6 mg Cr kg^{-1}) to determine chromium requirement because our aim was to assess the potential benefit from adding chromium to the practical diet of carp. Therefore, the use of a purified or semi-purified diet is needed in future experiments to determine the actual requirement of this element.

• Using ^{51}Cr-labeled or stable isotope tracers in human studies is possible to monitor Cr distribution in human subjects and in different experimental animals. Therefore, using a chromium isotope may produce definitive information in this aspect of fish nutrition research.

• The relationship between chromium action and HK activity needs further investigation. It is worth measuring the activity of HK and gene expression after feeding and during fasting for definitive conclusions.

• It is known that the hexokinase enzyme is regulated by insulin hormone; nevertheless, the insulin concentration in fish blood was not determined due to the limitation in the laboratory facilities. The effect of different levels and sources of dietary chromium on insulin level and the insulin receptor system along with hexokinase activity is a useful point for further research.

• Different advantages have been reported as a result of dietary chromium supplementation in other animals such as fecundity improvement in pigs, egg quality and production in birds, and disease resistance and immunity system enhancement in cows. These parameters should be highlighted as new projects in chromium and fish nutrition research.

• Since the aim of the fish nutrition studies is to produce a high quality protein for human consumption, the effect of chromium on the quality of market size fish would be beneficial.

Finally, Cr should now be re-assessed as being a vital trace element in diet for farmed fish based on the evidence presented in this thesis. It is envisaged that chromium will become recognized as being important as other established trace elements in premix formation. This work will hopefully support revision of legislation and regulations concerning the inclusion of chromium in various forms in aquafeed formulation for both fish and crustaceans farmed species to optimize both performance and health.

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