Biological and toxicological effects of dietary organic and inorganic forms of cobalt on *Tor putitora*

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A thesis submitted in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Dedicated To:

My Parents

Whose encouragement and motivation made it possible

To my loving husband

Without whom none of my success would be possible

IN THE NAME OF ALLAH

THE MOST MERCIFUL

THE MOST BENEFICIENT

AND

THE MOST COMPASSIONATE

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This is to certify that the research work presented in this thesis, entitled "Biological and Toxicological Effects of Dietary Organic and Inorganic form of Cobalt on *Tor putitora*" was conducted by **Ms. Naima Younus** under the supervision of **Prof. Dr. Amina Zuberi**. No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Zoology of Quaid-i-Azam University, Islamabad in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Field of Fisheries and Aquaculture.

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LIST OF ABBREVIATIONS

Abbreviation	Full Names
%WG	Percentage weight gain
µg	Micro-gram
ADG	Average daily gain
AWG	Average weight gain
BSA	Bovine Serum albumin
CFU	Colony forming unit
CMC	Carboxymethyl cellulose
$CoCl_2$	Cobalt chloride
Co-Met	Cobalt methionine complex
Co-NPs	$CoCl_2$ nanoparticles
DNS	Di-nitro salicylic acid
DO	Dissolved oxygen
EDS	Energy dispersive spectroscopy
FAO	Food and Agriculture Organization
HB	Hemoglobin
HSP-70KDa	Heat shock protein 70 KDA
IgM	Immunoglobulin M
ln	Natural log
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MS222	Tricaine methane-sulfonate
MyoD	Myoblast determination protein 1 homolog
MyoG	Myogenin
Na_2CO_3	Sodium carbonate
NBT	Nitro-blue tetrazolium
RBC	Red blood cells
Rpm	Revolutions per minute
SEM	Scanning electron microscope

SGR	Specific growth rate
T. putitora	*Tor putitora*
UV	Ultra violet
WBC	White blood cells
W_f	Final weight
XRD	X-rays diffraction
βME	B-Mercaptoethanol

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All praises and glories of Almighty Allah who says in the Holy Quran, “And your Lord is the most gracious Who taught by the pen. Taught man (those things) which he did not know”. Countless Darood on the Prophet of mercy Hazrat Muhammad (ṣallā -llāhu ʿalayhī wa-ʾālihī wa-sallam), who showed the path of knowledge to mankind and gave the lessons of seeking knowledge from cradle to the grave.

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ABSTRACT

Minerals play important biological and physicochemical functions inside living organisms. However, their efficiency depends on many factors, including chemical form, particle size, and dosage level. Cobalt is one of the important essential trace minerals, required in low quantity in the diet for normal body functioning. Unfortunately, information regarding the optimal dietary supplementation level of cobalt in most fish species, including Mahseer *Tor putitora,* is not documented. Here an attempt has been made to determine the optimum dietary supplementation level of different chemical forms of cobalt in *Tor putitora*. First, a feeding trial was executed to determine the optimum dietary dosage level of the conventional inorganic form of cobalt (macro-scale). Advanced fry of *T. Putitora* were fed graded levels of cobalt chloride supplemented diets (0.5-3mg/kg diet) for 90 days. Results indicated the positive effect of cobalt supplemented diet at dosage level up to 2mg/kg feed on the growth performance and proximate body composition of fish. However, Co supplemental level above 2mg/kg reduced the growth rate of fish, indicating a dose-dependent toxic effect. Moreover, a curvilinear relationship was observed with the increasing cobalt supplementation in diets between whole body crude protein (%) content and weight gain (%) of fish. Furthermore, at higher supplementation levels, results indicated the inhibitory effect of cobalt on the accumulation of iron and manganese in the muscle of fish. To validate the results, a nutrigenomic study was designed and the effect of dietary cobalt supplementation on the expression of growth-related genes, i.e., myoblast determination protein 1 homolog MyoD and myogenin MyoG in the muscle and stress response gene (heat shock protein 70KDa) in the liver was also studied. Results indicated the positive effect of a 2mg Co/kg diet on the mRNA levels of growth genes in the muscle. However, cobalt supplementation at a higher dosage level (3mg/kg diet), down-regulated the growth-related genes in the muscle and up-regulated the expression of stress response gene (heat shock protein 70KDa) in the liver of fish. Results of our preliminary experiment revealed that $CoCl_2$ supplementation at 2 mg/kg diet is optimal for the muscle growth

and body composition of *T.putitora*. We then further extended our investigation by studying the effect of graded levels of different chemical forms of cobalt, i.e., cobalt chloride nanoparticles (Co-NPs) and cobalt methionine complex (Co-Met) on *T. putitora* fingerlings. First, Co-NPs and Co-Met complex was prepared by using ball-milling and precipitation methods respectively, and then characterized by using X-rays diffraction spectroscopy (XRD), scanning electron microscope (SEM), and energy dispersive spectroscopy (EDS) for their size, surface morphology, and elemental composition. Characterization of particles indicated the crystalline nature of Co-NPs with particle size <20nm. However, the XRD spectrum of the Co-Met complex did not show any peak, indicating the amorphous nature of the complex. SEM image of Co-Met complex appeared as a honeycomb-like octahedral structure with an average size of 82.69nm. Furthermore, EDS spectrums indicated 21% oxygen, 40.33 % chloride and 37% cobalt in Co-NPs, and 31% carbon, 1.32% nitrogen, 48.53 % oxygen, 2.06% cobalt and 0.31% chlorine in Co-Met complex. In the next phase of the study, feeding trials were conducted to study the effect of graded levels of Co-NPs and Co-Met complex supplemented diets on *T. putitora* fingerlings. Results indicated the significant effect ($P<0.05$) of both chemical forms of cobalt on the growth performance, hemato-immunological indices, intestinal enzyme activities, the proximate composition of muscle, and metals accumulation in muscle. Co-NPs supplemented diet showed a positive effect on fish up to the inclusion level of 1.5mg/kg diet. However, further inclusion of Co-NPs in diet-induced toxicological effects, i.e., negative effect on growth, hemato-immunological indices, intestinal enzyme activities, and proximate composition of the muscle. Contrary to that, the Co-Met complex supplemented diet did not show any toxicity even at the maximum dosage level, i.e., 3mg /kg diet. Moreover, after exposure to a pathogen, *Aeromonas hydrophila*, maximum mortality was observed in a group of fish reared on 3mgCo-NPs /kg diet. However, the Co-Met fed-group of fish reared on same dosage level (3mgCo-Met complex /kg diet), showed no mortality. Two way ANOVA indicated a significant effect of chemical forms and dosage levels on all studied parameters of fish. Moreover, a

significant ($P<0.05$) interaction between both variables (chemical forms × dosage levels) indicated how both of them affected the fish. Furthermore, a pairwise comparison revealed that the organic form of cobalt (Co-Met) at every dosage level showed a comparatively stronger positive effect than the inorganic nanoform of cobalt. For further validation of results and practical application of both chemical forms of cobalt supplemented diets, a further completely randomize feeding experiment was designed and conducted in earthen ponds in semi-intensive culture conditions. Based on previous experiment results, two dosage levels of both organic and inorganic forms of cobalt, i.e., 1.5mg and 3mg /kg diet were selected and fed to fish for 90 days. Results indicated a dosage-dependent positive effect of Co-Met complex supplemented diets on growth performance (W_f, %WG, AWG, ADG, %SGR), hematological indices (RBC, HB, HCT, MCV, MCH, MCHC immunological indices (plasma protein, IgM level, serum lysozyme activity, WBCs count, respiratory burst and phagocytic activity), liver function indices (serum AST and ALT activities), nutritive value (muscle proximate composition and amino acids profile) and expression of MyoD and MyoG genes in the muscle of fish. However, in accord with the previous results, Co-NPs supplemented diet showed a positive effect up to inclusion level of 1.5mg/kg diet, and at higher inclusion level (3mg/kg diet), showed a toxic effect on all studied parameters of fish. Overall, Co-NPs appeared more efficient and required in less quantity for the beneficial and toxicological effects than conventional $CoCl_2$. However, Co-Met complex as compared to Co-NPs showed the most pronounced positive effects without any toxicity at all dosage levels on all studied parameters of fish. Based on the results, we could recommend an organic form of Co (Co-Met complex) as a dietary supplement for improving growth, immunity, health status, and nutritive value of *T.putitora.*

INTRODUCTION

INTRODUCTION

In the animal production system, good nutrition is essential because it not only influences the production cost but also affects the growth and health status of fish (Hardy et al., 2002). In fish farming, nutrition is critical because feed represents 40-50% of the production costs. In recent years, fish nutrition has advanced noticeably with the development of new, balanced commercial species-specific diets that promote optimal fish growth and health (Hixson, 2014). The primary function of fish food is to supply adequate energy for growth and reproduction. Therefore, the development of new species-specific diet formulations that could support the aquaculture industry as it expands is a prerequisite for affordable, safe, and high-quality fish and seafood products.

Fish feed formulation for different species generally depends on knowing how much energy and quantity of each nutrient is required (Craig et al., 2017). For instance, fish utilize protein and fat as a source of energy followed by carbohydrates. However, considerable variations in the utilization of nutrients as an energy source at the species level exist. For instance, carnivorous species most efficiently utilize dietary protein and lipid for energy, but less efficient at using dietary carbohydrates. Lipids are also considered as a rich source of energy along with dietary proteins, especially in carnivorous fish as they cannot efficiently utilize carbohydrates (Tseng and Hwang, 2008), however, tilapia and carps performed well on a diet high in carbohydrate (Paul et al., 2015). According to Jafri (1998), *Catla catla* fry showed improved growth performance and food conversion efficiency when fed a diet having 40% protein and 40% carbohydrate contents. Generally, fish do not have a specific dietary requirement for carbohydrates, but it is essential in feed, as it improves the palatability, serves as an inexpensive source of energy and source of carbon for constructing non-essential amino acids. When carbohydrate present at an adequate level in feed, it displays a protein-sparing effect, i.e., can

be burnt for energy and allowing the protein to retain and use for growth. The inadequate (deficient or excess) dietary energy levels cause a reduction in the growth rate of fish (Catacutan et al., 1995).

Protein is the most expensive part of fish feed. It plays an important role in the growth and the regulation of many physiological functions (El-Sayed, 2014). Like other animals, fish also synthesize body proteins from amino acids in the diet, and from some other sources. Though more than 200 amino acids occur in nature, only about 20 are common. Among these 20 amino acids, about 10 are essential or indispensable one that must be provided in the diet, while others are "nonessential" or "dispensable that the body can synthesize from other sources. The quantitative dietary requirements for most of the indispensable amino acids of several fish species have been evaluated and available in the literature (Wilson, 2003). A deficiency of any one of the essential amino acids can limit protein synthesis, thus reduce growth rates, impaired immune functions, and also show other specific symptoms.

Fish meal is the rich source of protein in carnivorous fish feeds due to the presence of adequate amino acid profile, non-existence of anti-nutrient factors, high palatability, and higher digestibility by the fish (Li et al., 2011). Other animal sources of proteins include meat meal, meat and bone meal, blood meal, feather meal, poultry by-product meal, etc. also contain the proper level of essential amino acids. Besides the animal source of protein, the plant source of protein like soybean meal, cottonseed meal, wheat germ meal, corn gluten meal, etc. is also used in fish feed formulations. The alternative sources of protein are variable in crude protein contents, digestibility, essential amino acid contents, minerals, and ash contents, etc. For instance, feather meal has a lower protein digestibility, meat meal and bone meal have higher ash contents and plant sources of proteins are deficient in some essential amino acids (e.g., methionine, lysine, etc.) and contains several anti-nutritional factors (Bora, 2014). An adequate level of dietary protein is required for growth, protection from infectious diseases and to maintain non-specific immune responses in fish (Li et al., 2007). Therefore, during

feed formulation, it is important to know the protein requirements of each fish species and select the protein source carefully by considering species, fish size, cost, etc. (Guillaume et al., 2001).

In addition to macronutrients, micronutrients including vitamins and minerals are also essential for the growth and health of fish. Therefore, their adequate level in fish feed is also essential for fish production. Vitamins are required in trace amounts for improving fish growth performance, reproduction, and health status (Hasan, 2001). They are of two types i.e., water-soluble and fat-soluble vitamins. Warm-water fish can synthesize water-soluble vitamins from their intestinal microbiota. However, in most of the cold water carnivorous fish, intestinal microflora is not considered as a significant source of vitamins (Lall, 2000). Vitamin deficiency in fish shows different symptoms such as scoliosis and lordosis due to ascorbic acid deficiency and dark skin coloration due to folic acid deficiency (Anderson et al., 2003). Vitamin A and D play an important role in the stimulation of immune responses in fish. Vitamin A deficiency reduces both innate and adaptive immunity by impairing the ability to counteract extracellular pathogens (Deluca et al., 2001), and deficiency of vitamin D results in defective antigen-specific immunity due to impaired localization of innate immune responses (DeLuca, 2004).

All living organisms require minerals along with other dietary ingredients such as protein, carbohydrates, lipids, and essential vitamins for their normal body functioning (Pond et al., 2004). Minerals can be classified as macro minerals that are required in a relatively large amount in the diet (g/kg) e.g., calcium, sodium, phosphorus, magnesium, potassium, and sulfur (Suttle, 2010). Whilst other metals which are required in quite a low quantity (mg/kg) are termed as micro minerals e.g. selenium, chromium, zinc, iron, manganese, cobalt, iodine, and copper. Minerals play important roles in fish like involve in cellular metabolism e.g. respiration, different enzymatic activities, and oxygen transport (iron being a basic part of hemoglobin) (Teles, 2012). They play an important role in maintaining cellular integrity and regulation of different physiological and biological functions like

growth, immunity, reproduction, digestion, etc. (Candlish, 2000). For instance, calcium and phosphorus play an important role in bone formation, while sulfur, zinc, manganese, fluorine are important constituents of soft tissues, and take part in the regulation of different body processes (Craig et al., 2017). Moreover, minerals also play an important role in energy generation, i.e., taking part as a cofactor for enzymes involves in the conversion of food into metabolites, synthesis, and the formation of high energy bonds such as ATP (Maggini et al., 2007). They are also required for the biosynthesis of certain hormones like iodine is required for thyroid hormone biosynthesis (Lall, 2003).

Micro-minerals possesses several catalytic functions and takes part in several metalloenzymatic processes e.g. iron is involved in the aerobic oxidation of carbohydrates and oxidation, zinc is the part of carbonic anhydrase (involve in CO_2 formation), alcohol dehydrogenase (alcohol metabolism), carboxypeptidase (digestion of protein), manganese activates the pyruvate carboxylase which is involved in pyruvate metabolism, etc. (Lall, 2003). Additionally, these minerals are also an integral part of biologically important compounds such as iron is an integral part of hemoglobin while cobalt is the component of vitamin B_{12} (Zhang et al., 2009). Selenium is required for optimal immune responses (both innate and acquired immunity), maintenance of cellular membrane integrity, and protection of DNA against oxidative damage (Maggini et al., 2007). Also important for the functioning of selenoproteins and its deficiency reduces the glutathione peroxidase (GPx) activity (Weeks et al., 2012).

The importance of trace minerals in humans and animals is well documented. However, information regarding the optimum dietary mineral requirement in fish is rare, which may be due to their less amount required in the diet (Guillaume et al., 2001). Literature revealed variable requirements in different fish species for a specific mineral. For instance, the optimum dietary requirement reported for Fe/kg diet was 33-100mg for Atlantic salmon, 30mg for channel catfish, 150

mg for red sea bream, and 170 mg for eel (Chanda et al., 2015). The optimum dietary requirement of Cu for rainbow trout was a 3mg/kg diet while it was a 5 mg/kg diet for channel catfish and Atlantic salmon (Shiau et al., 2003). Similarly, other micro-minerals also showed variable dietary requirements in different fish species or same species in different developmental stages like dietary manganese requirement of channel catfish, rainbow trout and Atlantic salmon was 2.4mg, 12-13mg and 7.5-10.5 mg/kg diet respectively. Moreover, the juvenile stage of salmon and trout required a 2-15mg/kg diet as compared to brood-stock which showed optimum performance at 30mg/kg diet (Antony et al., 2016).

Fish can absorb minerals from surrounding water and also taken from the diet, while incorporation in their body is highly selective and depends on the body's ability to utilize the mineral properly (Suttle, 2010). Fish maintain the internal balance of any mineral by integrating different mechanisms such as uptake, storage, and excretion (Evans et al., 2005). Generally, feed formulation, especially from plant-based feed ingredients requires mineral supplementation, since they have depleted or imbalanced mineral profiles (Lall et al., 2009). Furthermore, plant-based feed ingredients also have anti-nutritional factors, such as phytic and oxalic acid which affect mineral absorption inside the body e.g. phytate binds to zinc in the gastrointestinal tract making it unavailable for absorption (Francis et al., 2001).

Generally, all living organisms require mineral supplementation in the diet to overcome mineral deficiencies that occur either due to the suboptimal concentration level of specific important minerals in the diet or due to the presence of metabolic antagonist which hinder mineral absorption across the gastrointestinal tract (Lall, 2003; McDowell, 2008). According to Lall (2003), minerals are required at their optimum concentration level for proper functioning. For instance, deficiency of phosphorus affects the growth performance of *Anguilla japonica*, reduced bone mineralization, lower phosphate level in the blood, and reduce food conversion efficiency in *Oreochromis aureus* (Sugiura

et al., 2000). Similarly, Fe deficiency resulted in hypochromic microcytic anemia in *Salvelinus fontinalis* (Własow et al., 2004), low hemoglobin levels in *Ictalurus punctatus* (Camus et al., 2014), low plasma iron and Fe transferrin concentration in *Salmo salar* (Naser, 2000). Zinc deficiency results in impaired growth, high mortality, reduced food intake in *Ictalurus punctatus,* and reduced protein and carbohydrate digestibility due to low carboxypeptidase activity and skin/fins erosions (Lim et al., 2001). Likewise, copper deficiency affects the Cu-Zn superoxide dismutase activities of the liver and cytochrome oxidase activities in the heart of grouper (Lin et al., 2010) while deficiency of chromium impaired glucose utilization in carps (Pan et al., 2003).

Many intrinsic and extrinsic factors influence the bioavailability of minerals. The intrinsic factors include fish species, age, sex, genetic makeup, physiological condition, and feeding habitats (Lall, 2003), while extrinsic factors include the chemical form of the mineral, particle size, metal's electronic configuration and it's competitive antagonism, dietary supplementation, route of administration and solubility of mineral (Harvey, 2001). Inside the gastrointestinal tract, there is an interaction of minerals with macronutrients (protein, lipids, and carbohydrates), other metals, and anions, while at the intestinal membrane, there is a competition of mineral with other metal transporting ligands for absorption (Ashmead et al., 1992). Several endogenous mediated ligands and solubility of the product affect the bioavailability and release of metals to target cells (Kratzer, 2018). For instance, the soluble form of the mineral is normally ionized and the acidic medium of the stomach further increases their solubility. However, the basic medium of the intestine reduces their solubility by binding mineral ions with ligands or anions. This process occurs in the ileum and jejunum, and these metal-ligand/acid radicals' complexes are quite stable and highly insoluble which affects mineral absorption (Ashmead et al., 2012). Furthermore, integral membrane proteins which are embedded in the plasma membrane of the intestine also affect the absorption and bioavailability of minerals (Paik, 2001).

Trace minerals play an important role in cellular metabolism and are required relatively in low quantity while their excessive dosage level causes toxicity in fish (Sevcikova et al., 2011). Metal toxicity in fish results in morphological, behavioral, and physiological changes such as reduced growth, impairment in respiration, swimming activity, and reproductive disorder of the fish (Lall, 2003; Jezierska et al., 2009). The mechanisms of metal toxicity involve the blocking of biologically important enzymatic groups and the displacement of metal ions from functional cellular units and biomolecules (Atli et al., 2007). Dietary essential elements show toxic signs when they are fed above their optimum dietary requirement. For instance, iron above the optimum level impaired the growth performance, lower FCR, and caused histopathological destruction in the liver of rainbow trout (Carriquiriborde et al., 2004), while excessive dietary copper negatively affects the growth performance and FCR, damages gills, and resulted in the kidney, intestinal apoptosis and liver necrosis in Atlantic salmon (Berntssen et al., 1999).

The level of metal to become toxic to fish depend on various external and internal factors e.g., chemical form and dosage level of metals, route of exposure (dietary or through gills), fish age, genetic makeup, nutritional status, and developmental stage of fish (Kratzer, 2018). Depending upon concentration and exposure period, metal toxicity could be chronic (exposure of low concentration of metals for longer duration) or acute (exposure of the high concentration for a short period) (Jaishankar et al., 2014). At higher concentration levels, some of the essential dietary minerals exert an adverse effect on animals, including livestock and aquatic species, and also affect human health by entering the food chain. Hence, during feed formulation, it is importat to consider dietary factors that influence metal toxicity like management practices, feed additives, accidental contamination, and food ingredients (Pandey et al., 2014).

Metals are toxic due to their bio-accumulation and then further bio-magnification inside the living tissue where they are stored easily instead of being excreted out of the body. Rahman et al.

(2012) reported a positive correlation between dietary iron and manganese and their bioaccumulation level in different organs such as skin, muscle, and liver of broiler chickens. Copper is an essential micro-mineral but its excessive intake results in Cu poisoning in different organs such as gills, liver, kidney, and ultimately fish mortality (Mishra et al., 2008). Vaquero et al. (2011) reported reduced feed intake, low growth performance, excessive production of reactive oxygen species, and high accumulation level in farmed cattle in response to excessive dietary copper. According to Nnaji et al. (2011), fish can be the source of excessive metal toxicity in humans due to metal bioaccumulation in their n-3 polyunsaturated fatty acids and proteins (Sándor et al., 2011). Heavy metal bioaccumulation in fish depends on their concentration in the surrounding water and their diet (Khaled, 2004). Some of the metals such as mercury and arsenic are carcinogenic while others are accumulated in visceral organs such as the intestine and liver of fish which are normally discarded during fish processing, thus do not pose health risks for human consumption (Tchounwou et al., 2003; Gall et al., 2015). According to Qiu et al. (2011), lipid content greatly affects the metal bioaccumulation in fish and reported a direct relationship between copper and zinc accumulation and lipid content of two farmed fish, i.e., *Trachinotus carolinus* and *Lutjanus campechanu*.

Different factors such as species, size, diet composition, mineral interactions (either positive or negative), feed ingredients and mineral bioavailability must be considered to establish an optimal mineral dosage level in the feed (Soetan et al., 2010). Optimal mineral concentration in feed represents a band between inadequate/deficient and adequate/excessive level, which is established based on the dietary mineral supplementation level and its response in fish species at specific developmental stages (Craig et al., 2017). Additionally, metal speciation, their solubility index, and complexation with other molecules/elements also influence their optimum level and toxicity in the aquatic environment (Förstner et al., 2012). According to Heydarnejad et al. (2013), elevated levels of dietary cadmium in rainbow trout affect the growth performance and survival of fish. Clearwater

et al. (2002) studied the diet-borne toxicity of zinc and copper in fish. According to them, copper is toxic above the 1 µg/g/day dietary supplementation level in *Ictalurus punctatus* while dietary supplementation level above 1-15 µg/g/day is toxic to *Salmo salar* at different developmental stages. Mostly micro-minerals are essential at an optimum level to perform different functions inside the body, but beyond that level or in excessive amounts cause toxicity (Chanda et al., 2015; Prabhu et al., 2019). For instance, Zn is required in optimum quantity to improve the growth performance, immune response in fish. Moreover, it is involved in several enzymatic processes such as carboxypeptidase, glutamic dehydrogenase, carbonic anhydrase, etc. Zinc also possesses antioxidant properties and helps to protect the tissues against oxidative damage (Kumar et al., 2017), yet excessive zinc concentration causes hypocalcemia by competing with calcium ions for absorption (McRae et al., 2016). Oxidative stress in response to higher levels of Zn in *Fundulus heteroclitus* and manganese in *Carassius auratus* is well documented (Loro et al., 2012; Vieira et al., 2012)

The chemical form of the mineral is one of the main extrinsic factors which affect its utilization in living organisms. In the feed of aquaculture animals, minerals are mostly supplemented in their inorganic forms such as chlorides, sulfates, carbonates, and oxides. These inorganic forms of minerals are poorly absorbed due to the formation of a complex with several anti-nutritional factors present in the diet or in the intestinal lumen which ultimately hinders their utilization in fish (Miles and Handy, 2000; Lall, 2003). Nowadays, research is in progress to improve the bioavailability of minerals and utilization in humans and animals including fish (Gharibzahedi et al., 2017).

One way of improving the bioavailability of minerals involves the formation and utilization of chelates. Chelates are stable, low molecular weights organic metal complexes as compared to their inorganic salts thus making them more available inside the living body (Paripatananont et al., 1995; Katya et al., 2017). The word ‘chelate’ was formerly derived from the Greek word meaning claw. It is the type of bond between metal ions with a ligand having a minimum of two functional groups

(OH^-, N_2, NH_2, etc.). Metal chelation is a process in which positively charged polyvalent metal cations are sequestered with highly electronegative chemical compounds, i.e., ligands in a ring structure which enhances their absorption across membranes (Paik, 2001).

Different types of organic metal chelates are available in animal nutrition, which varies with respect to ligand type and ligand used for forming metal complex (Kratzer, 2018). Chelate release the incorporated metal ion at the site of absorption by adsorption at the intestinal luminal surface or sometimes being readily absorbed as an intact molecule and increase their absorption across intestinal lumen, thus inhibits the formation of insoluble collides of metal ion (Apines et al., 2004). For instance, copper chelate with HMTBA (2-hydroxy-4-methylthio butanoate) has a far more stable configuration, which resists its binding to antagonists present in the GI tract and precipitation in the intestinal lumen. This property makes the metal chelates molecules to reach the intestinal epithelial layers without hindrance and bind with receptor sites, where HMTBA-Cu chelate is cleaved and metal ions are transported via enterocytes into the blood circulation and HMTBA by diffusion passed through the epithelial cells. The transport of chelated copper makes the Cu more bio-available to animals and consequently decreases its requirement in the diet (Leeson, 2009). Consequently, less Cu is lost through the body and accumulated in the production system, thus protecting wild and farmed aquatic animals from contamination (Guo et al., 2001; Richards et al., 2010; Manangi et al., 2012). Moreover, it is also reported that phytic acid, phosphorus, calcium, and oxalic acid in commercially available salmon and catfish feed make insoluble and indigestible compounds with dietary supplemented zinc in the gastrointestinal tract, which hinders the absorption of zinc and makes it less bio-available to the body and more lost to the environment as waste (Francis et al., 2001; Rider et al., 2010; Prabu et al., 2017). Organic mineral supplements as metal chelates had gained importance in the feed industry due to their high bio-availability and similarity to naturally occurring bio-molecules as compared to their inorganic form (Fernandes et al., 2008). Since the utilization of mineral in the body depends on

the body's ability to convert inorganic minerals into their biologically activated organic form, therefore supplementing highly bio-available organic mineral chelates appeared as a more natural method for supplementation of trace mineral in the feed (Vieira et al., 2008; Abdallah et al., 2009)

Three types of metal chelates are important for biological functioning, including chelates in which metal itself has no function while ligand is used to transport and store the metal ions (Boling, 1993). Metal incorporated in such ligands does not modify its properties, instead takes benefits from ligand's physical and chemical property which helps them to absorb across the membranes and transport through the bloodstream and get deposit where require e.g. transferrin, iron-binding plasma glycoprotein act as ligand and helps to transport chelated iron throughout the body. Normally, amino acids act as an important carrier for minerals in the living body. These amino acids do not behave as individual amino acids, instead of being part of chelates, help their absorption across the intestinal mucosal wall and their stores throughout the body (Kratzer, 2018). The second group of chelates includes those which play important physiological functions inside the body, allowing the chelated metal to perform its function properly (Andrieu, 2008) e.g. chelated Fe in hemoglobin and Co in vitamin B_{12}. Without cheated iron, hemoglobin cannot efficiently bound and release oxygen for metabolic use, thus could not perform its function properly i.e., transport of oxygen (Olivieri et al., 2000). Another example of such types of chelates includes in which metals as a constituent of important enzymes (metalloenzymes) play important metabolic functions in the cells like manganese and zinc in pyruvate carboxylase and carboxypeptidase respectively (Sousa et al., 2007; Wood, 2009). The third and last group of chelates includes those which interfere with the utilization of important cations. These chelates are formed accidentally and do not perform any function inside the body.

Commercially, different types of metal amino acid chelates are available as a nutrient supplement such as zinc methionine/lysine (Jahanian et al., 2008), manganese methionine, Fe-methionine and Co-lysine (Uchida et al., 2001), Se- methionine (Ørnsrud et al., 2002; Berntssen et

al., 2017). The higher bioavailability, digestibility, and efficacy of the organic metal complex in contrast to the inorganic form of metals in mammals and fish are well documented. Many investigators reported the higher bioavailability (Berntssen et al., 2017), digestibility (Ørnsrud et al., 2002), and efficacy of the Se-Met complex as compared to the inorganic form of Se in different fish species. For instance, Mansour et al. (2017) reported the improved growth performance, feed utilization, the relative survival rate, immunological indices, and antioxidant status of *Argyrosomus regius* in response to dietary organic selenium. Similarly, Ilham et al. (2016) also observed improved growth performance and antioxidant enzyme activities of *Lates calcarifer* when fed a diet supplemented with organic selenium in contrast to the inorganic form of selenium. Moreover, other scientists reported the higher bioavailability and digestibility of the organic–selenium as compared to inorganic form in *Salmo salar* (Ørnsrud et al., 2002) and comparatively higher efficiency in improving resistance against *Edwardsiella ictaluri* infection in *Ictalurus punctatus* (Wang et al., 1997). Like Se, significantly higher bioavailability of organic forms of Zinc (Zinc gluconate) and manganese (Mn-amino acid chelate) in contrast to inorganic form (sulfated form) and positive effect on larval growth and feed conversion ratio of *Oncorhynchus mykiss* is also well documented (Apines et al., 2004).

Besides chemical form, the size of the supplemented mineral is another important extrinsic factor that affects the mineral absorption and its utilization in animals. Nowadays, nanotechnology is gaining importance in every field of life and is considered as a tool to improve the absorption, bioavailability, and efficiency of minerals inside the body (Sharif et al., 2017). Nanotechnology involves the synthesis and application of materials/elements at their molecular levels i.e., at their nanometer scales (Momin et al., 2013). Micronutrient bioavailability and efficiency can be improved by the utilization of advanced nanotechnology. Nanoparticles possess a large surface area to volume

ratio and high surface catalytic activities which make them more effective as compared to their macro forms (Joye et al., 2014).

Metallic nanoparticles (MNPs) comprise pure metals and their compounds as oxides, sulfates, phosphates, chlorides, etc. in different forms and shapes exhibiting distinct physicochemical properties which make them a potential candidate in biomedical and diagnostic techniques (Mody et al., 2010; Rosarin et al., 2011). NPs possess distinct physiochemical properties that enable them to perform under different thermodynamic and physicochemical conditions as compare to their macro forms. Moreover, they can conjugate with different types of drugs, antibodies, and ligands thus displaying antibacterial activities (Jiang et al., 2007). According to Pal et al. (2007) their mobility, reactivity, and biological activities depend on their size, physical and chemical forms, and ionic charge.

Nowadays, research is ongoing to change the state of free radicals by converting them into their nano form, having unique properties, and improved redox potentials (Kumar et al., 2020). It was observed that nanoform of Fe, Zn, and Se are more bioavailable and get easily absorbed in the body without their excessive fecal loss as compared to their macro-counterpart (Sen et al., 2016). Many investigators reported the higher bioavailability and efficiency of nanoform of metals as compared to microform of metals. For instance, Chris et al. (2018) reported growth-stimulating effect of dietary iron nanoparticles i.e., 30% and 24% faster growth rate in *Carassius auratus* and *Acipenser gueldenstaedtii* respectively, while other investigators had reported improved growth performance, highest survival rate, improved biochemical and hematological indices and digestive activities of *Macrobranchium rosenbergii* postlarvae (Srinivasan et al., 2016), *Oreochromis niloticus* (Abdel-Hammed et al., 2019), and *Labeo rohita* (Behera et al., 2014) in response to dietary Fe_2O_3-NPs as compared to macro forms of Fe_2O_3. Like this, some other scientists had utilized selenium in its nanoform as a feed supplement and reported the improved growth performance, increased

Glutathione Peroxidase (GSH- Px) activity and improved activities of antioxidant enzymes status in different fish species e.g., *Tor putitora* (Khan et al.,2016), *Cyprinus carpio* (Ashouri et al., 2015) and *Carassius auratus gibelio* (Wang et al., 2013), while Khan et al. (2017) reported the improved feed conversion ratio and weight gain of *T. putitora* when fed diet supplemented with 0.68mg Se-NPs/kg of feed as compared to those fed basal diet. The high bioavailability, antibacterial activity, growth-promoting, and immune-stimulating effect of ZnO-NPs in contrast to conventional ZnO is well documented (Swain et al., 2016). Different scientists reported growth-stimulating effect of ZnO-NPs supplemented diet in fish e.g. *Oreochromis niloticus* (Tawfik et al., 2017; Kishawy et al., 2020), *Oncorhynchus mykiss* (Shahpar et al., 2019) *Labeo rohita* (Thangapandiyan et al., 2019; Mondal et al., 2020), *Clarias gariepinus* (Onuegbu et al., 2018) and *Ctenopharyngodon idella* (Faiz et al., 2015), while others reported the antibacterial and antifungal activity of ZnO-NPs against fish pathogens, *Vibrio harveyi*, *Yersinia ruckeri*, *Aeromonas hydrophila* and *Aeromonas salmonicida,* and *Aphanomyces invadans* (Shaalan et al., 2016). Recently, Dawood et al. (2020) reported the beneficial effect of dietary copper nanoparticles on the weight gain and immunological indices of *Cyprinus carpio.* Though nanoparticles supplementation improves the effectiveness of mineral as compared to their macro forms, however, their high dosage level negatively affects the growth performance and health status of the fish (Shaw and Handy, 2011).

Fish can absorb minerals and other compounds from the surrounding water. Nevertheless, due to low concentration in water, freshwater fish generally require minerals as dietary supplements to fulfill body requirements (Beveridge et al., 2013). Supplementation of any particular mineral in foodstuffs should be carefully screened to avoid metal accumulation at toxic levels (Malomo and Ihegwuagu, 2017). The current knowledge about the supplementation of metallic minerals in fish is mostly limited to iron, manganese, zinc, and selenium, which are the main elements of body fluids and the basic components of non-enzymatic macromolecules and can also act as cofactors in

enzymatic reactions (Teles, 2012). However, various spiteful symptoms associated with deficiencies of other minerals like chromium, copper, fluorine, iodine, and molybdenum, are well known (Lall, 2002; Terech-Majewska et al., 2016).

Cobalt is considered an important essential micro-mineral in fish and other vertebrates (Perrault et al., 2014). It is required in a low quantity in the diet for the normal body functioning of an organism (Yılmaz et al., 2010). It plays an important role in the regulation of the activity of many enzymes, intestinal microbial synthesis of vitamin B_{12}, blood glucose levels, DNA synthesis, fatty acid synthesis, and energy metabolism (Blust, 2011). Structurally vitamin B_{12} or cobalamin is an organometallic compound containing a Corrin ring with a central cobalt atom. Co can be linked to the methyl group in methylcobalamin, cyanide group in vitamin B_{12}, and to 5'-deoxyadenosine at the 5' position in a coenzyme B_{12} (adenosylcobalamin). These links are important for particular B_{12}-dependent enzyme action (Kräutler, 2019). Cobalamin is structurally similar to hemoglobin, cytochrome, and chlorophyll porphyrin ring. Out of six coordination sites, four of them are provided by cobalt while the other two by dimethyl-benzimidazole (Hapke et al., 2020). Vitamin B_{12} is a cofactor of two important enzymes, i.e., methylmalonyl- CoA mutase (MCM) and methionine synthase (MS) (Blust, 2011). MCM plays an important role in the Krebs cycle by contributing to the synthesis of succinyl-CoA from methyl-malonyl coenzyme A (Kräutler, 2005), an important step in energy extraction from fats and protein. The second enzyme, MS or 5-methyl-tetrahydrofolate-homocysteine methyltransferase plays an important role in the synthesis of nucleic acids (McDowell, 2008) and processing of amino acids, especially the regeneration of methionine from homocysteine (Kräutler, 2005). In addition to these, the presence of Co in some other proteins like methionine aminopeptidase 2 (METAP2) and nitrile hydratase (NHases) is also documented (Blust, 2011). METAP2 is a cytosolic metalloenzyme (binds two Co or Mn ions), present in all organisms including fish. It is responsible for the cleavage of N-terminal methionine residues from nascent protein (Blust,

2011) and is playing a critical role in protein degradation and tissue repair (Bennett and Holz, 1997). N-Hases are the single Co^{+2} ion or Fe^{+2} containing enzymes present in bacteria and eukaryotic systems. These enzymes catalyze the hydration of numbers of different types of nitriles into their respective amides (Chang et al., 2010).

Cobalt as a component of vitamin B_{12} plays an important role in DNA synthesis, cell division, and the modulation of immune functions in humans and animals by facilitating the production of T-cell lymphocytes in the blood, maintaining the CD4/CD8 ratios, and keeping the lymphocyte count in the average range (Todorova et al., 2017). Vitamin B_{12} is involved in the concanavalin-A dependent production of T-cells and stimulates the immunoglobulin synthesis, thus protects the animals against pathogenic bacteria, virus, and other foreign toxic intruders (Erkurt et al., 2008). Worldwide Co abundance in the Earth's crust and water is relatively low about 0.0025% (weight/volume) and 4-10% respectively (Swanner et al., 2014). Therefore, it must be supplied with the feed (Mukherjee and Kaviraj, 2009).

Dietary Co requirement in fish varies with age, size, species, and culture conditions (Wilson, 2003). Although plant- and animal-based proteins in aquafeeds are a source of Co, some freshwater fish require an additional Co in their diet for optimum growth (Wilson, 2003). Though Co is ranked as an essential dietary mineral, knowledge about the optimum dietary requirement of cobalt in many fish species is limited. Some investigators had reported the positive effect of dietary supplement of Co on the intestinal production of vitamin B_{12} and the survival, growth, and immunity of different fish species. For instance, Lin et al. (2010) had observed a significant increase in plasma and hepatic cobalamin concentration when fed a 10mg/kg cobalt chloride supplemented diet to *Epinephelus malabaricus.* Viswanath (1989) had also reported improved growth performance i.e., net increased in 108% total length and 229% total percentage weight gain and survival rate of *Liza parsia* at lower supplemented level, i.e., 1mg/kg cobalt chloride, while Mukherjee and Kaviraj (2009) reported

improved growth performance of *C. carpio* fingerlings when they were fed diet supplemented with cobalt chloride at different dosage level i.e., 0.05%, 0.10%, and 1.0%. Conversely, the dietary optimum cobalt requirement of *Tilapia zillii* was somewhat higher. According to Anadu et al. (1990), a dietary supplementation level of 250 mg Co/kg diet is sufficient for growth and other physiological and biochemical functioning of *T. zillii*.

Nowadays, cobalt and its compounds are considered a promising candidate for drug developments due to their unique therapeutic physiochemical properties. Bruggraber et al. (2004) reported the antibacterial activity of cobalt chloride alone at a concentration level of 0.03–1.0 mg or a mixture of 2mg/kg $CoCl_2$with 1 mg/L of amoxicillin against *Helicobacter pylori* and suggested the role of cobalt ions in the interfere of oxidative mechanisms in bacteria. Furthermore, Heffern et al. (2013), recommended the use of coordination complexes of cobalt as a therapeutic agent due to their antibacterial anti-fungal activities against a wide range of pathogenic bacteria and fungi. Antibacterial activities of di-valent cobalt complexes with chitosan have also been reported by Adewuyi et al. (2011) against *Escherichia coli, Staphylococcus aureus, and Streptococcus faecalis.*

Cobalt is toxic to living organisms at a higher dosage and long-term exposure levels, which results in adverse effects on different body organs and their functioning (Jaishankar et al., 2014). For instance, cobalt salts are mutagenic during multiple exposures at higher concentrations and may cause inheritable damage to the exposed living body (Beyersmann et al., 1992). The carcinogenic effect of high cobalt doses may appear due to the interference of cobalt with the oxygen sensor in the hypoxia response pathway (Beyersmann et al., 2008). According to Elbetieha et al. (2008), chronic cobalt exposure in a time and dose-dependent effects fertility, reduced testicular weight and results in low concentration of epididymal sperm count in rats. Cobalt toxicity results in increased testosterone levels in treated rats and directly interferes with the testosterone synthesis regulatory mechanisms and spermatogenesis (Chen et al., 2013). According to Additives et al. (2009), cobalt toxicity occurs due

to its high affinity with SH (sulfhydryl) groups which is commonly involved in the inhibition of important mitochondrial enzymes involved in respiration, displacement of divalent metal ion from metal activated enzymes such as iron, zinc, etc. (Additives et al., 2009). Furthermore, cobalt toxicity results in generations of ROS (reactive oxygen species) by Fenton reaction which causes oxidative stress and damages DNA, protein, and fats (Tewari et al., 2002).

Cobalt is an essential important micro mineral in fish, but it became toxic above certain optimal concentrations e.g. *Oncorhynchus mykiss* showed toxic signs and reduced growth performance when exposed to Co for 28 days at a concentration level of 490 µg/L (Little et al., 2007). Moreover, the interaction of dietary cobalt with other metals in the body is well documented (Lison, 2015; Miroshnikova et al., 2015; Lopez et al., 2018). According to Comhaire et al. (1994) cobalt competitively inhibits the calcium absorption from the gills, most likely affects Ca regulations by blocking voltage- gated calcium channels (Blust, 2011).

Cobalt toxicity in fish involves the inhibition of important enzymes due to the displacement of divalent metal cations from a functional cellular unit and alteration of normal biochemical and physiological functioning of the body (Kubrak et al., 2011; Yaqub et al., 2012). Furthermore, a higher level of cobalt exposure results in activation of reactive oxygen species which causes oxidative damage to DNA, fatty acids, and proteins. Unfortunately, a scanty of literature is available regarding diet-borne cobalt toxicity in fish, thus research regarding diet-borne cobalt toxicity in fish is required (DeForest et al., 2015). Yaqub et al. (2012) had studied the dual exposure of cobalt (water and diet borne) on three fish species, i.e., *Catla catla, Labeo rohita*, and *Cirrhinus mrigala* of three different age groups (60, 90, and 120 days). Moreover, Abbas et al. (2016) had also studied the effect of dual exposure of cobalt on *Labeo rohita* of different age groups. They had reported a significant effect of dietary cobalt on the growth performance of fish with higher FCR during acute cobalt exposure. Furthermore, Al-Ghanem (2011) had reported an adverse effect of a 2% cobalt supplemented diet on

growth, proximate body composition, and digestive enzyme activities in *Cyprinus carpio.* According to them, higher dosage levels of cobalt are not only detrimental to fish health and growth performance, but also affect the aquatic ecosystem, since it is highly toxic to zooplankton (Mukherjee and Kaviraj, 2011).

Mahseer *(Tor putitora)* is indigenous freshwater fish of the Indian sub-continent which is the most common inhabitant of Himalayan cold waters. It is commonly known as golden mahseer, mighty mahseer, or sometimes water tiger due to its appearance, size, and sporting value. It has high nutritional and commercial value and an important game fish (Desai, 2003). It is rank as the national fish of Pakistan (Ayub, 2007). *T. putitora* is omnivorous fish, however feeding habit changes at different developmental stage i.e., adult fish mostly depend on plant matter (Ogale, 2002). External mouth morphology, i.e., suctorial and protrusible shows their ability to rapidly pick food from water bottom (Bhatt et al., 2016). In the culture system, mostly shows omnivorous and bottom-feeding behavior at different developmental stages (Kishor et al., 1998). In the natural environment, some scientists reported its opportunistic feeding habit, i.e., variable feed preference such as mollusks, algae, larvae, etc. (Chatta et al., 2015).

In the past few years, commercial fish farmers and conservational anglers have expressed their concern about the decreasing populations in natural water bodies. Multiple factors contributing to decreasing the population like low fecundity of this important fish and anthropogenic activities, i.e., overfishing, the introduction of different exotic species, pollution, construction of dams, etc., which adversely affect their natural habitat and breeding ground (Ogale, 2002). Depletion of natural stocks of *T. putitora* has generated interest for its conservation and aquaculture production. Contrary to natural bodies of water, culturing fish in captivity requires fortified nutrition and adequate feeding (Teles, 2012), since a lack of efficient and balanced diet is the main bottleneck for the rearing of this species.

Nowadays, mahseer is cultured in a semi-intensive culture environment. The average growth performance of mahseer is comparatively slower than the other carps, but it performs somewhat better when cultured on artificial diet. Knowledge about the optimal requirement of macronutrients (protein, lipids, and carbohydrates) and micronutrients (vitamins and minerals) is important to formulate and prepare a balanced diet (Sarma et al., 2010). Although some work on the nutrition of *T. putitora,* i.e., the dietary requirement of protein, vitamin C, Zn, and Se has still done but information on the optimum dietary requirement of other macro and micronutrients of this species is not available. The literature reveals that Hossain et al. (2002) worked on the dietary requirement of protein for this species and observed optimal growth performance of mahseer fingerlings on a 40% protein diet. Significantly improved growth performance, immune response, and antioxidant status of mahseer were also observed when fingerlings fed dietary vitamin C at the rate of 300 mg/kg diet (Khan et al., 2019). Dietary supplementation of Se-NPs at the rate of 0.68 mg/kg die t also showed a beneficial effect on growth and biochemical indices of *T. putitora* (Khan et al., 2016). The dietary requirement of Zn was determined by Bhagawati et al. (2015) and reported 46.73mg Zn/kg diet for the optimal growth performance, survival, and metabolic enzyme activity of the fingerlings and 40mg Zn/kg diet for the fry of this species.

The dietary requirement of Co as compared to other micronutrients in fish is not well documented. Therefore, information regarding the optimal cobalt requirement of mahseer *Tor putitora* is also not available. Keeping in view the limited information about the essentiality of dietary Co in fish as well as the impact of chemical form and size of metals on their bioavailability and efficiency, the present study was designed to determine the optimum supplementation level of different particle size and chemical forms of dietary cobalt in *T. putitora.*

We hypothesize that the nanoform of dietary cobalt (inorganic) in contrast to its microform would have a lower dietary requirement, while an organic form of cobalt may have a profound effect

on the overall growth performance, health status, and nutritive value of fish. To check our hypothesis, we designed different experiments by taking into consideration two main extrinsic factors, i.e., particle size and the chemical form of cobalt. The study was conducted in four phases. In the first phase, the optimal supplementation level of dietary inorganic form of cobalt was determined by feeding graded level of $CoCl_2$ (0.5, 1, 1.5, 2, 2.5, and 3mg/kg diet) to advanced fry of *T. putitora* for 90 days and determined the effect on the growth performance, proximate body composition, and metal bioaccumulation level of *T. putitora*.

In the second phase, for nutrigenomics study, growth and stress-related genes of *T. putitora* were cloned to study the effect of the inorganic form of dietary cobalt on the expression of muscle growth genes, i.e., myoblast determination protein 1 homolog MyoD and myogenin MyoG and heat shock protein (70KDa) genes in the liver was determined. Since mahseer genome is not sequenced; therefore, information related to the nucleotide coding sequence of genes of our interest was not available. First genes of interest were cloned and then sequenced.

In the third phase of the study, inorganic cobalt chloride nanoparticles (Co-NPs), and organic form of cobalt i.e., cobalt methionine complex (Co-Met) were prepared and for the evaluation of their comparative efficiency and requirement. A 90 days feeding trial under controlled condition was conducted and fingerlings of *T. putitora* were fed graded levels of these forms of cobalt and their effect on growth performance, hematological indices, immunological Indices (plasma protein, IgM level, serum lysozyme activity, WBCs count and respiratory burst activity), disease resistance after pathogen challenge, intestinal enzyme activities (proteases and amylase and cellulase), muscle proximate composition and bioaccumulation of metals in the muscle was determined.

In the fourth phase of the study, the practical application of dietary Co-Met complex (organic) and nano-scale cobalt chloride Co-NPs (inorganic) as feed supplements was evaluated by feeding

dietary Co-Met and Co-NPs supplemented diets for 90 days to fingerlings of *T.putitora* reared in earthen ponds under semi-intensive culture condition and observed their effect on growth performance, hemato-immunological indices, serum AST and ALT activities, nutritive value of fish (proximate composition and amino acids profile of muscle), and expression of growth and stress-related genes (MyoD, MyoG, and HSP-70kDa) in muscle and liver of fish.

MATERIAL AND METHODS

CHAPTER 1

Effect of cobalt supplemented diet on early rearing of advanced fry of *Tor putitora*

Acquisition of advanced fry of *T. putitora*

Advanced fry of *T. Putitora* (weight range =1-2g) were procured from the Mahseer Fish Seed Hatchery, Garyala, and transported live in well-aerated plastic bags to Fisheries and Aquaculture research station Quaid-i-Azam University, Islamabad. Upon arrival, after tempering, they were shifted to circular fiberglass tanks, having a flow-through system (500L capacity). Before the commencement of the feeding trial, advanced fry were acclimatized for 7 days to laboratory conditions. During this period, they were fed a 40% crude protein basal diet.

Diet preparation

Diet was prepared from the locally available feed ingredients. The dry feed ingredients were first finely ground and passed through a fine mesh. For dietary supplementation, Cobalt chloride hexahydrate (CAT# 255599, ACS reagent, 98 %, Sigma-Aldrich) was purchased from a local supplier and the supplemented amount of cobalt was calculated in the following way:

Molecular mass of $CoCl_2.6H_2O$ = 237.93

The Atomic mass of cobalt = 58.93

I.e.,

1 mg of $CoCl_2.6H_2O$ contains cobalt = 58.93 ÷ 237.93 = 0.247mg

2mg of $CoCl_2.6H_2O$ contains cobalt = 0.5mg

The feed was formulated by taking each feed ingredient in the proper portion. The formula and chemical composition of the test diets are shown in Table 1. Based on the basic formula, 7 isonitrogenous (40% crude protein) and isoenergetic (2909.63k calories/kg of feed) diets were prepared by adding cobalt with increasing level, i.e., 0.5-3mg Co/kg diet.

The feed was prepared by adopting the standard protocols reported earlier (Amir et al., 2018). Briefly, after thorough mixing of all the dry ingredients as well as cobalt in respective diet, the dough was prepared by hand blending with oil and a small amount of water, and then feed pellets were prepared by passing the dough through a locally available meat grinder. Feed pellets were dried in a pre-heated oven at 60ºC. After drying, the pellets were saved in zip lock bags and stored in the refrigerator till further use.

Fresh feed was prepared after every 15 days during the feeding trial. Before providing feed to *T.putitora* advanced fry, the pellets were finely crushed by using a hand-held pestle and motor and then passed through a fine mesh to adjust the particle size of feed approximately between 150 µm to 250 µm.

Experimental design

The whole experiment was conducted in triplicate. After acclimatization to laboratory conditions, advanced fry (initial average body weight, 1.36 ± 0.02g), in good health with no sign of infection, were randomly netted from the tank and stocked in twenty-one rectangular fish rearing fiberglass tanks at the same stocking density, i.e., 1.5 g/L. The tanks well fitted with an aeration system to maintain dissolved oxygen level in water up to 6.0 mg/L and heaters to keep the water temperature at optimum, i.e., 22.5°C during the feeding trial. These tanks were divided into seven groups with three replicates in each group and thirty fish in each tank. During the experiment, advanced fry were fed a basal diet without Co supplement to control group while experimental groups A-F were fed diets containing increasing levels of cobalt (A=0.5 mg/kg diet; B=1.0 mg/kg diet; C=1.5 mg/kg diet; D=2.0 mg/kg diet; E=2.5 mg/kg diet; F=3.0 mg/kg diet).

At the start of the feeding experiment, advanced fry were fed three times a day, i.e., 9:00 am, 1:00 pm, and 4:00 pm with a feeding frequency of 8% of body weight. Feeding rate and frequency

were adjusted fortnightly and replace with a final rate of 4% body weight, twice a day (at 9:00 am and 6:00 pm). On daily basis, the unconsumed feed was removed after 2 hours of the feeding while the fish fecal matter was removed daily by simple siphoning, filtered, and then separately collecting it for later calculating feed conversion ratio. After siphoning, water volume was adjusted by adding fresh water to the tanks.

Water quality parameters including temperature and dissolved oxygen were checked daily by using a Multi-parameter (Hanna HI 9829-01102 Woonsocket, USA), while water, ammonia level was checked weekly by using the ammonia Kit (Hanna: HI3824). During 90 days feeding trial, temperature and DO levels were slightly fluctuated, i.e., water temperature ±0.2°C and dissolved oxygen ± 0.35 mg/L, while total ammonia remained < 0.35 mg/L.

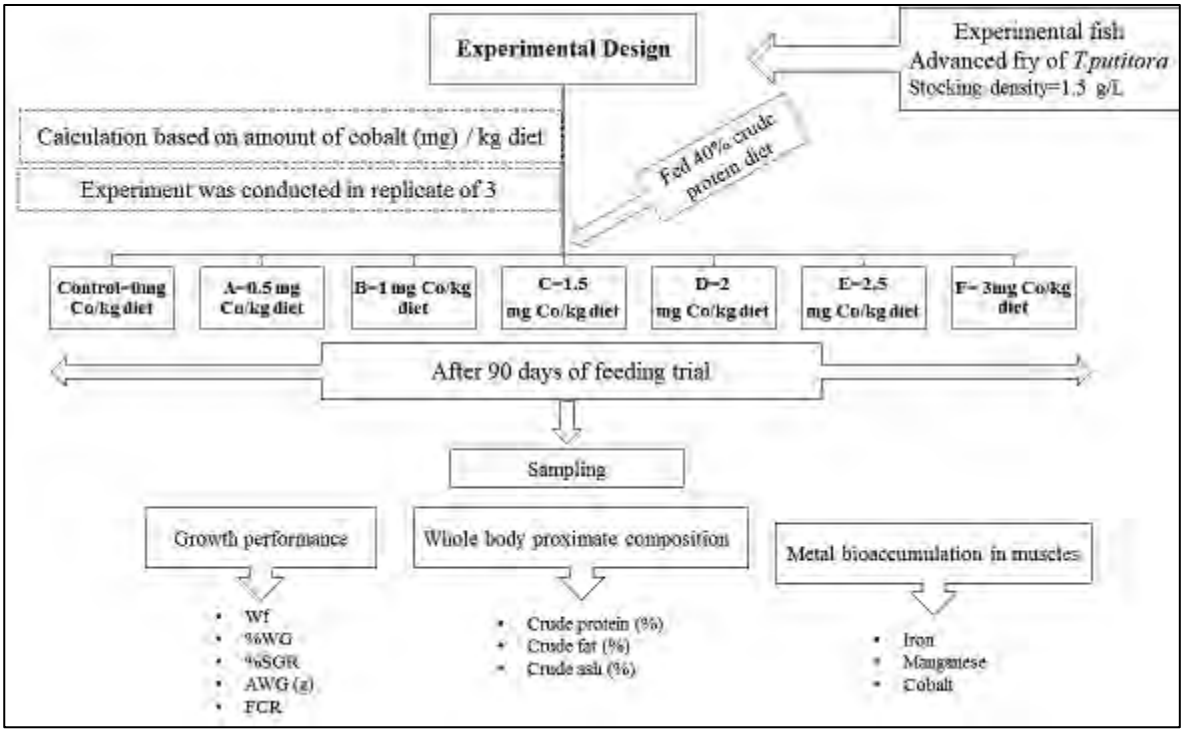

Figure 1. Experimental design to evaluate the effect of cobalt supplemented diet on early rearing of advanced fry of *Tor putitora*

Table 1. Formulation of 40 % crude protein feed for *Tor putitora*

Ingredients	Quantity (g/kg)
White fish meal	450
Soybean meal	150
Sunflower meal	150
Gluten	150
Rice polish	40
Wheat bran	40
CMC[a]	10
Vitamin premix[b]	10
Proximate composition (%)	
Gross energy (k calories)	2909.63
Crude protein	39.42
Crude fat	12.57
Ash	8.73

[a] Carboxy-methyl-cellulose; [b] Vitamin premix contains vitamins, amino acids, and minerals premix/100g

Growth performance

At the end of the experiment, i.e., after 90 days, fish were starved for 24 hours before sampling. On the day of sampling, fish were captured from each tank collectively with the help of a scoop net and weighed on a top-loading balance (SHIMADZU-ELB3000, Japan) and the number of fish was counted for the calculation of the average weight of each individual. Growth performance and FCR determined by adopting standard formulas reported earlier (Khan et al., 2017; Amir et al., 2018)

Weight gain= Average final weight of fish (W_f) - Average initial weight of fish (W_i)

% Weight gain (%WG) = $\frac{(W_f - W_i)}{W_i} \times 100$

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

Feed conversion ratio (FCR) = $\frac{\text{Total dry feed consumed (g)}}{\text{Total wet weight gain (g)}}$

Sampling

After weighing, 21 fish from each tank were anesthetized with buffered MS222 (0.1mg/L, buffered with Na_2CO_3) and used for whole-body proximate composition analysis. Since the size of fish were not enough for proximate analysis, the 21 fish /tank were divided into 3 pools, each having 7 fish, i.e., 3 fish samples/tank and 9 samples/ treatment (n=9). Whilst 3 fish/treatment were used for metal bioaccumulation analysis.

Furthermore, for the nutrigenomics studies, 6 fish /tank(18/treatment) were anesthetized with buffered MS222 (0.1mg/L, buffered with Na_2CO_3) and dissected at low temperature on an ice pad,

and their muscle and liver tissues were collected and preserved in RNA later™ (Thermo scientific CAT# AM7020) and stored at -20ºC till further analysis.

Whole-body proximate composition

The whole-body proximate composition was determined by adopting standard procedures approved by AOAC 2000 (Horwitz, 2000) at ISO 17025 certified laboratory of Poultry Research Institute (PRI), Islamabad.

Crude protein

Crude protein is usually determined by measuring nitrogen, the characteristic element in protein, rather than the protein itself. Crude protein in the sample was determined by micro Kjeldahl's method.

Briefly, 2g of sample was mixed with 5g of a digestion mixture (1g w/w Na_2SO_4 in 10g $CuSO_4$) and 30ml of concentrated H_2SO_4 to digest the samples. Subsequently, the reaction mixture was heated at 250°C for 2-3 hours until the light green color appeared. In the next step, the reaction mixture was cooled and distilled water was added to make the final volume of 250 ml in a volumetric flask. Then 10 ml of the mixture was taken in Kjeldahl's apparatus and 10 ml of 40 % NaOH was added to it. The funnel was plugged firmly, heated for 2-3 minutes. After heating, 10ml of 2% boric acid (H_3BO_3) solution was added to the funnel. The liberated ammonia (NH_3) was collected and titrated against 0.1N H_2SO_4 by using methyl red as an indicator.

Total crude protein was determined by multiplying the amount of nitrogen by 6.25 (appropriate factor). The percentage of nitrogen was determined by using the following formula.

$$\text{Nitrogen (\%)} = \frac{\text{Normality of } H_2SO_4 \times \text{volume of } H_2SO_4 \text{ used} \times 250 \times 0.014}{\text{Weight of sample} \times 10} \times 100$$

Where,

- 250 = Dilution of digested mixture
- 0.014= Standard volume of H_2SO_4 to neutralize 1ml of ammonia
- 10= used volume of the diluted mixture
- 100= percentage of nitrogen

Crude protein (%) = Nitrogen (%) × 6.25

Where,

- 6.25 = Assumed factor to calculate crude protein from nitrogen (%)

Crude fat

For crude fat determination, 3g of sample was placed in the thimble which is placed in Soxhlet extractor. After that, the solvent (150 ml of ether) was added to a round bottom flask and connected to a Soxhlet extractor and condenser. Subsequently, the temperature of the Soxhlet apparatus was increased and the whole extraction process was preceded for about 10 hours at 100ºC at the rate of 3-4 drops per second. In the end, the thimble was removed from the extractor, dried and its weight was noted. The crude fat in the whole body was then determined by using the following formula.

$$\text{Crude fats (\%)} = \frac{\text{Wt. of thimble after evaporation} - \text{Wt. of empty thimble before heating}}{\text{Wt. of the sample used}} \times 100$$

Crude ash

For the determination of crude ash content, a clean crucible was placed first in a muffle furnace oven at 100^0C for an hour. After that, the crucible was cooled by placing it in a desiccator and cooled down at room temperature and its weight was noted. In the next step, 5g of sample was placed in a pre-weighted crucible and then the crucible was again placed in a furnace and heated at

550-600^{0}C for 24 hours. After heating, the crucible was cooled in a desiccator at room temperature. To avoid moisture absorption, ash in a crucible was weighed immediately.

$$\text{Crude ash (\%)} = \frac{\text{Weight of ash}}{\text{Wt. of the sample used}} \times 100$$

Metal bioaccumulation

For the determination of metal bioaccumulation (iron, cobalt, and manganese), 1g of the frozen sample from each treatment was thawed at room temperature and then placed in conical flasks separately. After that, nitric acid HNO_3 (5ml) and perchloric acid $HClO_4$ (1ml) were added into the conical flasks having tissue samples. In the next step, the reaction mixture was digested at 250˚C on a hot-plate until the appearance of a clear transparent solution. The obtained clear solution was cooled at room temperature and then filtered by passing through Whatman No.42 filter paper. In the final step, distilled water (50ml) was added to the filtered solution. Fast Sequential Atomic Absorption Spectrometer AA240 FS (AA240FS (Palo Alto, CA, USA) was then used for the determination of cobalt (Co; (λ=240 nm), Iron (Fe; λ=248 nm) and Manganese (Mn λ=279.5 nm). To calculate the metal concentration, the standard calibration curve of each studied metal was plotted by making several dilutions of standards purchased from Merck.

Statistical Analysis

Results were analyzed using the statistical package program SPSS (version 20). One-way ANOVA followed by LSD test was applied to determine significant differences ($P < 0.05$) among experimental groups (A-F) for growth performance, proximate body composition, and the muscle iron, cobalt, and manganese bioaccumulation levels of *T. putitora*.

CHAPTER 2

Effect of cobalt supplemented diet on the expression of myoblast determination protein 1 homolog (MyoD) myogenin (MyoG) in muscle and HSP-70KDa in liver of *Tor putitora*

Extraction of RNA

Muscle

RNA from muscle was extracted by using a High Pure RNA tissue kit (Roche, Basel, Switzerland). Briefly, RNA later™ from muscle samples was removed, and the tissue was crushed into fine powder in the liquid nitrogen, and then the tube having sample was placed on dry ice. Subsequently, a mixture of 300 µl of lysis buffer plus 3.0 µl of 2-Mercaptoethanol βME was prepared and added to the 40mg of powdered sample. The mixture was homogenized immediately by using BRINKMAN POLYTRON®PT 1200 starter hand-held homogenizer until no visible pieces of the sample were seen. Afterward, to digest the homogenized sample, it was mixed with the 600µl of protein K (10 µl) and 590µl milli-Q water (MQ) solution, vortexed vigorously, and incubated at 55ºC in a water bath for 10 minutes.

After incubation, a homogenized mixture was transferred into the gene elute filtration column and centrifuged at 1300 rpm (SCILOGEX LLC) for 2 minutes. The filtered sample was taken in a new Eppendorf tube and 600 µl of absolute alcohol (C_2H_5OH) was added and mixed vigorously. Afterward, the sample was transferred to the nuclease binding column, and centrifuged at 13000 rpm (SCILOGEX LLC) for 15 seconds. Then 250µl of wash solution-1 was added to the nuclease binding column and centrifuged at 13000 rpm (SCILOGEX LLC) for 15 seconds.

DNase digestion

The DNase digestion mixture was prepared by mixing 140µl of DNase digestion buffer with 20µl of DNase enzyme. After that, the DNase digestion mixture was added to the center of the nuclease binding column, and the column was incubated at room temperature for 30 minutes.

After incubation, 250µl of wash solution-1 was added to the column, to stop the reaction and centrifuged for 15 seconds at 13000 rpm (SCILOGEX LLC). Subsequently, 500 µl of wash solution-2 was added to the column and centrifuged at 13000 rpm (SCILOGEX LLC) for 15 seconds. The liquid was discarded and 500µl of wash solution was added again and centrifuged at maximum speed for 2 minutes. Again liquid was discarded and centrifuged to dry the column. In the final step, 40µl elusion solution was added to the column and centrifuged at 13000 rpm (SCILOGEX LLC) for 1 minute. The extracted RNA was quantified using NanoDrop ND-1000 (Thermo Scientific, Waltham, MA, USA) by using the elusion solution as blank.

Liver

RNA from liver samples was extracted using Illustra RNA spin Mini Isolation Kit (GE Healthcare, Chicago, IL, USA). Briefly RNA later ™ from liver samples was removed and 30 mg of sample was crushed into fine powder in liquid nitrogen and later homogenized by using BRINKMAN POLYTRON ® PT 1200 -starter handheld homogenizer. After that, 350µl of RA1 buffer and 3.5µl of β-mercaptoethanol was added to the sample and vortexed to dissolve cells until a clear (light yellow color) lysate solution appeared. The viscosity of a clear lysate solution was reduced by placing the lysate solution in RNA-spin mini-filter units and centrifuged at 11000 rpm (SCILOGEX LLC) for 1 minute. The obtained filtrate was then transferred into a new tube, and 350µl of 70% ethanol was added and vortexed for 5 seconds. After that, the lysate was loaded into RNA spin-mini filters placed in a 2ml microcentrifuge tube, and centrifuged at 8000 rpm (SCILOGEX LLC) for 30 seconds. The filtrate was discarded and the column was again placed in the collecting tube. In the next step, 350µl of membrane desalting buffer was added to the column and centrifuged at 11000 rpm (SCILOGEX LLC) for 1 minute.

DNase digestion

DNase digestion was done by mixing 2µl of reconstituted DNase 1 with 40µl DNase reaction buffer, and then adding this solution to the center of the column and incubated at room temperature for 30 minutes. After that, the column was washed three times, first by adding 200µl of RA2 buffer and centrifugation at 11000rpm (SCILOGEX LLC) for 1 minute, then with 600µl of RA3 solution and centrifugation at 11000rpm (SCILOGEX LLC) for 1 minute, while the third wash was with 250µl of RA3 buffer and centrifugation at 11000rpm (SCILOGEX LLC) for 2 minutes. In the final step, RNA was eluted from the column by adding 30µl of RNase free water and centrifuged at 11000rpm (SCILOGEX LLC) for 1 minute. The extracted RNA was quantified by using NanoDrop ND-1000 (Thermo Scientific, Waltham, MA, USA), while RNase free water was used as a blank. Furthermore, the quality of RNA was checked by measuring absorbance at 260/280nm and 230/280nm.

Synthesis of complementary DNA (cDNA)

RNA obtained from liver and muscle was converted into cDNA by using reverse transcriptase. At first, the reverse transcriptase master mix was prepared (composition is shown in table 2) and then added to the RNA. Then, the solution was first incubated at 37ºC in a water bath for 1 hour and subsequently at 55ºC for 5 minutes. The synthesized cDNA was stored at -20ºC for further use.

Table 2. Reverse transcriptase master mix composition

Ingredient	Quantity (µl)
Buffer 5X	4
dNTPs 10mM	1
Random Primers	2.5
MLV-RT (Promega, Madison, WI, USA).	1
RNAsin	0.5
Total	9µl

Reverse transcriptase Polymerase chain reaction (RT-PCR)

Quality of synthesized cDNA was determined through RT-PCR by using primers designed through oligo Explorer 1.1.2 software (RT-PCR reaction mixture composition and reaction conditions are shown in tables 3 and 4 respectively).

Since the genome of *T. putitora* was not sequenced, thus no information was available for the nucleotide sequence of the selected studied genes (MyoD, Myogenin, and HSP-70kDa), therefore primers were designed by using the local alignment sequence tool Clustal Omega. Four related species i.e., *Danio rerio, Ctenopharyngodon idella*, *Cyprinus carpio,* and *Labeo rohita* were selected and their gene sequence was subjected to BLAST. The conserved sequence in multiple related species for a specific gene was used to design primers. Since the β actin gene (KU714644.1) is already sequenced in *T. putitora,* therefore primers were directly designed by using oligo Explorer 1.1.2 software. Two reverse primers having different lengths of PCR products i.e., 99bp and 567bp were designed for β-actin (Table 5).

Table 3. Reverse transcriptase-polymerase chain (RT-PCR) reaction mixture composition

Ingredient	Quantity (µl)
cDNA (RT)	0.5
Oligo (10mM) forward	0.3
Oligo (10mM) reverse	0.3
Buffer 10X	1.5
DNTPs	0.3
Taq. Polymerase	0.09
Water	12.01
Final volume	15µl

Table 4. Reverse transcriptase polymerase chain (RT-PCR) reaction conditions

Temperature (ºC)		Time duration (minutes)
94		5
94		0.5
65	40 cycles	0.5
72		2
72		5

Table 5. Primers used for reverse transcriptase Polymerase chain reaction (RT-PCR)

Gene	Name of primer		Sequence 5´ to 3´
MyoD	NY1801	Foreword	TTTCTACGACGACCCTTGCTTC
	NY1802	Reverse	TGCCATCAGAGCAGTTGGATC
Myogenin	NY1803	Foreword	CCAGCGTTTTTACGAAGGCG
	NY1804	Reverse	ACGTCAGAGACCTCAGGTTGG
HSP-70kDa	NY1814	Foreword	ATGGTCCTGGTGAAGATGAAG
	NY1815	Reverse	GATGTCCTTCTTGTGCTTCCTC
β-actin	KU714644.1	Foreword	GCTGTGCTGTCCCTGTATGC
		Reverse	GGCGTAACCCTCGTAGATGG (PCR Product=99bp)
		Reverse	TTTCATTGTGCTGGGGGC (PCR Product=567bp)

Agarose gel electrophoresis

To visualize and confirm the quality of cDNA and efficiency of the designed primers, the RT-PCR product was run on the agarose gel. Briefly, 1% agarose gel was prepared by taking 0.5g of agarose powder in a beaker, and then 1ml of 50X Tris-acetate-EDTA buffer (TAE buffer) and 49 ml of water was added to it. The ingredients were mixed and boiled in a microwave for 1-3 minutes and then placed at room temperature for 10 minutes for cooling. Subsequently, 10µl red DNA gel Stains

(Thermo Fisher Scientific) was added. Afterward, the gel was poured into a gel tray which already had a well-placed comb. After approximately 30 minutes, the comb was removed carefully, and the cooling gel was placed in a gel electrophoresis tank having 1X TAE buffer. It was made sure that the gel was completely dipped in the buffer solution.

At the end of RT-PCR, 7µl of Gel Loading Dye, Orange (Thermo Fisher Scientific) was added to the final PCR products. Then, 5µl of the mixture was loaded carefully into the gel along with 5µl of the 1kb ladder as reference. The gel was run for 15-30 minutes and then visualized under U.V light (Fig. 2).

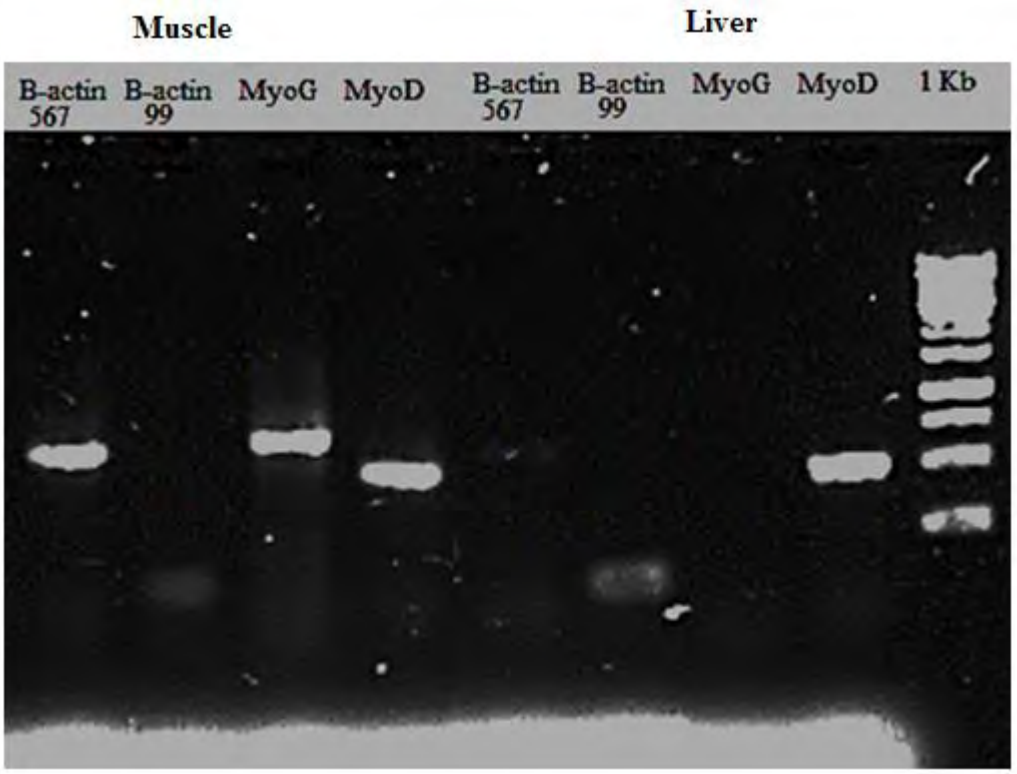

Figure 2. Gel picture of MyoD, Myogenin, β-actin 99 and, β-actin 567 cDNA (PCR) final products after agarose gel electrophoresis

Gene cloning and nucleotide sequencing

For gene expression analysis, a 100% nucleotide sequence must be known to design accurate primers. Since the whole genome of mahseer is not sequenced yet, so to study growth and stress response genes expression in advanced fry of *T. putitora* reared on graded level of dietary cobalt chloride, these genes were first cloned in *E.coli* and then sequenced.

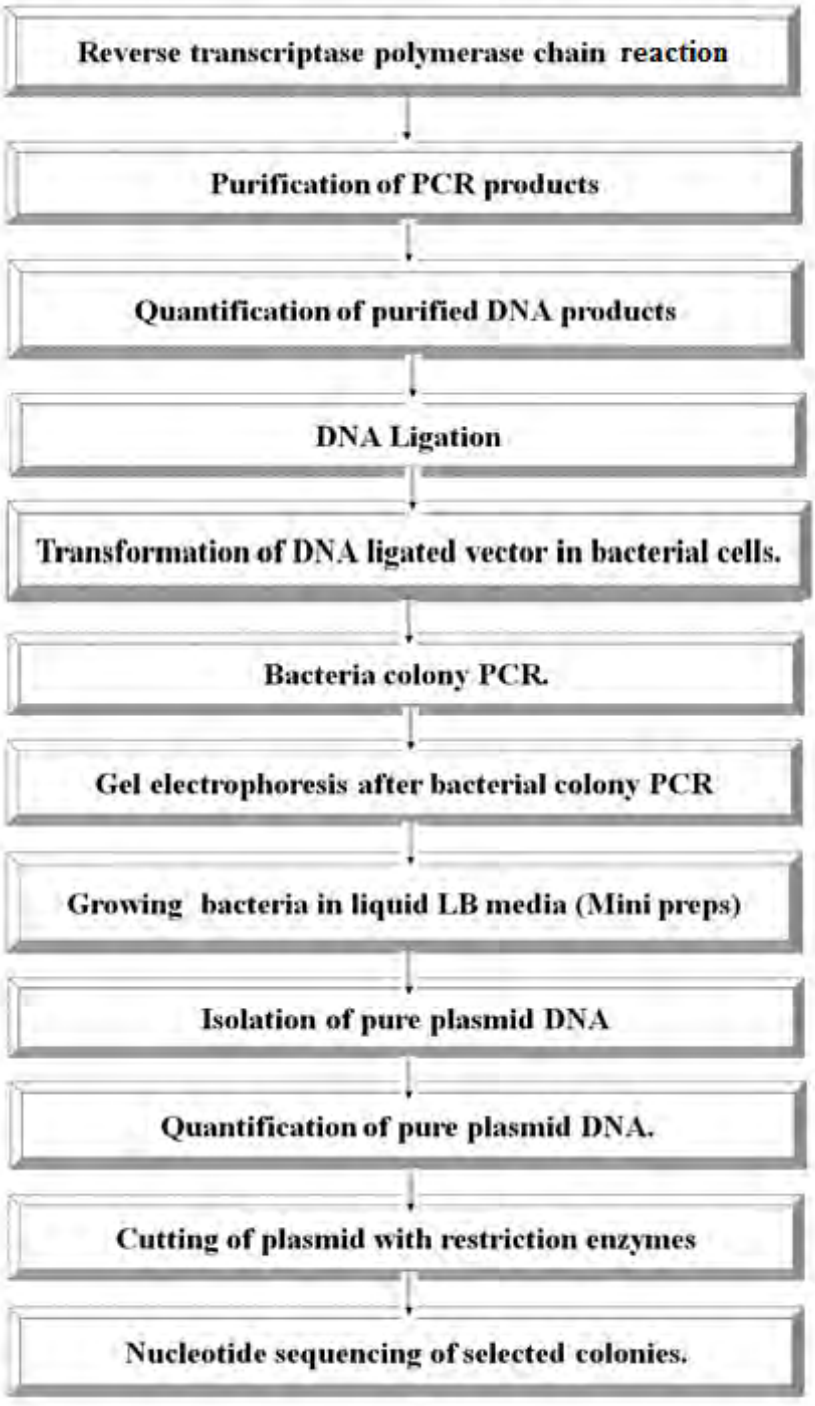

Figure 3. Steps followed for the molecular cloning of growth and stress response genes in muscle and liver.

Reverse transcriptase-polymerase chain RT-PCR reaction conditions

PCR was performed having a final volume of 150µl for each MyoD, Myogenin (MyoG), and HSP-70kDa. (RT-PCR reaction conditions and reaction mixture composition is shown in table 6 and 7 respectively) At the end of the PCR reaction, 5µl PCR product (from a sample) and 1µl of reaction buffer were added to perform gel electrophoresis (protocol as mentioned earlier). After that, the gel was visualized under UV light (Fig. 4)

Table 6. Reverse transcriptase polymerase chain (RT-PCR) reaction conditions (cloning)

Temperature ºC		Time (minutes)
94		2
94		0.5
68	40 cycles	0.5
72		1.15
72		5

Table 7. Reverse transcriptase-polymerase chain (RT-PCR) reaction mixture composition (for cloning)

Ingredients	Quantity (µl)
cDNA (RT)	5
Oligo (10mM) forward	3
Oligo (10mM) reverse	3
Buffer 10X	15
dNTPs	3
Taq. Polymerase	0.9
Water	120
Final volume	150(µl)

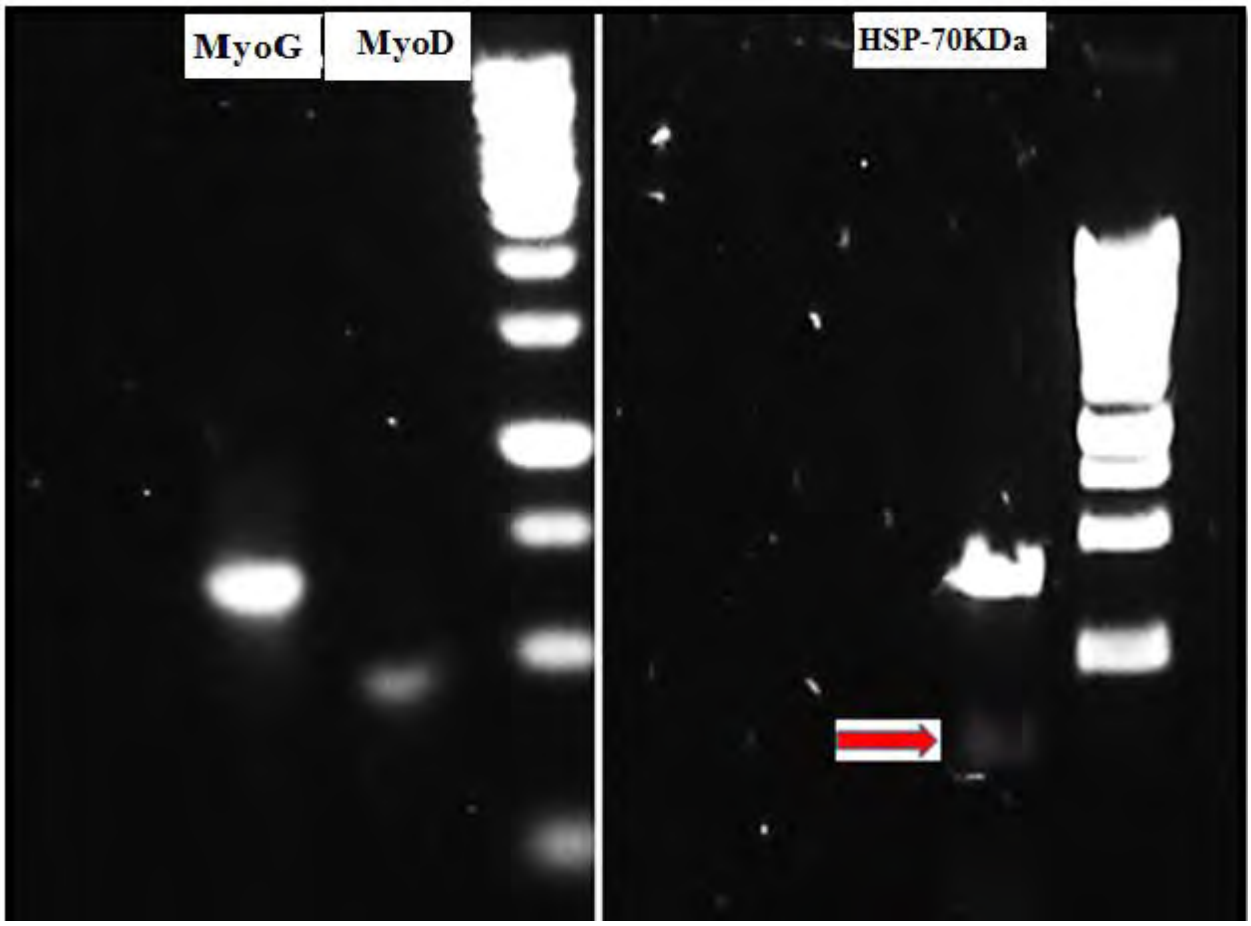

Figure 4. Gel picture of final products after agarose gel electrophoresis (for cloning of MyoD, MyoG, and HSP-70KDa genes: Red arrow showing impure band)

Purification of PCR products

PCR products (cDNA) were purified using a High pure PCR purification Kit (Roche; version 9, Ref. # 12-033-674-001) by using the following protocol:

Briefly, 50µl of PCR product and 50µl of Milli-Q water (MQ) water were mixed in an eppendorf tube, and 250µl of binding buffer was added. Afterward, the above solution was added to a high pure filter tube and centrifuged for 1 minute at 13000rpm (SCILOGEX LLC). To collect the DNA, the liquid was passed through the filter. Subsequently, 250µl binding buffer was added to the filter tube having cDNA and centrifuged at 13000rpm (SCILOGEX LLC) for 1 minute. The flow-through was discarded. After that, 100 µl of wash solution was added to filter for second wash and centrifuged at 13000 rpm (SCILOGEX LLC) for 1 minute and flow-through was again discarded. In the final step, 30µl of elusion buffer was added to the filter and centrifuge for 1 min at 13000 rpm (SCILOGEX LLC) to get highly purified DNA. The purified DNA products were quantified using NanoDrop. Furthermore, due to the appearance of impurity in the HSP-70kDa gel picture (presence of the unexpected lighter band, along with one band of the expected size), we had cut the expected band from the gel and purified it by adopting the following protocol.

Briefly, at first, DNA from the gel was released by placing the band in a micro-centrifuge tube having 300µl of binding buffer and vortexed for 15-30 seconds and then incubated at 56ºC for 10 minutes. Afterward, for purification, the released cDNA was transferred to the high pure filter tube and centrifuged at 8000rpm (SCILOGEX LLC) for 1 minute. After discarding the flow-through, 400 µl of wash buffer was added and again centrifuged at 8000 rpm (SCILOGEX LLC) for 1 minute. For the second wash, the 300µl wash solution was added and centrifuged at 8000 rpm (SCILOGEX LLC) for 1 minute and flow-through was again discarded. In the final step, 20 µl of elution buffer was used to get highly purified DNA. Purified DNA products were quantified using NanoDrop (Table 8).

Table 8. Concentration (ng/µl) of DNA after purification of PCR products

Gene	Concentration (ng/µl)	260/280	260/230
MyoD	36.4	1.58	0.77
	41.9	1.50	0.79
	19.1	1.78	0.85
Myogenin	133.8	1.51	0.79
	144.5	1.59	0.36
	49.4	1.80	1.24
HSP-70KDa	8.3	1.90	0.06
	5.5	4.51	0.06
	22.6	1.25	0.04

After DNA purification, gel electrophoresis was done to get purified bands of expected size by using 5µl of sample and 1µl of sample buffer (gel electrophoresis protocol mentioned above).

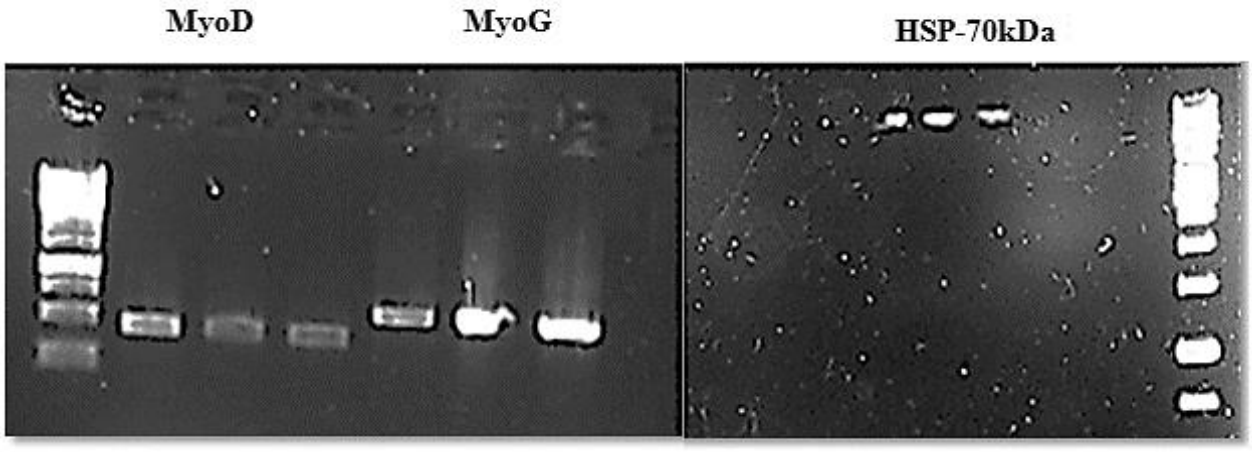

Figure 5. Gel picture of cDNA after purification of PCR products

DNA Ligation

Purified DNA was ligated with the plasmid pGemt Easy vector (3015bp). Ligation was performed having a final reaction volume of 10µl i.e., 5µl of 2X buffer, 1µl of ligase enzyme, while remaining 4µl contained DNA and plasmid vector having 3:1 ratio respectively. The ligation mixture was placed at room temperature for 15 minutes and then placed in the refrigerator overnight.

Transformation of DNA ligated vector in bacterial cells.

After overnight incubation of ligated cDNA, it was introduced into the bacteria.

Preparation of Petri plates for bacteria culturing

At first, LB agar media was prepared by dissolving 10g of NaCl, 10g of Tryptone, 5g of Yeast extract, and 20g of Agar in 1L of de-ionized water. Afterward, 3g of ampicillin was dissolved in 30 ml of water and filtered by using Whatman filter paper to sterilize it. Subsequently, the ampicillin stock solution was stored in a refrigerator. After that, ampicillin from stock solution was added to previously prepared LB agar media at the concentration level of 100µg/ml. Then, 20ml of LB agar

media + ampicillin mixture was poured into the petri dish and waited till it solidified. Later plates were incubated at 37ºC overnight.

Subsequently, an aliquot of "competent DH5α *E.coli* cell (stored at -80 ºC) was taken and its temperature was slowly reduced on ice (de-freeze) for 10 minutes. After that, 5µl of ligated DNA was mixed with 75µl of bacterial cells in a 1.5ml Eppendorf tube, mixed well, and incubated for 15 minutes on ice. Afterward, for thermal shock, it was incubated at 42ºC in a water bath for 1.5 minutes (this thermal shock had made a thick bacterial cell wall porous which allowed ligated DNA to enter inside the bacteria). After the thermal shock, the Eppendorf was immediately placed on ice for 2-3 minutes and its temperature was reduced from 42 ºC to 37 ºC (optimal temperature for bacterial cell growth). After that, 400µl LB was added to the tube and incubated at 37 ºC for 50 minutes in a water bath. In the final step, after the incubation period, Eppendorf was fast spin. The supernatant was discarded while the bottom layer having plasmid containing bacteria was selected.

Afterward, the suspended bacterial cells were tapped with a micropipette and spread on a Petri dish containing LB media + Ampicillin. Plates were placed at room temperature for 15 minutes and then incubated at 37ºC overnight. The next day, bacterial colonies were selected randomly and marked to perform bacterial colony PCR. To pick the bacterial colonies from a plate, a micropipette was set at 10µl and touched the tip of the micropipette to the colony. Then this tip was taken inside the PCR tubes having PCR master mix (Table 9) and moved up and down to mix it with the master mix.

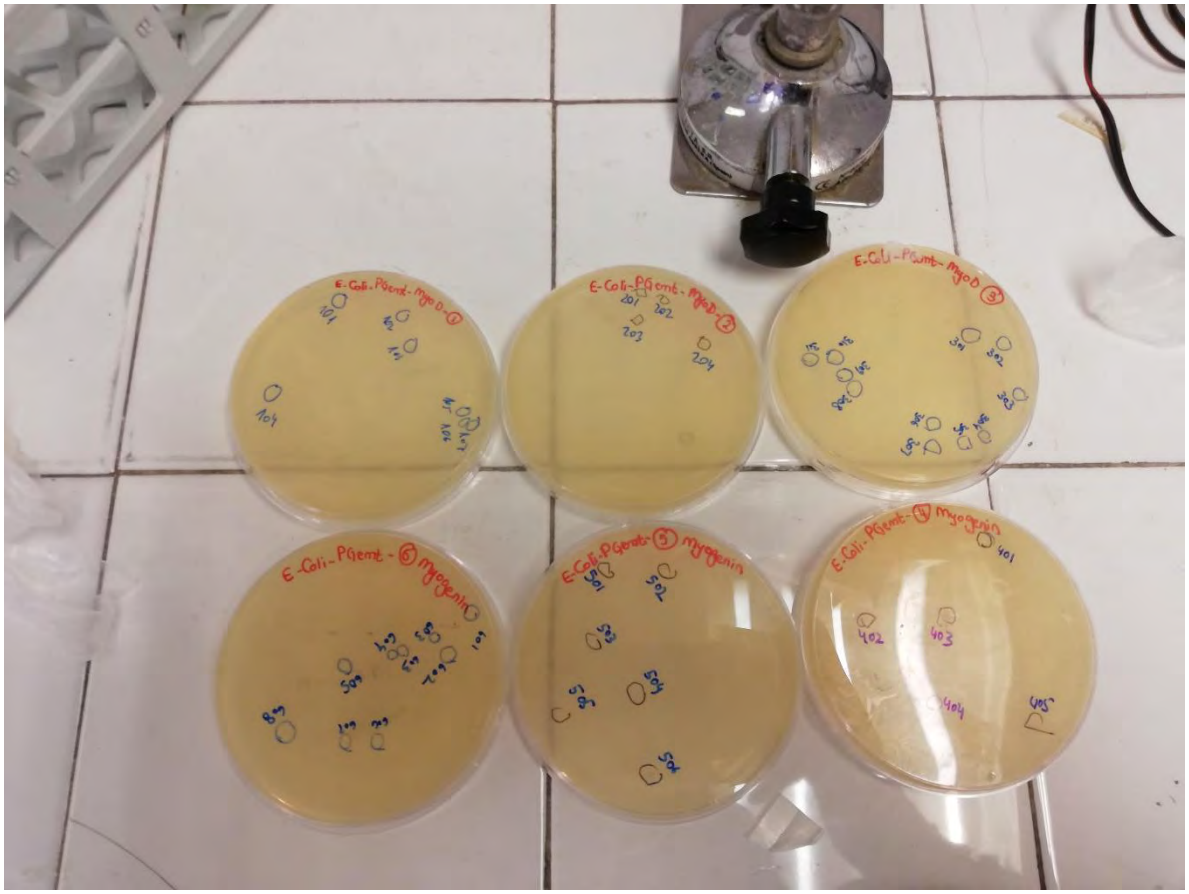

Figure 6. Petri dish having E.coli-pGemt-DNA

Bacteria colony PCR.

Bacteria colony PCR was performed from selected colonies (Fig.6) by adopting the same protocol as mentioned above (bacteria colony PCR and reaction conditions is shown in table 9 and 10 respectively).

Table 9. PCR reaction mix for bacteria colony amplification

Ingredient	Quantity (µl)
Bacteria	0.1
Oligo T7	0.3
Oligo SP6	0.3
Buffer 10X	1.5
d NTPs	0.3
Taq. Polymerase	0.09
Water	12.41
Final volume	15(µl)

Table 10. PCR program for bacteria colony PCR

Temperature (ºC)		Time (minutes)
94		5
94		0.5
55	35 cycles	0.5
72		1.15
72		5

Gel-electrophoresis after bacterial colony PCR

At the end of bacterial colony PCR, gel electrophoresis was done using bacterial colony PCR product (protocol mentioned above for electrophoresis).

After bacterial colony PCR, positive clones were selected from each plate

- ***E.coli*-pGemt-MyoD**

  103,104,203, 204, 310, 311 (Fig. 7)

- ***E.coli*-pGemt-MyoG**

  403, 404, 502, 506, 607, 608 (Fig. 8)

- ***E.coli*-pGemt-HSP-70kDa**

  701, 703, 704, 709, 711, 714, 803, 804, 805, 813, 905, 908, 912, 913 (Fig. 9)

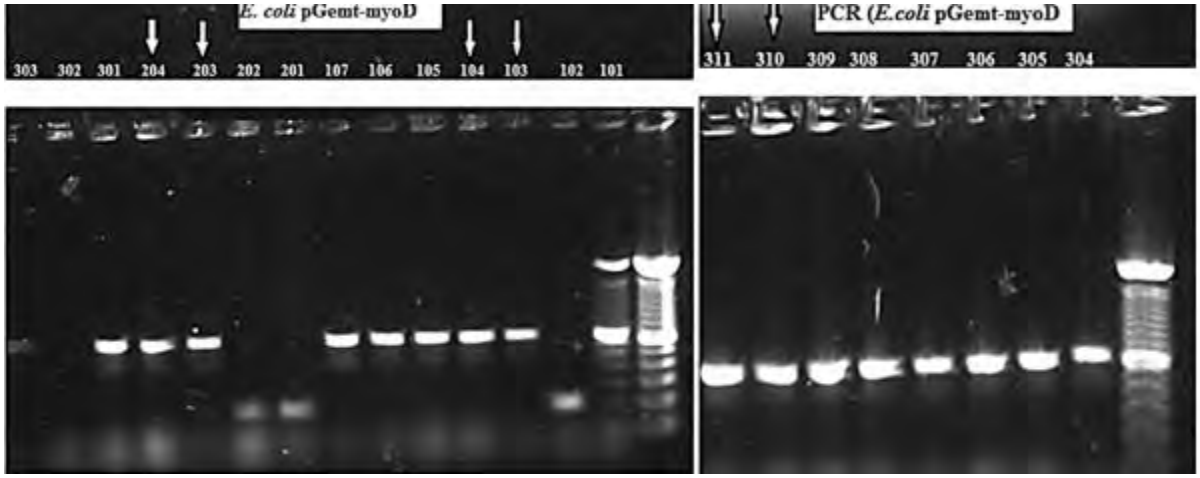

Figure 7. Gel picture of the final product of bacterial colony PCR (*E.coli* pGemt-myoD)

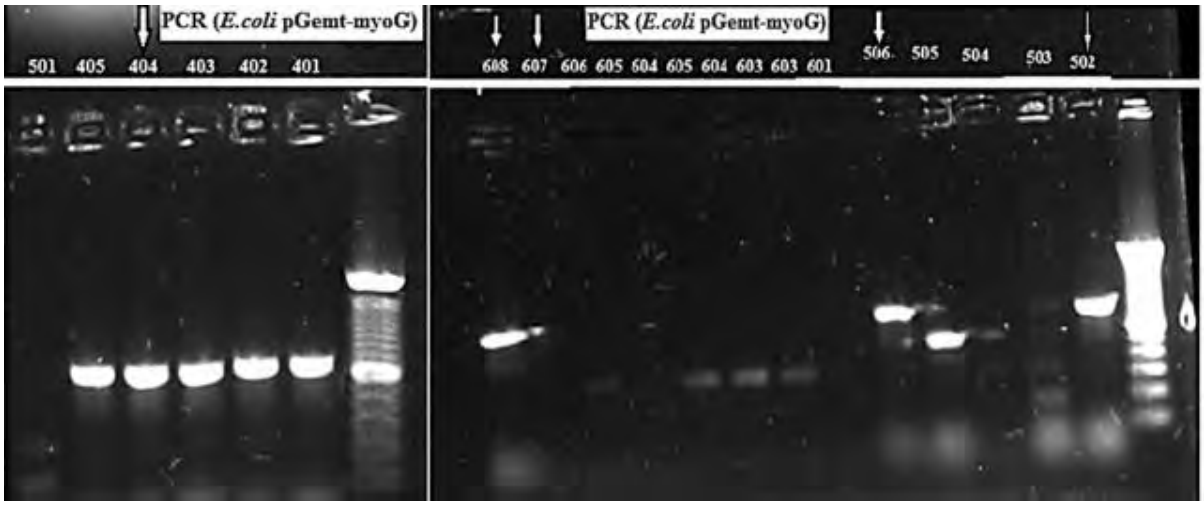

Figure 8. Gel picture of the final product of bacterial colony PCR (*E.coli* pGemt-MyoG)

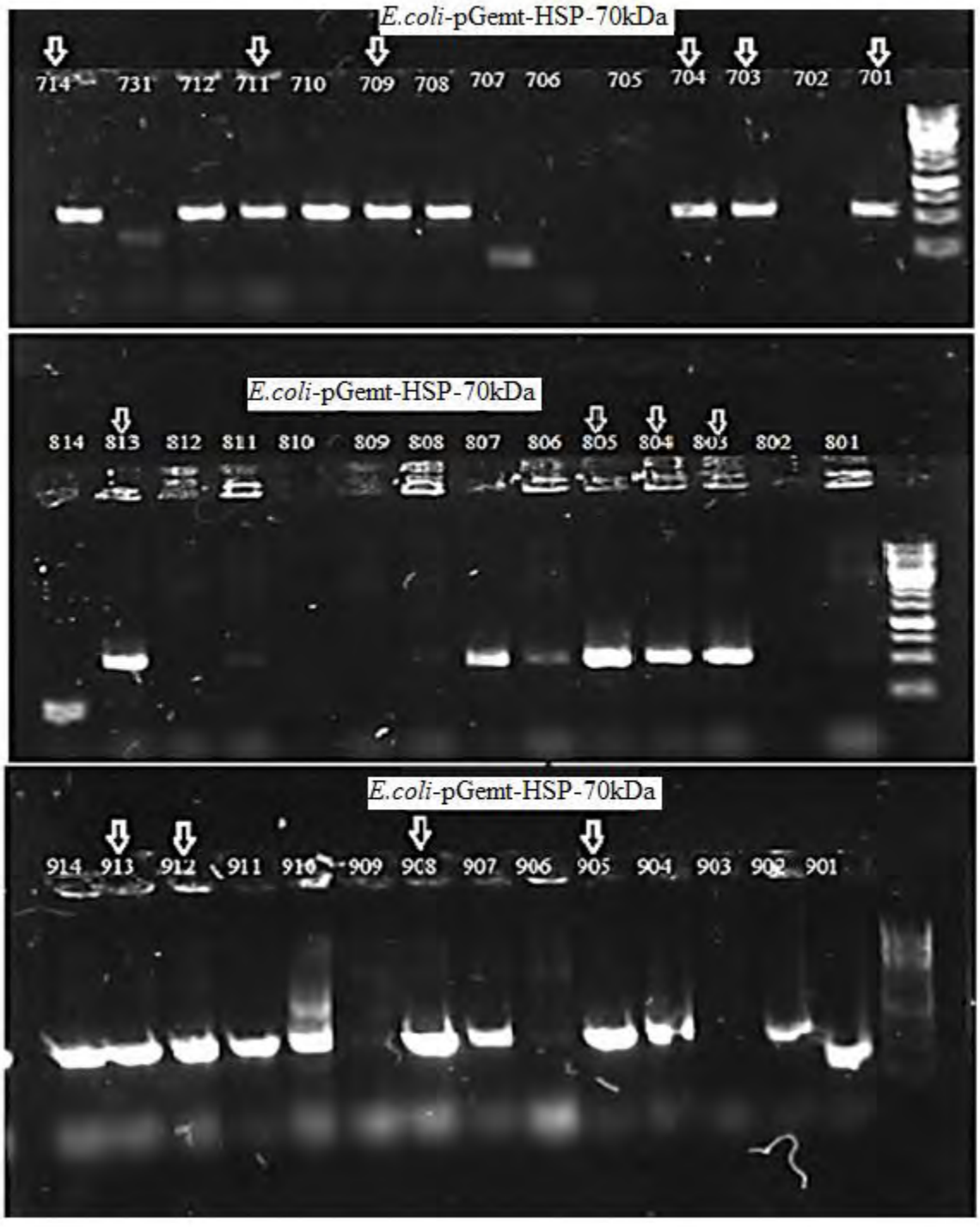

Figure 9. Gel picture of the final product of bacterial colony PCR (*E.coli* pGemt-HSP-70kDa)

Growing bacteria in liquid LB media (Mini preps)

Isolation of pure plasmid DNA

The next day, Pure plasmid DNA was isolated from bacteria cultures using Gene elute plasmid miniprep kit (CAT# PLN350, Sigma) using the following protocol.

Cultured cells were taken in a 1.5ml Eppendorf tube and centrifuged at 13000 rpm (SCILOGEX LLC) for 1 minute to get a small pellet. After centrifugation, the liquid was removed with a suction volume pump, and 200µl of suspension buffer was added and mixed by simple pipetting, then 200µl of lysis buffer was added to the tube and mixed by hand. After 5 minutes, 350µl of neutralization solution was added to the tube, mixed with hands, and then centrifuged at 13000 rpm (SCILOGEX LLC) for 10 minutes (side by side, the binding column was prepared by adding 500µl of column preparation solution to the column and centrifuged at 13000 rpm (SCILOGEX LLC) for 1minute). After centrifugation, the whole lysate was loaded into the binding column and centrifuged at 13000rpm (SCILOGEX LLC) for 1 minute. After centrifugation, flow-through was discarded, and 500µl of wash solution was added to the column and centrifuged. The flow-through was again discarded and the column was dried by spinning it for 1 minute. Afterward, the column was placed in a new collecting tube, and pure plasmid DNA was isolated with 30µl elution solution and quantified by using Nanodrop (Table 11).

Table 11. Quantification of pure plasmid DNA

Colony Number	Concentration ng/µl	O.D 260/280	O.D 260/230
103	425.5	1.87	2.31
104	231.1	1.88	2.30
203	288.2	1.89	2.45
204	268.2	1.89	2.36
310	528.6	1.88	2.30
311	168.6	1.87	3.68
403	241.3	1.86	2.41
404	161.7	1.88	2.52
502	271.3	1.86	2.3
506	409.3	1.84	2.32
607	257.3	1.88	2.28
608	213.6	1.89	2.72
701	402.8	1.94	2.32
703	363.6	1.95	2.32
704	230.9	1.95	2.57
709	434.1	1.92	2.41
711	318.6	1.96	2.39
714	245.7	1.95	2.34
803	281.3	1.95	2.42
804	501.6	1.93	2.41
805	390.5	1.94	2.42

813	421.7	1.92	2.43
905	445.0	1.91	2.16
908	439.5	1.92	2.33
912	436.9	1.94	2.28
913	375.9	1.92	2.32

Cutting of plasmid with restriction enzymes

In this step, the plasmid was cut with the help of a restriction enzyme i.e., EcoR1 (CAT No. ER0271, Thermo Scientific™) to release ligated DNA. All the ingredients mentioned in table 12 were mixed and incubated at 37ºC for 90 minutes and stored at -20ºC. Later on, the gel was run to look for the bands (Fig. 10).

Table 12. The reaction mixture for plasmid cutting with restriction enzymes

Ingredients	**Quantity µl**
Plasmid	1
EcoR1 Buffer	1
EcoR1 enzyme	0.3
Water	7.7
Total	10µl

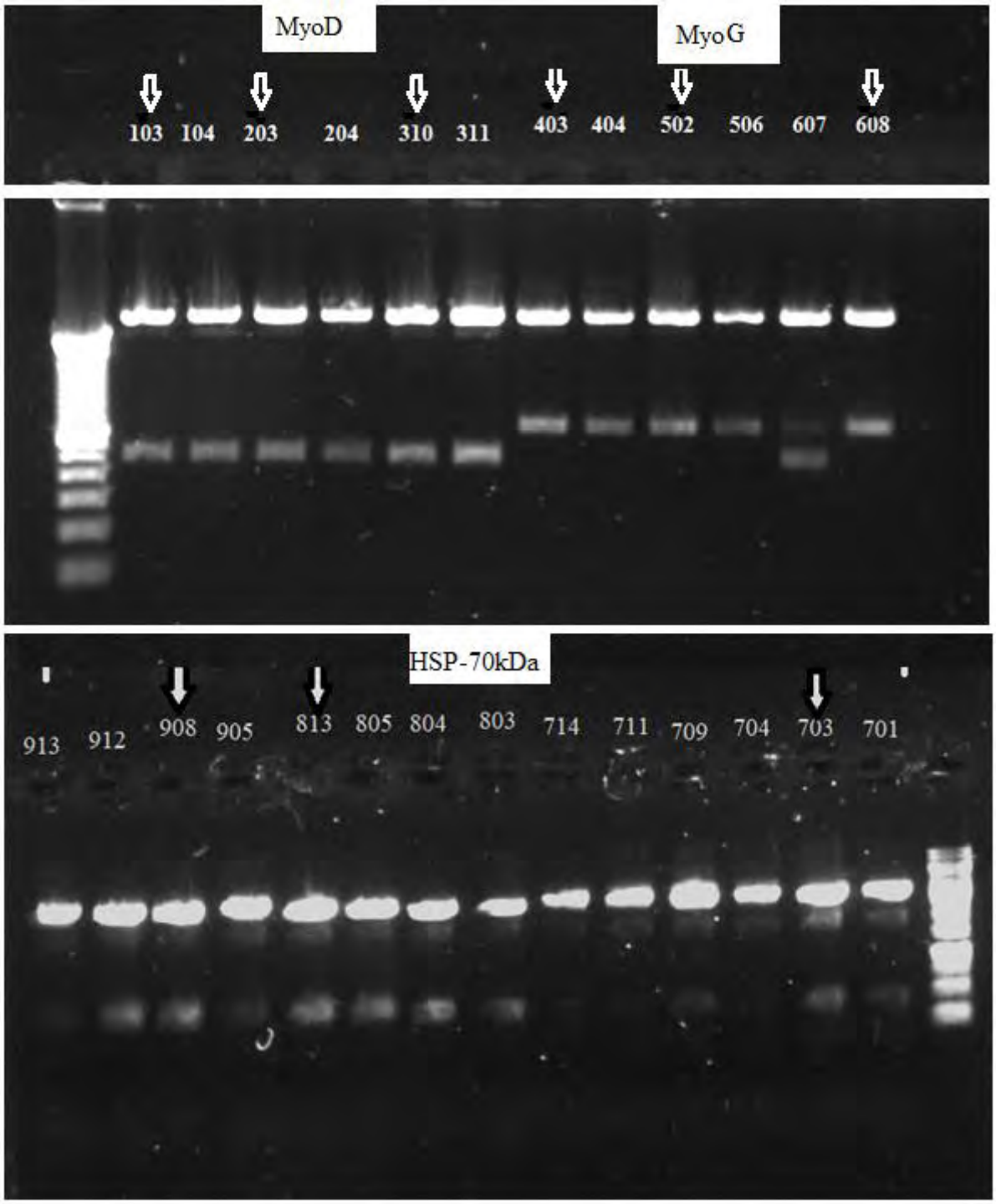

Figure 10. Gel picture after cutting plasmid with restriction enzyme

Nucleotide sequencing of selected colonies

In the final step, DNA was sent for gene sequencing. For this purpose, samples were dried with forward and reverse oligos separately. Briefly, DNA samples were added in each tube (2tubes for each sample, one for forward primer while the other for reverse primer) and dried on the the thermostat at 85ºC for 15 minutes. The tubes were sent for gene sequencing to Serveis Cientificotecnis, Unitat de Genomica, University of Barcelona, Spain.

As per calculation,

Quantity of plasmid-DNA which was added into each tube=100ng/1kb of DNA

Size of pGemt= 3015bp

Size of PCR product (MyoD) = 464bps

(MyoG) = 665bps

(HSP-70kDa)= 396bps

Total length of plasmid DNA fragment = 3479bp (MyoD); 3680bps (MyoG); 3411bps (HSP-70kDa)

Table 13. Samples used for gene sequencing

Gene	Sample	Primer	Sample code for sequencing
MyoD	103	F	NYF-1
		R	NYR1
MyoD	203	F	NYF-2
		R	NYR-2
MyoD	310	F	NYF-3
		R	NYR-3
Myogenin	403	F	NYF-4
		R	NYR-4
Myogenin	502	F	NYF-5
		R	NYR-5
Myogenin	603	F	NYF-6
		R	NYR-6
HSP-70kDa	703	F	NYF-7
		R	NYR-7
HSP-70kDa	813	F	NYF-8
		R	NYR-8
HSP-70kDa	908	F	NYF-9
		R	NYR-9

MyoD gene sequncing

Obtained sequences were analyzed through bioedit 7.0.5.3 software.

>NYF1

TTTCTACGACGACCCTTGCTTCAACACAAACGATATGCACTTCTTTGAAGACCTGGA
CCCCAGGCTCGTCCACGTGAGTCTGCTCAAGCCCGACGAGCATCACCACATCGAGGAC
GAGCACGTAAGGGCGCCCAGCGGGCATCATCAGGCCGGCAGGTGCCTGCTGTGGGCAT
GCAAAGCCTGCAAGAGAAAAACTACCAACGCTGACCGCCGCAAAGCCGCCACCATGA
GGGAGAGGAGAAGACTGAGCAAGGTCAACGATGCTTTCGAGACCCTCAAGAGATGCA
CGTCCACCAACCCCAACCAGAGGCTGCCCAAAGTGGAGATTCTGAGAAACGCCATTAG
TTACATTGAGTCTCTG
CAGGCGCTGCTCAGGAGTCAAGAGGAAAACTACTACCCTGTTCTGGAACATTACAGTG
GAGACTCCGATGCCTCCAGCCCGA**GATCCAACTGCTCTGATGGCA**

>NYF3

TTTCTACGACGACCCTTGCTTCAACACAAACGATATGCACTTCTTTGAAGACCTGGA
CCCCAGGCTCGTCCACGTGAGCCTGCTCAAGCCCGACGAGCATCACCTCGAGGACGAG
CACGTGAGGGCGCCCAGCGGGCATCATCAGGCCGGCAGGTGCCTGCTGTGGGCATGCA
AAGCCTGCAAGAGAAAAACCACCAACGCTGACCGCCGCAAAGCCGCCACCATGAGGG
AGAGGAGAAGACTGAGCAAAGTCAACGACGCTTTCGAGACCCTCAAGAGATGCACGT
CCACCAACCCCAACCAGAGGCTGCCCAAAGCGGAGATTCTGAGAAACGCCATTAGTTA
CATTGAGTCTCTGCAAGCGCTGCTTAGGAGTCAAGAGGAAAACTACTACCCCGTTCTG
GAACATTACAGTGGAGACGCCGATGCCTCCAGCCCGA**GATCCAACTGCTCTGATGGC
A**

>NYR1

TTTCTACGACGACCCTTGCTTCAACACAAACGATATGCACTTCTTTGAAGACCTGGA
CCCCAGGCTCGTCCACGTGAGTCTGCTCAAGCCCGACGAGCATCACCACATCGAGGAC
GAGCACGTAAGGGCGCCCAGCGGGCATCATCAGGCCGGCAGGTGCCTGCTGTGGGCAT
GCAAAGCCTGCAAGAGAAAAACTACCAACGCTGACCGCCGCAAAGCCGCCACCATGA
GGGAGAGGAGAAGACTGAGCAAGGTCAACGATGCTTTCGAGACCCTCAAGAGATGCA

CGTCCACCAACCCCAACCAGAGGCTGCCCAAAGTGGAGATTCTGAGAAACGCCATTAG
TTACATTGAGTCTCTGCAGGCGCTGCTCAGGAGTCAAGAGGAAAACTACTACCCTGTTC
TGGAACATTACAGTGGAGACTCCGATGCCTCCAGCCCGA**GATCCAACTGCTCTGATG**
GCA

>NYR2

TTTCTACGACGACCCTTGCTTCAACACCAATGACATGCACTTCTTTGAAGACCTGGA
CCCCAGGCTCGTCCACGTGAGTCTGCTCAAGCCCGACGAGCATCACCACATCGAGGAC
GAGCACGTAAGGGCGCCCAGCGGGCATCATCAGGCCGGCAGGTGCCTGCTGTGGGCAT
GCAAAGCCTGCAAGAGAAAAACTACCAACGCTGACCGCCGCAAAGCCGCCACCATGA
GGGAGAGGAGACGACTGAGCAAAGTCAACGACGCTTTCGAGACCCTCAAGAGATGCA
CGTCCACCAACCCGAACCAGAGGCTGCCCAAAGTGGAGATTCTGAGAAACGCCATTAG
TTACATCGAGTCTCTGCAGGCGCTACTTAGGAGTCAAGAGGAAAACTACTACCCTGTTC
TGGAGCATTACAGCGGAGACTCTGATGCCTCCAGCCCGA**GATCCAACTGCTCTGATG**
GCA

>**NYR3**

TTTCTACGACGACCCTTGCTTCAACACAAACGATATGCACTTCTTTGAAGACCTGGA
CCCCAGGCTCGTCCACGTGAGCCTGCTCAAGCCCGACGAGCATCACCTCGAGGACGAG
CACGTGAGGGCGCCCAGCGGGCATCATCAGGCCGGCAGGTGCCTGCTGTGGGCATGCA
AAGCCTGCAAGAGAAAAACCACCAACGCTGACCGCCGCAAAGCCGCCACCATGAGGG
AGAGGAGAAGACTGAGCAAAGTCAACGACGCTTTCGAGACCCTCAAGAGATGCACGT
CCACCAACCCCAACCAGAGGCTGCCCAAAGCGGAGATTCTGAGAAACGCCATTAGTTA
CATTGAGTCTCTGCAAGCGCTGCTTAGGAGTCAAGAGGAAAACTACTACCCCGTTCTG
GAACATTACAGTGGAGACGCCGATGCCTCCAGCCCGA**GATCCAACTGCTCTGATGGC**
A

```
NYF3        TTTTCTACGACGACCCTTGCTTCAACACAAACGATATGCACTTCTTTGAAGACCTGGACC      60
NYR3        TTTTCTACGACGACCCTTGCTTCAACACAAACGATATGCACTTCTTTGAAGACCTGGACC      60
NYR2        TTTTCTACGACGACCCTTGCTTCAACACCAATGACATGCACTTCTTTGAAGACCTGGACC      60
NYF1        -TTTCTACGACGACCCTTGCTTCAACACAAACGATATGCACTTCTTTGAAGACCTGGACC      59
NYR1        TTTTCTACGACGACCCTTGCTTCAACACAAACGATATGCACTTCTTTGAAGACCTGGACC      60
             ************************** ** ** ************************

NYF3        CCAGGCTCGTCCACGTGAGCCTGCTCAAGCCCGACGAGCATCAC---CTCGAGGACGAGC      117
NYR3        CCAGGCTCGTCCACGTGAGCCTGCTCAAGCCCGACGAGCATCAC---CTCGAGGACGAGC      117
NYR2        CCAGGCTCGTCCACGTGAGTCTGCTCAAGCCCGACGAGCATCACCACATCGAGGACGAGC      120
NYF1        CCAGGCTCGTCCACGTGAGTCTGCTCAAGCCCGACGAGCATCACCACATCGAGGACGAGC      119
NYR1        CCAGGCTCGTCCACGTGAGTCTGCTCAAGCCCGACGAGCATCACCACATCGAGGACGAGC      120
            ****************** ************************    ************

NYF3        ACGTGAGGGCGCCCAGCGGGCATCATCAGGCCGGCAGGTGCCTGCTGTGGGCATGCAAAG      177
NYR3        ACGTGAGGGCGCCCAGCGGGCATCATCAGGCCGGCAGGTGCCTGCTGTGGGCATGCAAAG      177
NYR2        ACGTAAGGGCGCCCAGCGGGCATCATCAGGCCGGCAGGTGCCTGCTGTGGGCATGCAAAG      180
NYF1        ACGTAAGGGCGCCCAGCGGGCATCATCAGGCCGGCAGGTGCCTGCTGTGGGCATGCAAAG      179
NYR1        ACGTAAGGGCGCCCAGCGGGCATCATCAGGCCGGCAGGTGCCTGCTGTGGGCATGCAAAG      180
            **** ************************************************************

NYF3        CCTGCAAGAGAAAAACCACCAACGCTGACCGCCGCAAAGCCGCCACCATGAGGGAGAGGA      237
NYR3        CCTGCAAGAGAAAAACCACCAACGCTGACCGCCGCAAAGCCGCCACCATGAGGGAGAGGA      237
NYR2        CCTGCAAGAGAAAAACTACCAACGCTGACCGCCGCAAAGCCGCCACCATGAGGGAGAGGA      240
NYF1        CCTGCAAGAGAAAAACTACCAACGCTGACCGCCGCAAAGCCGCCACCATGAGGGAGAGGA      239
NYR1        CCTGCAAGAGAAAAACTACCAACGCTGACCGCCGCAAAGCCGCCACCATGAGGGAGAGGA      240
            *************** ******************************************

NYF3        GAAGACTGAGCAAAGTCAACGACGCTTTCGAGACCCTCAAGAGATGCACGTCCACCAACC      297
NYR3        GAAGACTGAGCAAAGTCAACGACGCTTTCGAGACCCTCAAGAGATGCACGTCCACCAACC      297
NYR2        GACGACTGAGCAAAGTCAACGACGCTTTCGAGACCCTCAAGAGATGCACGTCCACCAACC      300
NYF1        GAAGACTGAGCAAGGTCAACGATGCTTTCGAGACCCTCAAGAGATGCACGTCCACCAACC      299
NYR1        GAAGACTGAGCAAGGTCAACGATGCTTTCGAGACCCTCAAGAGATGCACGTCCACCAACC      300
            ** ********** ******** ************************************

NYF3        CCAACCAGAGGCTGCCCAAAGCGGAGATTCTGAGAAACGCCATTAGTTACATTGAGTCTC      357
NYR3        CCAACCAGAGGCTGCCCAAAGCGGAGATTCTGAGAAACGCCATTAGTTACATTGAGTCTC      357
NYR2        CGAACCAGAGGCTGCCCAAAGTGGAGATTCTGAGAAACGCCATTAGTTACATCGAGTCTC      360
NYF1        CCAACCAGAGGCTGCCCAAAGTGGAGATTCTGAGAAACGCCATTAGTTACATTGAGTCTC      359
NYR1        CCAACCAGAGGCTGCCCAAAGTGGAGATTCTGAGAAACGCCATTAGTTACATTGAGTCTC      360
            * ****************** ****************************** *******

NYF3        TGCAAGCGCTGCTTAGGAGTCAAGAGGAAAACTACTACCCCGTTCTGGAACATTACAGTG      417
NYR3        TGCAAGCGCTGCTTAGGAGTCAAGAGGAAAACTACTACCCCGTTCTGGAACATTACAGTG      417
NYR2        TGCAGGCGCTACTTAGGAGTCAAGAGGAAAACTACTACCCTGTTCTGGAGCATTACAGCG      420
NYF1        TGCAGGCGCTGCTCAGGAGTCAAGAGGAAAACTACTACCCTGTTCTGGAACATTACAGTG      419
NYR1        TGCAGGCGCTGCTCAGGAGTCAAGAGGAAAACTACTACCCTGTTCTGGAACATTACAGTG      420
            **** ***** ** ************************** ******** ******** *

NYF3        GAGACGCCGATGCCTCCAGCCCGAGATCCAACTGCTCTGATGGCA   462
NYR3        GAGACGCCGATGCCTCCAGCCCGAGATCCAACTGCTCTGATGGCA   462
NYR2        GAGACTCTGATGCCTCCAGCCCGAGATCCAACTGCTCTGATGGCA   465
NYF1        GAGACTCCGATGCCTCCAGCCCGAGATCCAACTGCTCTGATGGCA   464
NYR1        GAGACTCCGATGCCTCCAGCCCGAGATCCAACTGCTCTGATGGCA   465
            ***** * ************************************
```

Figure 11. Sequence alignments for MyoD (Clustal Omega Multiple Sequence Alignment tool)

Selected MyoD gene sequence for *Tor putitora*

AACACAAACGATATGCACTTCTTTGAAGACCTGGACCCCAGGCTCGTCCACGTGAGTC

TGCTCAAGCCCGACGAGCATCACCACATCGAGGACGAGCACGTAAGGGCGCCCAGCG

GGCATCATCAGGCCGGCAGGTGCCTGCTGTGGGCATGCAAAGCCTGCAAGAGAAAAA

CTACCAACGCTGACCGCCGCAAAGCCGCCACCATGAGGGAGAGGAGAAGACTGAGCA

AAGTCAACGACGCTTTCGAGACCCTCAAGAGATGCACGTCCACCAACCCCAACCAGAG

GCTGCCCAAAGTGGAGATTCTGAGAAACGCCATTAGTTACATTGAGTCTCTGCAGGCG

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CCGATGCCTCCAGCCCGA

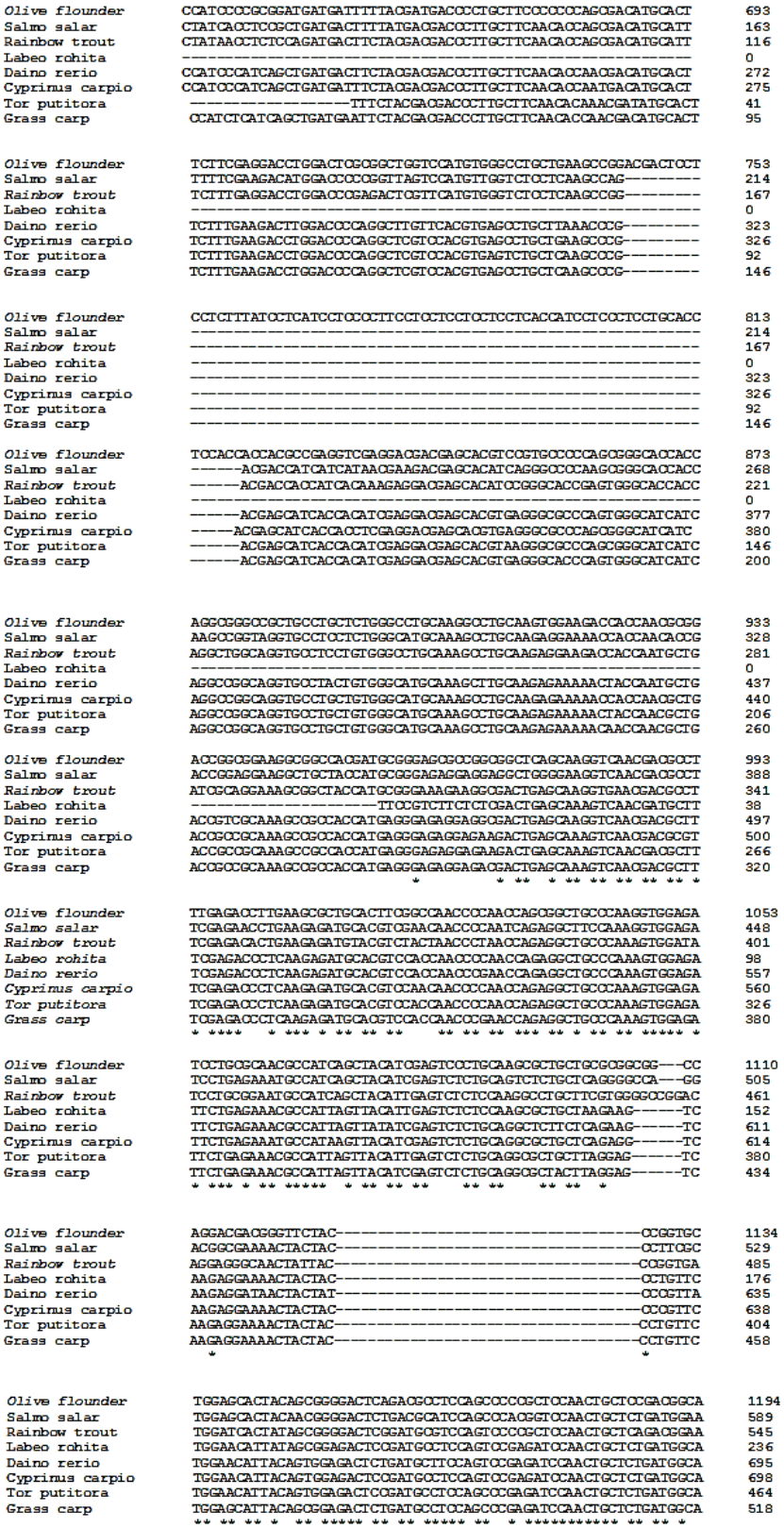


Figure 12. Múltiple species nucleotide alignment for MyoD gene

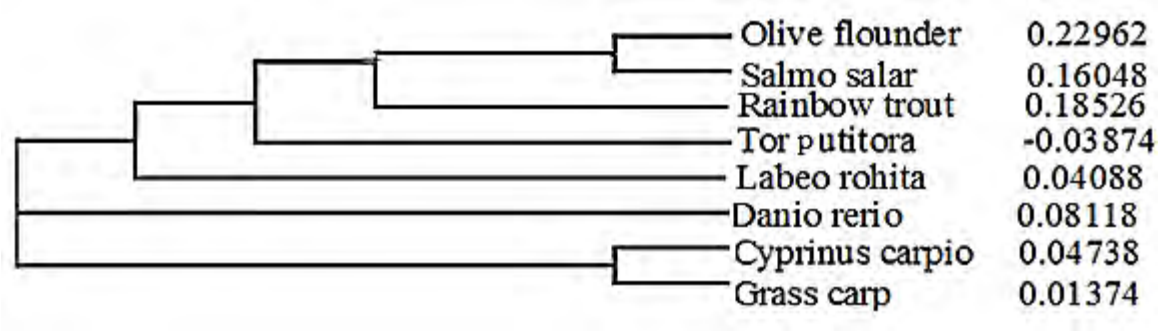


Figure 13. Phylogenetic tree (nucleotide alignments) for MyoD

MyoD Protein alignment

T. putitora protein sequence was translated from nucleotide sequence through the ExPASy bioinformatics Resource portal.

NTNDMHFFEDLDPRLVHVSLLKPDEHHHIEDEHVRAPSGHHQAGRCLLWACKACKRKTTNADRRKAATMRERRRLSKVN
DAFETLKRCTSTNPNQRLPKVEILRNAISYIESLQALLRSQEENYYPVLEHYSGDSDASSP

```
Mus                 MELLSPPLRDIDLTGPDGSLCSFETADDFYDDPCFDSPDLRFFEDLDPRLVHVGALLKPE        60
Homo                MELLSPPLRDVDLTAPDGSLCSFATTDDFYDDPCFDSPDLRFFEDLDPRLMHVGALLKPE        60
Paralichthys        MELS-------------DMSFPIPADDDFYDDPCFPPSDMHFFEDLDSRLVHVGLL-KPD        46
Salmo salar         MELS-------------DISFPITSADDFYDDPCFNTSDMHFFEDMDPRLVHVGLL-KPD        46
O.mykiss            MELP-------------DIPFPITSPDDFYDDPCFNTSDMHFFEDLDPRLVHVGLL-KPD        46
Cyprinus carpio     MELS-------------DIPFPIPSADDFYDDPCFNTNDMHFFEDLDPRLVHVSLL-KPD        46
Daino rerio         MELS-------------DIPFPIPSADDFYDDPCFNTNDMHFFEDLDPRLVHVSLL-KPD        46
Tor putitora        -----------------------------------NTNDMHFFEDLDPRLVHVSLL-KPD        24
C idella            MELS-------------DIPFPISSADEFYDDPCFNTNDMHFFEDLDPRLVHVSLL-KPD        46
                                                        *::****:* **:**. * **:

Mus                 EHAHFSTA--------------VHPGPGAREDEHVRAPSGHHQAGRCLLWACKACKRKTT       106
Homo                EHSHFPAA--------------VHPAPGAREDEHVRAPSGHHQAGRCLLWACKACKRKTT       106
Paralichthys        DSSSLSSSSPSSSSSSPSSLLHLHHHAEVEDDEHVRAPSGHHQAGRCLLWACKACKWKTT       106
Salmo salar         DHH-------------------------HNEDEHIRAPSGHHQAGRCLLWACKACKRKTT       81
O.mykiss            DHH-------------------------HKEDEHIRAPSGHHQAGRCLLWACKACKRKTT       81
Cyprinus carpio     EHH-------------------------HLEDEHVRAPSGHHQAGRCLLWACKACKRKTT       81
Daino rerio         EHH-------------------------HIEDEHVRAPSGHHQAGRCLLWACKACKRKTT       81
Tor putitora        EHH-------------------------HIEDEHVRAPSGHHQAGRCLLWACKACKRKTT       59
C idella            EHH-------------------------HIEDEHVRAPSGHHQAGRCLLWACKACKRKTT       81
                    :                             :***:**********************.***

Mus                 NADRRKAATMRERRRLSKVNEAFETLKRCTSSNPNQRLPKVEILRNAIRYIEGLQALLRD       166
Homo                NADRRKAATMRERRRLSKVNEAFETLKRCTSSNPNQRLPKVEILRNAIRYIEGLQALLRD       166
Paralichthys        NADRRKAATMRERRRLSKVNDAFETLKRCTSANPNQRLPKVEILRNAISYIESLQALLRG       166
Salmo salar         NTDRRKAATMRERRRLGKVNDAFENLKRCTSNNPNQRLPKVEILRNAISYIESLQSLLRG       141
O.mykiss            NADRRKAATMRERRRLSKVNDAFETLKRCTSTNPNQRLPKVDILRNAISYIESLQGLLRG       141
Cyprinus carpio     NADRRKAATMRERRRLSKVNDAFETLKRCTSNNPNQRLPKVEILRNAISYIESLQALLRG       141
Daino rerio         NADRRKAATMRERRRLSKVNDAFETLKRCTSTNPNQRLPKVEILRNAISYIESLQALLRS       141
Tor putitora        NADRRKAATMRERRRLSKVNDAFETLKRCTSTNPNQRLPKVEILRNAISYIESLQALLRS       119
C idella            NADRRKAATMRERRRLSKVNDAFETLKRCTSTNPNQRLPKVEILRNAISYIESLQALLRS       141
                    *:***************.***:***.****** *********:****** ***.**.***.

Mus                 QDAAPPGAAAFYAPGPLPPGRGSEHYSGDSDASSPRSNCSDGMMDYSGPPSGPRRQNGYD       226
Homo                QDAAPPGAAAFYAPGPLPPGRGGEHYSGDSDASSPRSNCSDGMMDYSGPPSGARRRNCYE       226
Paralichthys        G-QDD----GFY--------PVLEHYSGDSDASSPRSNCSDGMTDFNGPTCQSTRRGSYE       213
Salmo salar         QD-GE----NYY--------PSLEHYNGDSDASSPRSNCSDGMMEYNA------------       176
O.mykiss            AGQEG----NYY--------PVMDHYSGDSDASSPRSNCSDGMMDFNGQSCPPRRRNKYD       189
Cyprinus carpio     --QEE----NYY--------PVLEHYSGDSDASSPRSNCSDGMMDFMGPTCQSRRRNSYD       187
Daino rerio         --QED----NYY--------PVLEHYSGDSDASSPRSNCSDGIMDFMGPTCQTRRRNSYD       187
Tor putitora        --QEE----NYY--------PVLEHYSGDSDASSP------------------------       140
C idella            --QEE----NYY--------PVLEHYSGDSDASSPRSNCSDGMMDFMGPTCQSRRRNSYD       187
                          :*          :**.********
```

Figure 14. Múltiple species protein alignment for MyoD

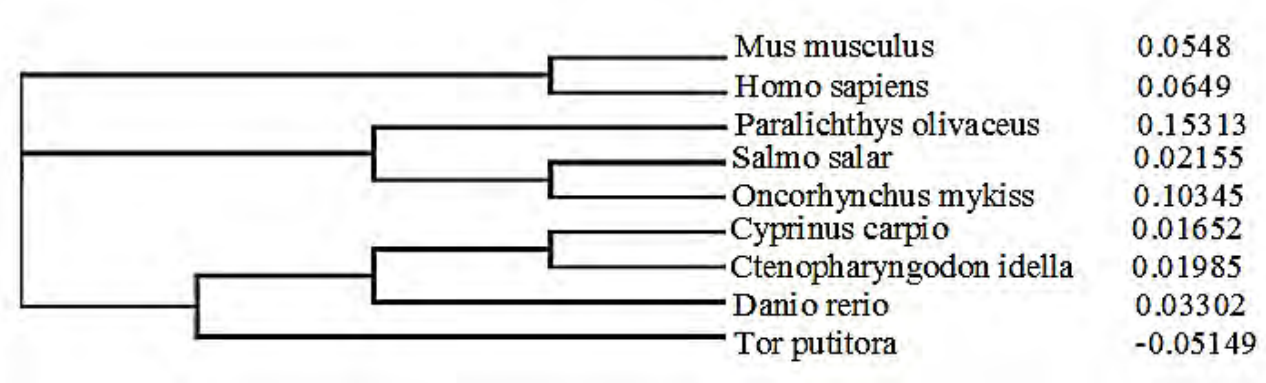


Figure 15. Phylogenetic tree (protein alignments) for MyoD

Myogenin gene sequncing

>**NYF4**

CCAGCGTTTTTACGAAGGCGGCGATAACTTCTTCCAGTCAAGGCTGACCGGAGGCTT
CGACCAAGCAGGATATCAGGACCGAAGCTCCATGGTGGGCTTGTGTGGCGACGGAAG
GCTGCTGTCAAACGGAGTGGGGATGGAGGACAAACCGTCTCCATCTTCTAGCCTCGGT
CTGTCCATGTCTCCTCACCAGGAGCAGCAGCACTGTCCGGGTCAGTGTCTGCCCTGGGC
CTGCAAGGTGTGTAAGCGCAAGTCGGTGACAATGGATCGACGGAAAGCCGCCACTTTG
AGGGAGAAGAGGAGGCTGAAGAAAGTCAACGAGGCCTTTGAGGCTCTTAAGAGGAGC
ACGCTCATGAATCCCAACCAGAGGCTGCCTAAGGTGGAGATCCTGCGCAGCGCCATCC
AGTACATCGAGAGACTCCAGGCGCTGGTCAGCTCTCTCAACCAGCAGGAACATGAGCA
GGGAAACATGCATTACAGAGCCGCGGCTCCTCAAGGGATGTCGTCCTCTAGCGATCAG
GGCTCTGGCAGCACCTGCTGTAGCAGTCCAGAGTGGAGCAGTACGTCTGAGCACTGTG
CCCCCGCCTACAGCTCCACCCATGAGGATCTCCTGAATGACGACTCCTCAGAGCAAA**C**
CAACCTGAGGTCTCTGACGT

>**NYF5**

CCAGCGTTTTTACGAAGGCGGCGATAACTTCTTCCAGTCAAGGCTGACCGGAGGCTT
CGACCAAGCAGGATATCAGGACCGAAGCTCCATGGTGGGCTTGTGTGGCGACGGAAG
GCTGCTGTCAAACGGAGTGGGGATGGAGGACAAACCGTCTCCATCTTCTAGCCTCGGT
CTGTCCATGTCTCCTCACCAGGAGCAGCAGCACTGTCCGGGTCAGTGTCTGCCCTGGGC
CTGCAAGGTGTGTAAGCGCAAGTCGGTGACAATGGATCGACGGAAAGCCGCCACTTTG
AGGGAGAAGAGGAGGCTGAAGAAAGTCAACGAGGCCTTTGAGGCTCTTAAGAGGAGC
ACGCTCATGAATCCCAACCAGAGGCTGCCTAAGGTGGAGATCCTGCGCAGCGCCATCC
AGTACATCGAGAGACTCCAGGCGCTGGTCAGCTCTCTCAACCAGCAGGAACATGAGCA
GGGAAACATGCATTACAGAGCCGCGGCTCCTCAAGGGATGTCGTCCTCTAGCGATCAG
GGCTCTGGCAGCACCTGCTGTAGCAGTCCAGAGTGGAGCAGTACGTCTGAGCACTGTG
CCCCCGCCTACAGCTCCACCCATGAGGATCTCCTGAATGACGACTCCACAGAGCAAA**C**
CAACCTGAGGTCTCTGACGT

>**NYF6**

CCAGCGTTTTTACGAAGGCGGCGATAACTTCTTCCAGTCAAGGCTGAGTGGAGGCTT
CGACCAAGCAGGATATCAGGACCGAAACTCCATGATGGGCTTGTGTGGCGACGGAAG
GCTGCTGTCAAACGGAGTGGGCTTGGAGGACAAACCATCTCCATCTTCTAGCCTCGGTC
TGTCCATGTCTCCTCACCAGGAGCAGCAGCATTGTCCGGGCCAGTGTCTGCCCTGGGCC
TGCAAGGTGTGCAAGCGCAAGTCGGTGACCATGGATCGACGGAAAGCCGCCACTTTGA
GGGAGAAGAGGAGGTTGAAGAAAGTCAACGAGGCCTTTGAGGCTCTTAAGAGGAGCA
CGCTCATGAATCCCAACCAGAGGCTGCCTAAGGTGGAGATCCTGCGCAGCGCCATCCA
GTACATCGAGAGACTCCAGGCGCTGGTCAGCTCTCTCAACCAGCAGGAGCACGAGCAG
GGAAACTTGCATTACAGAGCCGCGGCTCCTCAAGGGGTGTCGTCCTCTAGTGATCAGG
GCTCTGGCAGCACCTGCTGTAGCAGTCCAGAGTGGAGCAGTGCATCTGAGCACTGCGT
CCCCGCATACAGCTCCACCCATGAGGATCTCCTGAACGACGACTCCACAGAGCAAA**CC**
AACCTGAGGTCTCTGACGT

>**NYR4**

CGCCTTCGTAAAAACGCTGGACGAAGGCGGCGATAACTTCTTCCAGTCAAGGCTGACC
GGAGGCTTCGACCAAGCAGGATATCAGGACCGAAGCTCCATGGTGGGCTTGTGTGGCG
ACGGAAGGCTGCTGTCAAACGGAGTGGGGATGGAGGACAAACCGTCTCCATCTTCTAG
CCTCGGTCTGTCCATGTCTCCTCACCAGGAGCAGCAGCACTGTCCGGGTCAGTGTCTGC
CCTGGGCCTGCAAGGTGTGTAAGCGCAAGTCGGTGACAATGGATCGACGGAAAGCCGC
CACTTTGAGGGAGAAGAGGAGGCTGAAGAAAGTCAACGAGGCCTTTGAGGCTCTTAA
GAGGAGCACGCTCATGAATCCCAACCAGAGGCTGCCTAAGGTGGAGATCCTGCGCAGC
GCCATCCAGTACATCGAGAGACTCCAGGCGCTGGTCAGCTCTCTCAACCAGCAGGAAC
ATGAGCAGGGAAACATGCATTACAGAGCCGCGGCTCCTCAAGGGATGTCGTCCTCTAG
CGATCAGGGCTCTGGCAGCACCTGCTGTAGCAGTCCAGAGTGGAGCAGTACGTCTGAG
CACTGTGCCCCCGCCTACAGCTCCACCCATGAGGATCTCCTGAATGACGACTCCTCAGA
GCAAA**CCAACCTGAGGTCTCTGACGT**

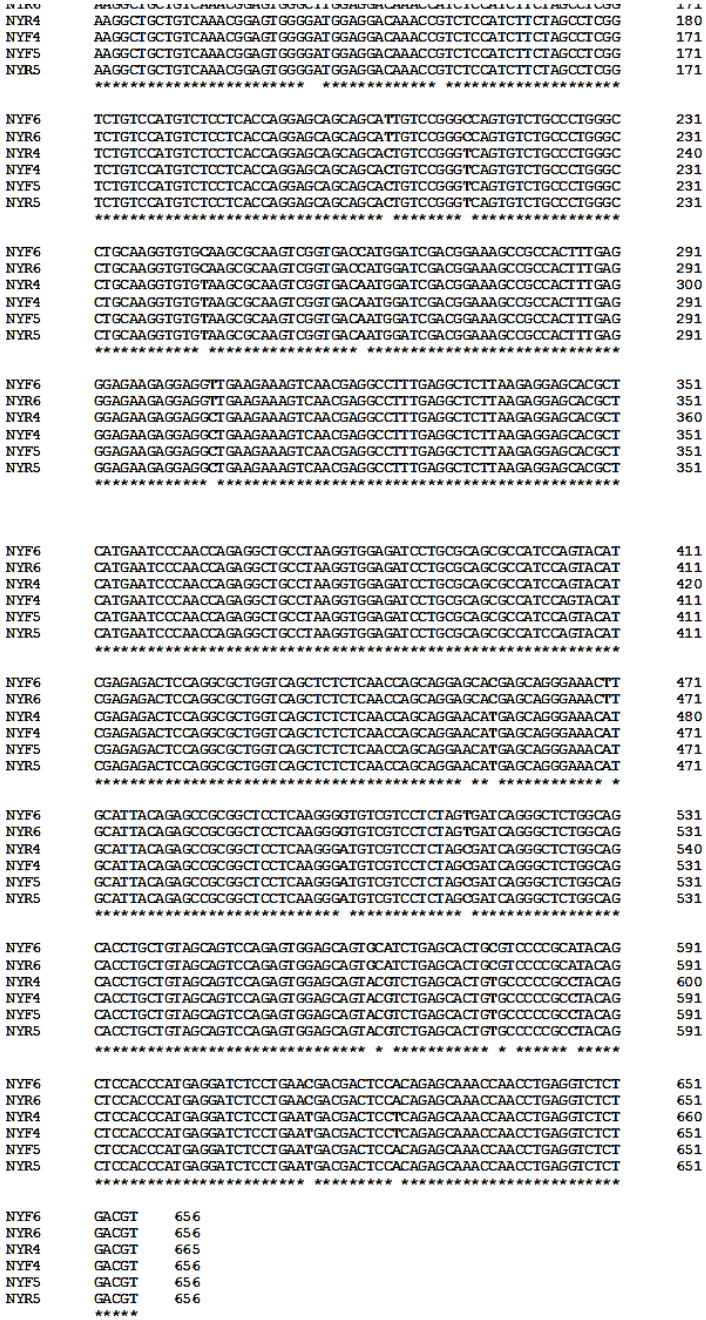
>**NYR5**

CCAGCGTTTTTACGAAGGCGGCGATAACTTCTTCCAGTCAAGGCTGACCGGAGGCTT
CGACCAAGCAGGATATCAGGACCGAAGCTCCATGGTGGGCTTGTGTGGCGACGGAAG
GCTGCTGTCAAACGGAGTGGGGATGGAGGACAAACCGTCTCCATCTTCTAGCCTCGGT
CTGTCCATGTCTCCTCACCAGGAGCAGCAGCACTGTCCGGGTCAGTGTCTGCCCTGGGC
CTGCAAGGTGTGTAAGCGCAAGTCGGTGACAATGGATCGACGGAAAGCCGCCACTTTG

AGGGAGAAGAGGAGGCTGAAGAAAGTCAACGAGGCCTTTGAGGCTCTTAAGAGGAGC
ACGCTCATGAATCCCAACCAGAGGCTGCCTAAGGTGGAGATCCTGCGCAGCGCCATCC
AGTACATCGAGAGACTCCAGGCGCTGGTCAGCTCTCTCAACCAGCAGGAACATGAGCA
GGGAAACATGCATTACAGAGCCGCGGCTCCTCAAGGGATGTCGTCCTCTAGCGATCAG
GGCTCTGGCAGCACCTGCTGTAGCAGTCCAGAGTGGAGCAGTACGTCTGAGCACTGTG
CCCCCGCCTACAGCTCCACCCATGAGGATCTCCTGAATGACGACTCCACAGAGCAAA**C**
CAACCTGAGGTCTCTGACGT

>**NYR6**

CCAGCGTTTTTACGAAGGCGGCGATAACTTCTTCCAGTCAAGGCTGAGTGGAGGCTT
CGACCAAGCAGGATATCAGGACCGAAACTCCATGATGGGCTTGTGTGGCGACGGAAG
GCTGCTGTCAAACGGAGTGGGCTTGGAGGACAAACCATCTCCATCTTCTAGCCTCGGTC
TGTCCATGTCTCCTCACCAGGAGCAGCAGCATTGTCCGGGCCAGTGTCTGCCCTGGGCC
TGCAAGGTGTGCAAGCGCAAGTCGGTGACCATGGATCGACGGAAAGCCGCCACTTTGA
GGGAGAAGAGGAGGTTGAAGAAAGTCAACGAGGCCTTTGAGGCTCTTAAGAGGAGCA
CGCTCATGAATCCCAACCAGAGGCTGCCTAAGGTGGAGATCCTGCGCAGCGCCATCCA
GTACATCGAGAGACTCCAGGCGCTGGTCAGCTCTCTCAACCAGCAGGAGCACGAGCAG
GGAAACTTGCATTACAGAGCCGCGGCTCCTCAAGGGGTGTCGTCCTCTAGTGATCAGG
GCTCTGGCAGCACCTGCTGTAGCAGTCCAGAGTGGAGCA
GCAAA**CCAACCTGAGGTCTCTGACGT**



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NYR6        AAGGCTGCTGTCAAACGGAGTGGGGATGGAGGACAAACCGTCTCCATCTTCTAGCCTCGG      171
NYR4        AAGGCTGCTGTCAAACGGAGTGGGGATGGAGGACAAACCGTCTCCATCTTCTAGCCTCGG      180
NYF4        AAGGCTGCTGTCAAACGGAGTGGGGATGGAGGACAAACCGTCTCCATCTTCTAGCCTCGG      171
NYF5        AAGGCTGCTGTCAAACGGAGTGGGGATGGAGGACAAACCGTCTCCATCTTCTAGCCTCGG      171
NYR5        AAGGCTGCTGTCAAACGGAGTGGGGATGGAGGACAAACCGTCTCCATCTTCTAGCCTCGG      171
            ************************  ************ ******************

NYF6        TCTGTCCATGTCTCCTCACCAGGAGCAGCAGCATTGTCCGGGCCAGTGTCTGCCCTGGGC      231
NYR6        TCTGTCCATGTCTCCTCACCAGGAGCAGCAGCATTGTCCGGGCCAGTGTCTGCCCTGGGC      231
NYR4        TCTGTCCATGTCTCCTCACCAGGAGCAGCAGCACTGTCCGGGTCAGTGTCTGCCCTGGGC      240
NYF4        TCTGTCCATGTCTCCTCACCAGGAGCAGCAGCACTGTCCGGGTCAGTGTCTGCCCTGGGC      231
NYF5        TCTGTCCATGTCTCCTCACCAGGAGCAGCAGCACTGTCCGGGTCAGTGTCTGCCCTGGGC      231
NYR5        TCTGTCCATGTCTCCTCACCAGGAGCAGCAGCACTGTCCGGGTCAGTGTCTGCCCTGGGC      231
            ********************************** ******** *****************

NYF6        CTGCAAGGTGTGCAAGCGCAAGTCGGTGACCATGGATCGACGGAAAGCCGCCACTTTGAG      291
NYR6        CTGCAAGGTGTGCAAGCGCAAGTCGGTGACCATGGATCGACGGAAAGCCGCCACTTTGAG      291
NYR4        CTGCAAGGTGTGTAAGCGCAAGTCGGTGACAATGGATCGACGGAAAGCCGCCACTTTGAG      300
NYF4        CTGCAAGGTGTGTAAGCGCAAGTCGGTGACAATGGATCGACGGAAAGCCGCCACTTTGAG      291
NYF5        CTGCAAGGTGTGTAAGCGCAAGTCGGTGACAATGGATCGACGGAAAGCCGCCACTTTGAG      291
NYR5        CTGCAAGGTGTGTAAGCGCAAGTCGGTGACAATGGATCGACGGAAAGCCGCCACTTTGAG      291
            ************ ***************** ****************************

NYF6        GGAGAAGAGGAGGTTGAAGAAAGTCAACGAGGCCTTTGAGGCTCTTAAGAGGAGCACGCT      351
NYR6        GGAGAAGAGGAGGTTGAAGAAAGTCAACGAGGCCTTTGAGGCTCTTAAGAGGAGCACGCT      351
NYR4        GGAGAAGAGGAGGCTGAAGAAAGTCAACGAGGCCTTTGAGGCTCTTAAGAGGAGCACGCT      360
NYF4        GGAGAAGAGGAGGCTGAAGAAAGTCAACGAGGCCTTTGAGGCTCTTAAGAGGAGCACGCT      351
NYF5        GGAGAAGAGGAGGCTGAAGAAAGTCAACGAGGCCTTTGAGGCTCTTAAGAGGAGCACGCT      351
NYR5        GGAGAAGAGGAGGCTGAAGAAAGTCAACGAGGCCTTTGAGGCTCTTAAGAGGAGCACGCT      351
            ************ ************************************************


NYF6        CATGAATCCCAACCAGAGGCTGCCTAAGGTGGAGATCCTGCGCAGCGCCATCCAGTACAT      411
NYR6        CATGAATCCCAACCAGAGGCTGCCTAAGGTGGAGATCCTGCGCAGCGCCATCCAGTACAT      411
NYR4        CATGAATCCCAACCAGAGGCTGCCTAAGGTGGAGATCCTGCGCAGCGCCATCCAGTACAT      420
NYF4        CATGAATCCCAACCAGAGGCTGCCTAAGGTGGAGATCCTGCGCAGCGCCATCCAGTACAT      411
NYF5        CATGAATCCCAACCAGAGGCTGCCTAAGGTGGAGATCCTGCGCAGCGCCATCCAGTACAT      411
NYR5        CATGAATCCCAACCAGAGGCTGCCTAAGGTGGAGATCCTGCGCAGCGCCATCCAGTACAT      411
            ************************************************************

NYF6        CGAGAGACTCCAGGCGCTGGTCAGCTCTCTCAACCAGCAGGAGCACGAGCAGGGAAACTT      471
NYR6        CGAGAGACTCCAGGCGCTGGTCAGCTCTCTCAACCAGCAGGAGCACGAGCAGGGAAACTT      471
NYR4        CGAGAGACTCCAGGCGCTGGTCAGCTCTCTCAACCAGCAGGAACATGAGCAGGGAAACAT      480
NYF4        CGAGAGACTCCAGGCGCTGGTCAGCTCTCTCAACCAGCAGGAACATGAGCAGGGAAACAT      471
NYF5        CGAGAGACTCCAGGCGCTGGTCAGCTCTCTCAACCAGCAGGAACATGAGCAGGGAAACAT      471
NYR5        CGAGAGACTCCAGGCGCTGGTCAGCTCTCTCAACCAGCAGGAACATGAGCAGGGAAACAT      471
            ****************************************** ** ************ *

NYF6        GCATTACAGAGCCGCGGCTCCTCAAGGGGTGTCGTCCTCTAGTGATCAGGGCTCTGGCAG      531
NYR6        GCATTACAGAGCCGCGGCTCCTCAAGGGGTGTCGTCCTCTAGTGATCAGGGCTCTGGCAG      531
NYR4        GCATTACAGAGCCGCGGCTCCTCAAGGGATGTCGTCCTCTAGCGATCAGGGCTCTGGCAG      540
NYF4        GCATTACAGAGCCGCGGCTCCTCAAGGGATGTCGTCCTCTAGCGATCAGGGCTCTGGCAG      531
NYF5        GCATTACAGAGCCGCGGCTCCTCAAGGGATGTCGTCCTCTAGCGATCAGGGCTCTGGCAG      531
NYR5        GCATTACAGAGCCGCGGCTCCTCAAGGGATGTCGTCCTCTAGCGATCAGGGCTCTGGCAG      531
            ****************************** ************ ******************

NYF6        CACCTGCTGTAGCAGTCCAGAGTGGAGCAGTGCATCTGAGCACTGCGTCCCCGCATACAG      591
NYR6        CACCTGCTGTAGCAGTCCAGAGTGGAGCAGTGCATCTGAGCACTGCGTCCCCGCATACAG      591
NYR4        CACCTGCTGTAGCAGTCCAGAGTGGAGCAGTACGTCTGAGCACTGTGCCCCCGCCTACAG      600
NYF4        CACCTGCTGTAGCAGTCCAGAGTGGAGCAGTACGTCTGAGCACTGTGCCCCCGCCTACAG      591
NYF5        CACCTGCTGTAGCAGTCCAGAGTGGAGCAGTACGTCTGAGCACTGTGCCCCCGCCTACAG      591
NYR5        CACCTGCTGTAGCAGTCCAGAGTGGAGCAGTACGTCTGAGCACTGTGCCCCCGCCTACAG      591
            ****************************** * *********** * ****** *****

NYF6        CTCCACCCATGAGGATCTCCTGAACGACGACTCCACAGAGCAAACCAACCTGAGGTCTCT      651
NYR6        CTCCACCCATGAGGATCTCCTGAACGACGACTCCACAGAGCAAACCAACCTGAGGTCTCT      651
NYR4        CTCCACCCATGAGGATCTCCTGAATGACGACTCCTCAGAGCAAACCAACCTGAGGTCTCT      660
NYF4        CTCCACCCATGAGGATCTCCTGAATGACGACTCCTCAGAGCAAACCAACCTGAGGTCTCT      651
NYF5        CTCCACCCATGAGGATCTCCTGAATGACGACTCCACAGAGCAAACCAACCTGAGGTCTCT      651
NYR5        CTCCACCCATGAGGATCTCCTGAATGACGACTCCACAGAGCAAACCAACCTGAGGTCTCT      651
            ************************ ********* ************************

NYF6        GACGT     656
NYR6        GACGT     656
NYR4        GACGT     665
NYF4        GACGT     656
NYF5        GACGT     656
NYR5        GACGT     656
            *****
```

Figure 16. Sequence alignments (Clustal Omega Multiple Sequence Alignment tool) for MyoG

Selected Myogenin (MyoG) gene sequence for *T. putitora*

CCAGCGTTTTTACGAAGGCGGCGATAACTTCTTCCAGTCAAGGCTGACCGGAGGCTTCGACCAAGCAGG
ATATCAGGACCGAAGCTCCATGGTGGGCTTGTGTGGCGACGGAAGGCTGCTGTCAAACGGAGTGGGGAT
GGAGGACAAACCGTCTCCATCTTCTAGCCTCGGTCTGTCCATGTCTCCTCACCAGGAGCAGCAGCACTGTC
CGGGTCAGTGTCTGCCCTGGGCCTGCAAGGTGTGTAAGCGCAAGTCGGTGACAATGGATCGACGGAAAG
CCGCCACTTTGAGGGAGAAGAGGAGGCTGAAGAAAGTCAACGAGGCCTTTGAGGCTCTTAAGAGGAGCA
CGCTCATGAATCCCAACCAGAGGCTGCCTAAGGTGGAGATCCTGCGCAGCGCCATCCAGTACATCGAGAG
ACTCCAGGCGCTGGTCAGCTCTCTCAACCAGCAGGAACATGAGCAGGGAAACATGCATTACAGAGCCGC
GGCTCCTCAAGGGATGTCGTCCTCTAGCGATCAGGGCTCTGGCAGCACCTGCTGTAGCAGTCCAGAGTGG
AGCAGTACGTCTGAGCACTGTGCCCCCGCCTACAGCTCCACCCATGAGGATCTCCTGAATGACGACTCCA
CAGAGCAAA**CCAACCTGAGGTCTCTGACGT**

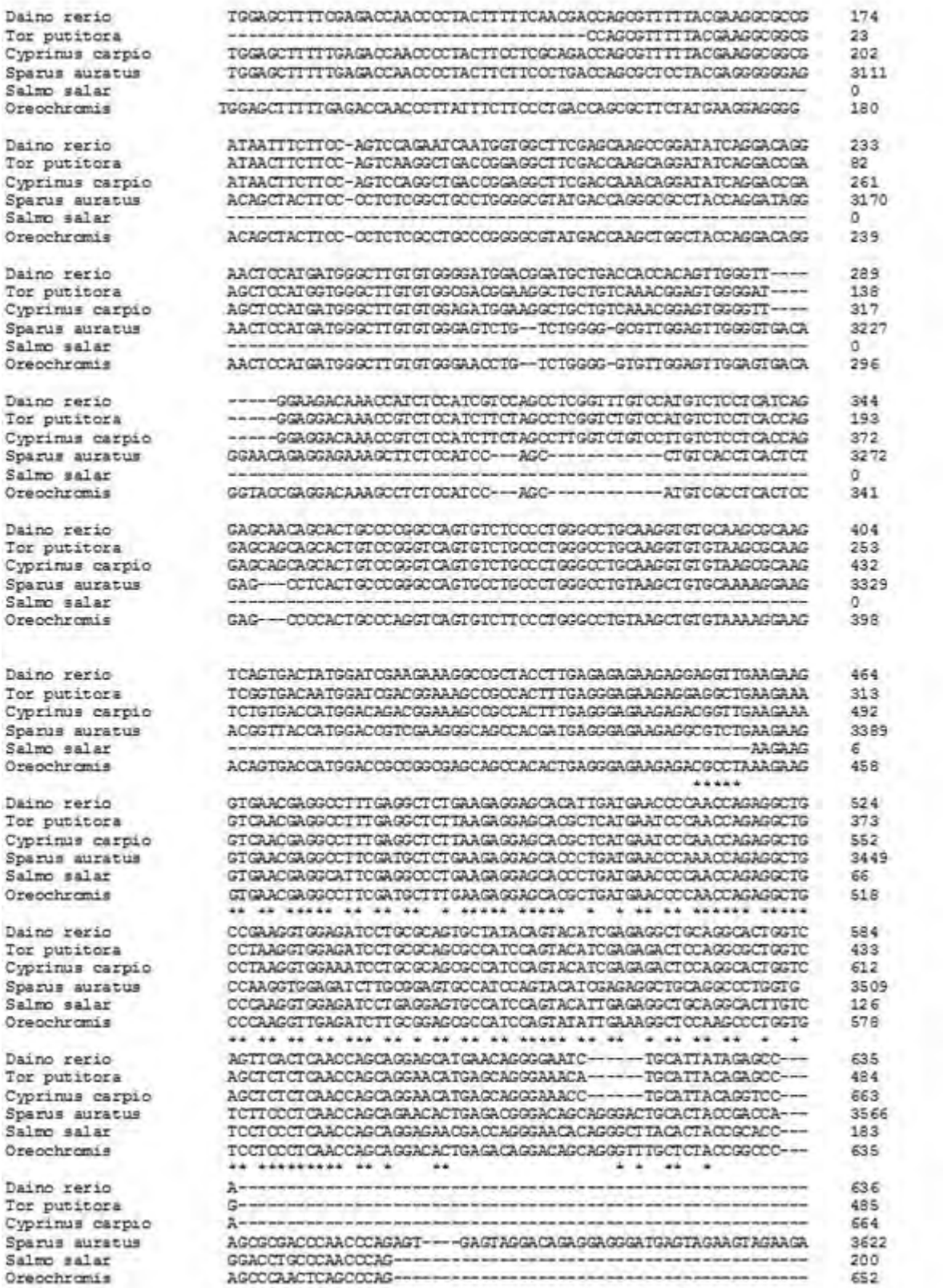

Figure 17. Múltiple species Nucleotide alignments for MyoG

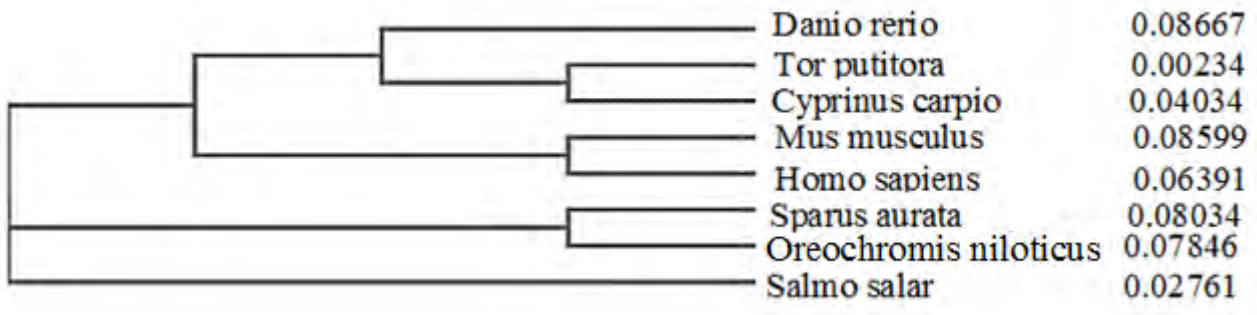

Figure 18. Phylogenetic tree (nucleotide alignments) MyoG

Myogenin (MyoG) Protein alignment

T.putitora protein sequence was translated from nucleotide sequence through the ExPASy bioinformatics Resource portal.

M V G L C G D G R L L S N G V G **M** E D K P S P S SS L G L S **M** S P H Q E Q Q H C P G Q C L P W A C K V C K R K S V T **M** D R R K A A T L R E K R R L K K V N E A F E A L K R S T L **M** N P N Q R L P K V E I L R S A I Q Y I E R L Q A L V S S L N Q Q E H E Q G N **M** H Y R A AA P Q G **M** S SSS D Q G S G S T C C S S P E W S S T S E H C A P A Y S S T H E D L L N D D S T E Q T N L R S L T

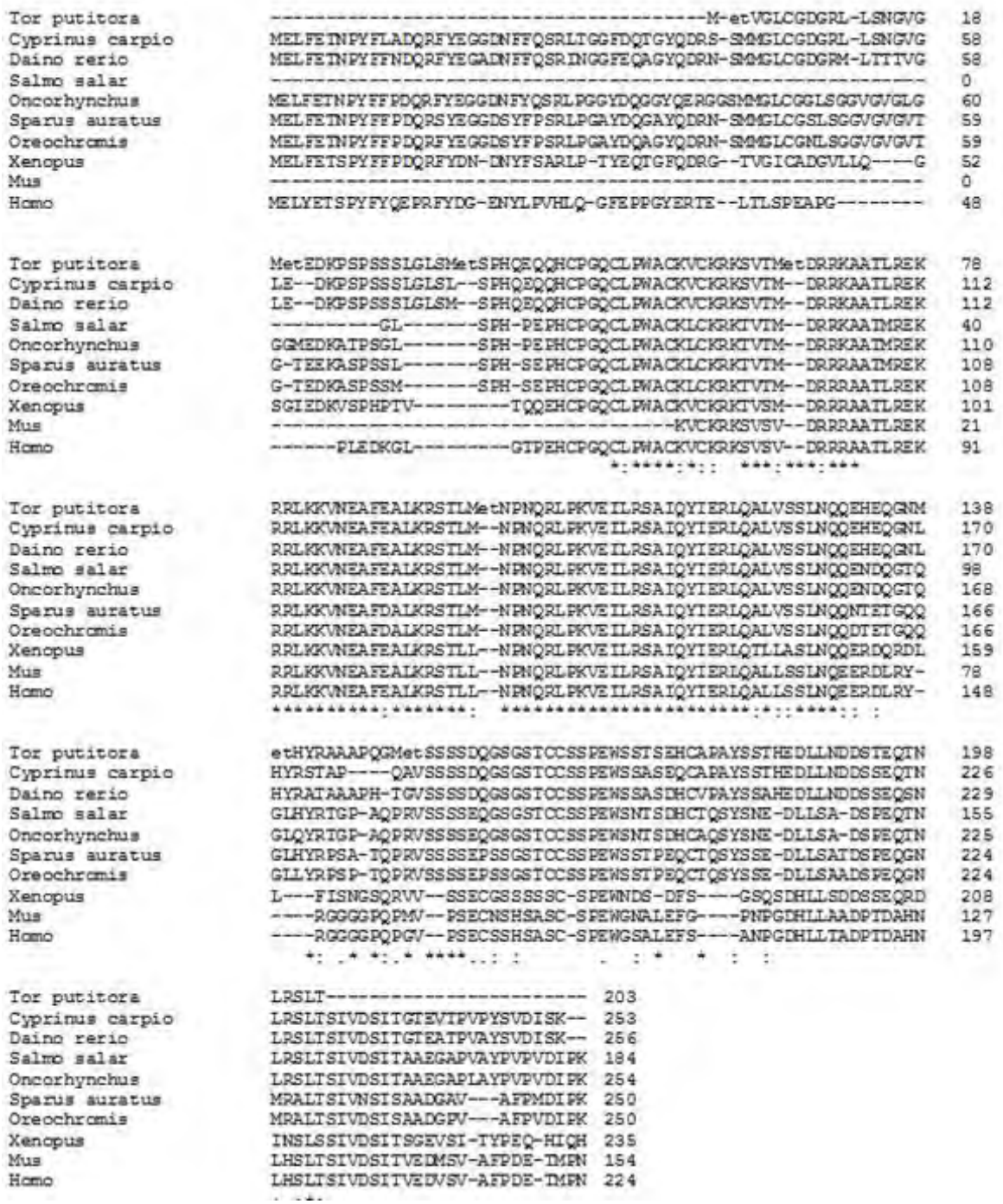

Figure 19. Multiple species protein alignment for MyoG

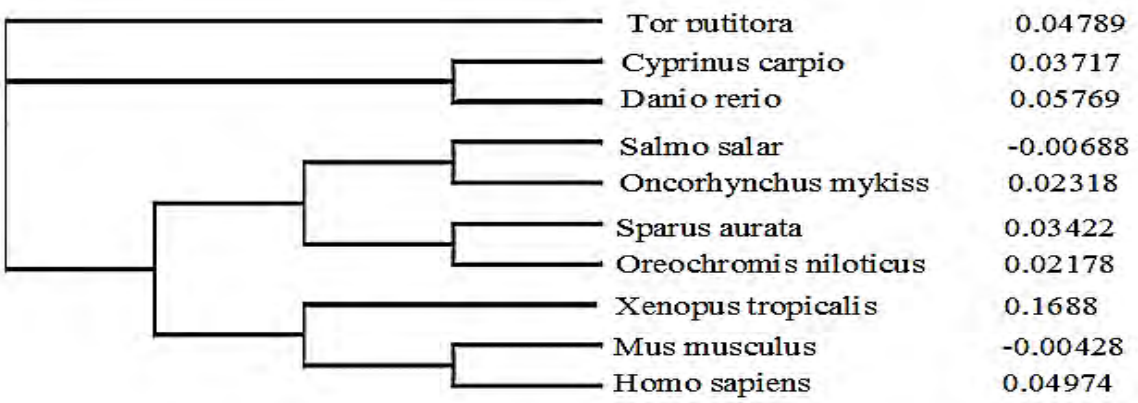

Figure 20. Phylogenetic tree (protein alignments) for MyoG

Heat shock protein-70 (HSP-70KDa) gene sequncing

>**NYF7**

ATGGTCCTGGTGAAGATGAAGGNCCTTTTTGAAGCCTATCTGGGGCAGAAGGTGACA
AATGCAGTTGTCACAGATTCCTGCCTATTTCAATGACTCCCAGAGGCAAGCGACTAAA
GATGCTGGAGTAATCGCTGGACTGAATGTCCTGAGAATCATCAACGAGCCCACAGCTG
CAGCTATTGCCTACGGCCTTGACAAAGGCAAAGCTTCAGAGCGCAACGTCCTGATCTTT
GACCTGGGCGGAGGCACCTTTGATGTGTCCATCCTGACCATTGAAGATGGCATCTTTGA
GGTGAAGGCCACAGCTGGAGACACTCATCTGGGTGGGGAGGACTTTGACAACCGCATG
GTGAATCACTTTGTTGAAGAATTCAA**GAGGAAGCACAAGAAGGACATC**

>**NYF8**

ATGGTCCTGGTGAAGATGAAGGAGATTGCTGAAGCTTATCTGGGGCAGAAGGTGAC
AAATGCAGTTATCACAGTTCCTGCCTATTTCAATGACTCCCAGAGGCAAGCGACTAAA
GACGCTGGAGTAATCGCTGGACTGAATGTCCTGAGAATCATCAACGAGCCCACAGCTG
CAGCCATTGCCTACGGCCTTGACAAAGGCAAAGCTTCAGAGCGCAACGTCCTGATCTT
TGACCTGGGCGGAGGCACCTTTGATGTGTCCATCCTGACCATTGAAGACGGCATCTTTG
AGGTGAAGGCCACAGCTGGAGACACTCATCTGGGTGGGGAGGACTTTGACAACCGCAT
GGTGAATCACTTTGTTGAAGAATTCAA**GAGGAAGCACAAGAAGGACATC**

>**NYF9**

ATGGTCCTGGTGAAGATGAAGGAGATTGCTGAAGCCTATCTGGGGCAGAAGGTGAC
AAATGCAGTTGTCACAGTTCCTGCCTATTTCAATGACTCCCAGAGGCAAGCGACTAAG
GATGCTGGAGTAATCGCTGGACTGAATGTCCTGAGAATCATCAACGAGCCCACAGCTG
CAGCTATTGCCTACGGCCTTGACAAAGGCAAAGCTTCAGAGCGCAACGTCCTGATCTTT
GACCTGGGCGGAGGCACCTTTGATGTGTCCATCCTGACCATTGAAGATGGCATCTTTGG
GGTGAAGGCCACAGCTGGAGACACTCATCTGGGTGGGGAGGACTTTGACAACCGCATG
GTGAATCACTTTGTTGAAGAATTCAA**GAGGAAGCACAAGAAGGACATC**

>**NYR7**

ATGGTCCTGGTGAAGATGAAGGAGATTGCTGAAGCCTATCTGGGGCAGAAGGTGAC
AAATGCAGTTGTCACAGTTCCTGCCTATTTCAATGACTCCCAGAGGCAAGCGACTAAA
GATGCTGGAGTAATCGCTGGACTGAATGTCCTGAGAATCATCAACGAGCCCACAGCTG

CAGCTATTGCCTACGGCCTTGACAAAGGCAAAGCTTCAGAGCGCAACGTCCTGATCTTT
GACCTGGGCGGAGGCACCTTTGATGTGTCCATCCTGACCATTGAAGATGGCATCTTTGA
GGTGAAGGCCACAGCTGGAGACACTCATCTGGGTGGGGAGGACTTTGACAACCGCATG
GTGAATCACTTTGTTGAAGAATTCAA**GAGGAAGCACAAGAAGGACATC**

>**NYR8**

ATGGTCCTGGTGAAGATGAAGGAGATTGCTGAAGCTTATCTGGGGCAGAAGGTGAC
AAATGCAGTTATCACAGTTCCTGCCTATTTCAATGACTCCCAGAGGCAAGCGACTAAA
GACGCTGGAGTAATCGCTGGACTGAATGTCCTGAGAATCATCAACGAGCCCACAGCTG
CAGCCATTGCCTACGGCCTTGACAAAGGCAAAGCTTCAGAGCGCAACGTCCTGATCTT
TGACCTGGGCGGAGGCACCTTTGATGTGTCCATCCTGACCATTGAAGACGGCATCTTTG
AGGTGAAGGCCACAGCTGGAGACACTCATCTGGGTGGGGAGGACTTTGACAACCGCAT
GGTGAATCACTTTGTTGAAGAATTCAA**GAGGAAGCACAAGAAGGACATC**

>**NYR9**

TATGGTCCTGGTGAAGATGAAGGAGATTGCTGAAGCCTATCTGGGGCAGAAGGTGA
CAAATGCAGTTGTCACAGTTCCTGCCTATTTCAATGACTCCCAGAGGCAAGCGACTAA
GGATGCTGGAGTAATCGCTGGACTGAATGTCCTGAGAATCATCAACGAGCCCACAGCT
GCAGCTATTGCCTACGGCCTTGACAAAGGCAAAGCTTCAGAGCGCAACGTCCTGATCT
TTGACCTGGGCGGAGGCACCTTTGATGTGTCCATCCTGACCATTGAAGATGGCATCTTT
GGGGTGAAGGCCACAGCTGGAGACACTCATCTGGGTGGGGAGGACTTTGACAACCGC
ATGGTGAATCACTTTGTTGAAGAATTCAA**GAGGAAGCACAAGAAGGACATC**

```
NYF7       -ATGGTCCTGGTGAAGATGAAGGNCCTTTTTGAAGCCTATCTGGGGCAGAAGGTGACAAA      59
NYF8       -ATGGTCCTGGTGAAGATGAAGGAGATTGCTGAAGCTTATCTGGGGCAGAAGGTGACAAA      59
NYR8       -ATGGTCCTGGTGAAGATGAAGGAGATTGCTGAAGCTTATCTGGGGCAGAAGGTGACAAA      59
NYF9       -ATGGTCCTGGTGAAGATGAAGGAGATTGCTGAAGCCTATCTGGGGCAGAAGGTGACAAA      59
NYR9       TATGGTCCTGGTGAAGATGAAGGAGATTGCTGAAGCCTATCTGGGGCAGAAGGTGACAAA      60
NYR7       -ATGGTCCTGGTGAAGATGAAGGAGATTGCTGAAGCCTATCTGGGGCAGAAGGTGACAAA      59
            **********************   **  ****** ************************

NYF7       TGCAGTTGTCACAGATTCCTGCCTATTTCAATGACTCCCAGAGGCAAGCGACTAAAGATG      119
NYF8       TGCAGTTATCACAG-TTCCTGCCTATTTCAATGACTCCCAGAGGCAAGCGACTAAAGACG      118
NYR8       TGCAGTTATCACAG-TTCCTGCCTATTTCAATGACTCCCAGAGGCAAGCGACTAAAGACG      118
NYF9       TGCAGTTGTCACAG-TTCCTGCCTATTTCAATGACTCCCAGAGGCAAGCGACTAAGGATG      118
NYR9       TGCAGTTGTCACAG-TTCCTGCCTATTTCAATGACTCCCAGAGGCAAGCGACTAAGGATG      119
NYR7       TGCAGTTGTCACAG-TTCCTGCCTATTTCAATGACTCCCAGAGGCAAGCGACTAAAGATG      118
            ******* ****** ****************************************** ** *

NYF7       CTGGAGTAATCGCTGGACTGAATGTCCTGAGAATCATCAACGAGCCCACAGCTGCAGCTA      179
NYF8       CTGGAGTAATCGCTGGACTGAATGTCCTGAGAATCATCAACGAGCCCACAGCTGCAGCCA      178
NYR8       CTGGAGTAATCGCTGGACTGAATGTCCTGAGAATCATCAACGAGCCCACAGCTGCAGCCA      178
NYF9       CTGGAGTAATCGCTGGACTGAATGTCCTGAGAATCATCAACGAGCCCACAGCTGCAGCTA      178
NYR9       CTGGAGTAATCGCTGGACTGAATGTCCTGAGAATCATCAACGAGCCCACAGCTGCAGCTA      179
NYR7       CTGGAGTAATCGCTGGACTGAATGTCCTGAGAATCATCAACGAGCCCACAGCTGCAGCTA      178
            ********************************************************** *

NYF7       TTGCCTACGGCCTTGACAAAGGCAAAGCTTCAGAGCGCAACGTCCTGATCTTTGACCTGG      239
NYF8       TTGCCTACGGCCTTGACAAAGGCAAAGCTTCAGAGCGCAACGTCCTGATCTTTGACCTGG      238
NYR8       TTGCCTACGGCCTTGACAAAGGCAAAGCTTCAGAGCGCAACGTCCTGATCTTTGACCTGG      238
NYF9       TTGCCTACGGCCTTGACAAAGGCAAAGCTTCAGAGCGCAACGTCCTGATCTTTGACCTGG      238
NYR9       TTGCCTACGGCCTTGACAAAGGCAAAGCTTCAGAGCGCAACGTCCTGATCTTTGACCTGG      239
NYR7       TTGCCTACGGCCTTGACAAAGGCAAAGCTTCAGAGCGCAACGTCCTGATCTTTGACCTGG      238
            ************************************************************

NYF7       GCGGAGGCACCTTTGATGTGTCCATCCTGACCATTGAAGATGGCATCTTTGAGGTGAAGG      299
NYF8       GCGGAGGCACCTTTGATGTGTCCATCCTGACCATTGAAGACGGCATCTTTGAGGTGAAGG      298
NYR8       GCGGAGGCACCTTTGATGTGTCCATCCTGACCATTGAAGACGGCATCTTTGAGGTGAAGG      298
NYF9       GCGGAGGCACCTTTGATGTGTCCATCCTGACCATTGAAGATGGCATCTTTGGGGTGAAGG      298
NYR9       GCGGAGGCACCTTTGATGTGTCCATCCTGACCATTGAAGATGGCATCTTTGGGGTGAAGG      299
NYR7       GCGGAGGCACCTTTGATGTGTCCATCCTGACCATTGAAGATGGCATCTTTGAGGTGAAGG      298
            ************************************** ********** ********

NYF7       CCACAGCTGGAGACACTCATCTGGGTGGGGAGGACTTTGACAACCGCATGGTGAATCACT      359
NYF8       CCACAGCTGGAGACACTCATCTGGGTGGGGAGGACTTTGACAACCGCATGGTGAATCACT      358
NYR8       CCACAGCTGGAGACACTCATCTGGGTGGGGAGGACTTTGACAACCGCATGGTGAATCACT      358
NYF9       CCACAGCTGGAGACACTCATCTGGGTGGGGAGGACTTTGACAACCGCATGGTGAATCACT      358
NYR9       CCACAGCTGGAGACACTCATCTGGGTGGGGAGGACTTTGACAACCGCATGGTGAATCACT      359
NYR7       CCACAGCTGGAGACACTCATCTGGGTGGGGAGGACTTTGACAACCGCATGGTGAATCACT      358
            ************************************************************

NYF7       TTGTTGAAGAATTCAAGAGGAAGCACAAGAAGGACATC          397
NYF8       TTGTTGAAGAATTCAAGAGGAAGCACAAGAAGGACATC          396
NYR8       TTGTTGAAGAATTCAAGAGGAAGCACAAGAAGGACATC          396
NYF9       TTGTTGAAGAATTCAAGAGGAAGCACAAGAAGGACATC          396
NYR9       TTGTTGAAGAATTCAAGAGGAAGCACAAGAAGGACATC          397
NYR7       TTGTTGAAGAATTCAAGAGGAAGCACAAGAAGGACATC          396
            ***********************************
```

Figure 21. Sequence alignments (Clustal Omega Multiple Sequence Alignment tool) for HSP-70kDa

Selected Heat shock protein- HSP-70kDa

gene sequence for *Tor putitora*

GAGATTGCTGAAGCCTATTCTGGGGCAGAAGGTGACAAATGCAGTTGTCACAGTTCCTGCCTATTTCAAT
GACTCCCAGAGGCAAGCGACTAAAGATGCTGGAGTAATCGCTGGACTGAATGTCCTGAGAATCATCAAC
GAGCCCACAGCTGCAGCTATTGCCTACGGCCTTGACAAAGGCAAAGCTTCAGAGCGCAACGTCCTGATCT
TTGACCTGGGCGGAGGCACCTTTGATGTGTCCATCCTGACCATTGAAGATGGCATCTTTGAGGTGAAGGC
CACAGCTGGAGACACTCATCTGGGTGGGGAGGACTTTGACAACCGCATGGTGAATCACTTTGTTGAAGAA
TTCAA

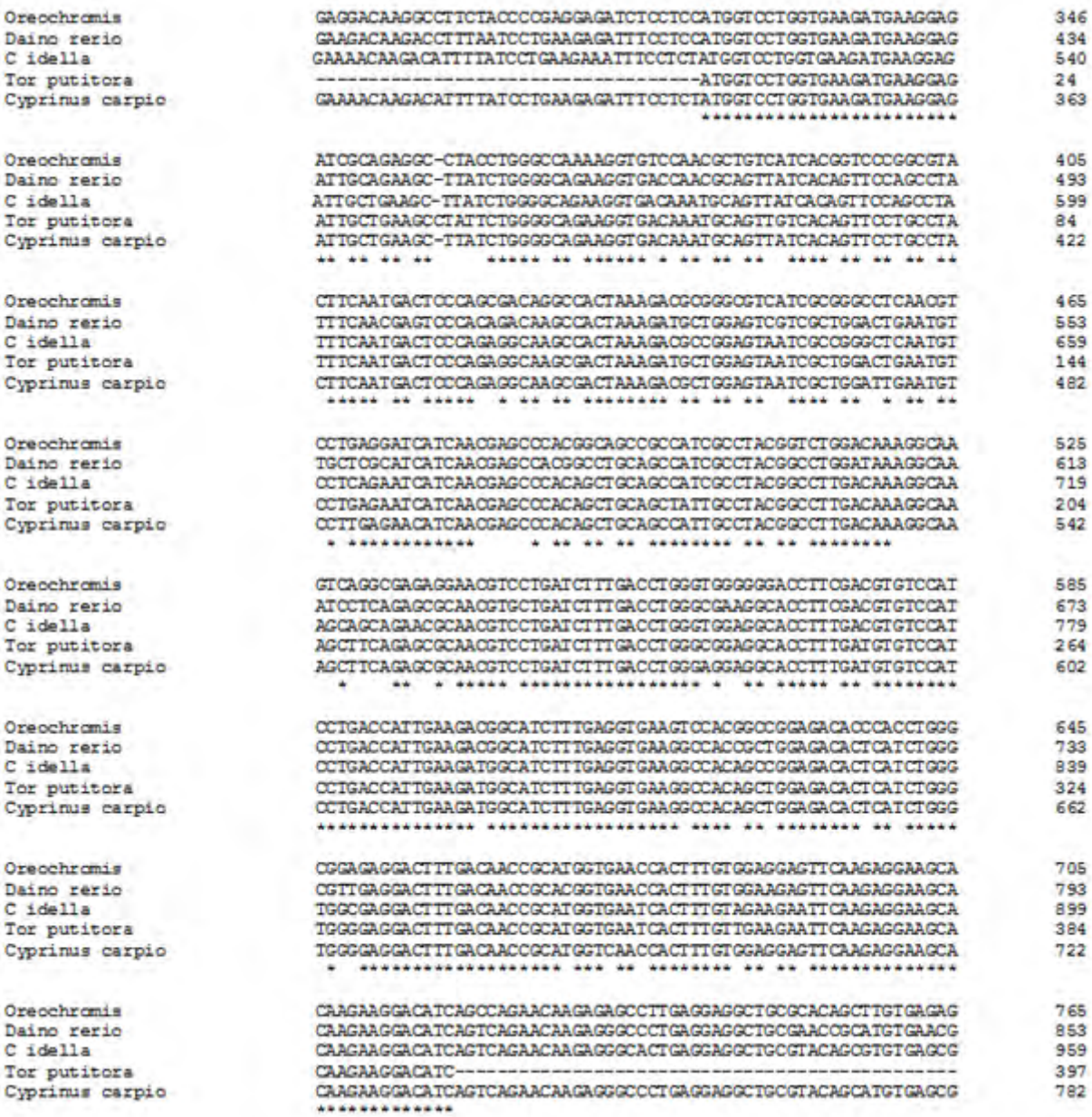

Figure 22. Multiple species nucleotide aligments for HSP-70kDa

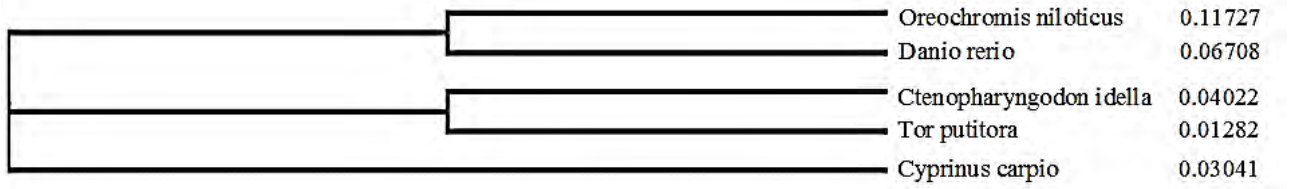

Figure 23. Phylogenetic tree (nucleotide alignments) for HSP-70kDa

Amino acid sequence of HSP-70kDa

RLLKPILGQKVTNAVVTVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTAAAIAYGLDKG

KASERNVLIFDLGGGTFDVSILTIEDGIFEVKATAGDTHLGGEDFDNRMVNHFVEEF

```
Cirrhinus              ----------AYLGQKVTNXVITVPAYFNDSQXQXTXXAGXJXGXNXLRIINEPTA   46
Catla                  ------------------------------------------------------   66
Tor putitora           ----------RLLKPILGQKVTNAVVTVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTA   50
Labeo                  ------------------------------------------------------    0
Daino rerio            ISSMVLVKMKEIAEAYLGQKVTNAVITVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTA  180
Carassius              ------------------------------------------------------    0
Cyprinus carpio        ------------------------------------------------------   74
C idella               ------------------------------------------------------    0
Hypopthalmichthys      ISSMVLVKMKEIAEAYLGQKVTNAVITVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTA  180

Cirrhinus              AAIAYGLDKGKASERNVLIFHLGGGTFDVSILTI----------------------   80
Catla                  ------------------------------------------------------   66
Tor putitora           AAIAYGLDKGKASERNVLIFDLGGGTFDVSILTIEDGIFEVKATAGDTHLGGEDFDNRMV  110
Labeo                  ------------------------------------------------MV    2
Daino rerio            AAHPYGLDKGKSSERNVLIFDLGGGTFDVSILTIEDGIFEVKATAGDTHLGGEDFDNRMV  240
Carassius              ---------------------------------IFEVKATAGDTHLGGEDFDNRMV   23
Cyprinus carpio        ------------------------------------------------------   74
C idella               ------------------------VSILTIEDGIFEVKATAGDTHLGGEDFDNRMV   32
Hypopthalmichthys      AAIAYGLDKGKAAERNVLIFDLGGGTFDVSILTIEDGIFEVKATAGDTHLGGEDFDNRMV  240

Cirrhinus              ------------------------------------------------------   80
Catla                  ------------------------------------------------------   66
Tor putitora           NHFVEEF--------------------------------------------------  117
Labeo                  NHFVEEFKRKHKKDISQNKRALRRLRTACERAKRTLSSSSQASIEIDSLYEGIDFYSSIT   62
Daino rerio            NHFVEEFKRKHKKDISQNKRALRRLRTACERAKRTLSSSSQASIEIDSLYEGIDFYTSIT  300
Carassius               NHFVEEFKRKHKKDISQNKRALRRLRTACERAKRTLSSSSQASIEIDSLYEGIDFYTSIT  83
Cyprinus carpio        ------------------------------------------------------   74
C idella               NHFVEEFKRKHKKDISQNKRALRRLRTACERAKRTLSSSSQASLEIDSLYEGIDFYTSIT   92
Hypopthalmichthys      NHFVEEFKRKHKKDISQNKRALRRLRTACERAKRTLSSSSQASLEIDSLYEGIDFYTSIT  300
```

Figure 24. Múltiple species protein alignment for HSP-70kDa

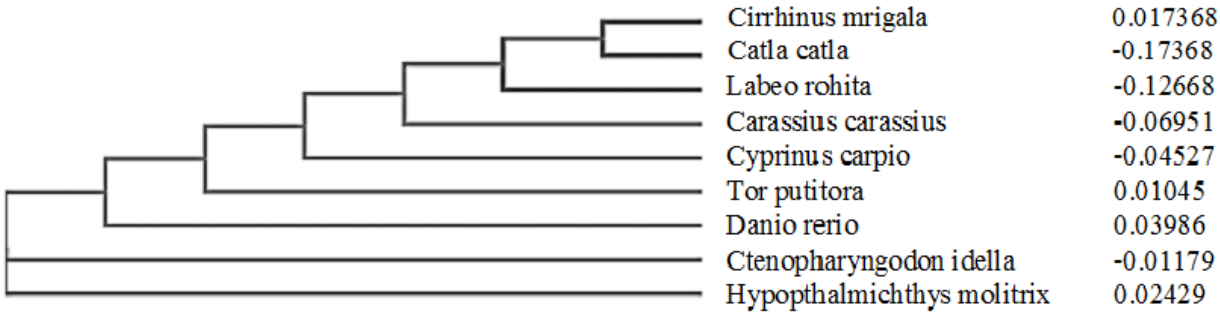

Figure 25. Phylogenetic tree (protein alignments) for HSP-70kDa

Submission of Data in NCBI gene bank

1) MyoD

Nucleotide sequence

AACACAAACGATATGCACTTCTTTGAAGACCTGGACCCCAGGCTCGTCCACGTGAGTCTGCTCAAGCCCGACGAGCAT
CACCACATCGAGGACGAGCACGTAAGGGCGCCCAGCGGGCATCATCAGGCCGGCAGGTGCCTGCTGTGGGCATGCAA
AGCCTGCAAGAGAAAAACTACCAACGCTGACCGCCGCAAAGCCGCCACCATGAGGGAGAGGAGAAGACTGAGCAAA
GTCAACGACGCTTTCGAGACCCTCAAGAGATGCACGTCCACCAACCCCAACCAGAGGCTGCCCAAAGTGGAGATTCT
GAGAAACGCCATTAGTTACATTGAGTCTCTGCAGGCGCTGCTTAGGAGTCAAGAGGAAAACTACTACCCTGTTCTGGA
ACATTACAGTGGAGACTCCGATGCCTCCAGCCCGA

Protein Sequence

M H F F E D L D P R L V H V S L L K P D E H HH I E D E H V R A P S G H H Q A G R C L L W A C K A C K

2) MyoG

Nucleotide sequence

GCGATAACTTCTTCCAGTCAAGGCTGACCGGAGGCTTCGACCAAGCAGGATATCAGGACCGAAGCTCCAT
GGTGGGCTTGTGTGGCGACGGAAGGCTGCTGTCAAACGGAGTGGGGATGGAGGACAAACCGTCTCCATC
TTCTAGCCTCGGTCTGTCCATGTCTCCTCACCAGGAGCAGCAGCACTGTCCGGGTCAGTGTCTGCCCTGGG
CCTGCAAGGTGTGTAAGCGCAAGTCGGTGACAATGGATCGACGGAAAGCCGCCACTTTGAGGGAGAAGA
GGAGGCTGAAGAAAGTCAACGAGGCCTTTGAGGCTCTTAAGAGGAGCACGCTCATGAATCCCAACCAGA
GGCTGCCTAAGGTGGAGATCCTGCGCAGCGCCATCCAGTACATCGAGAGACTCCAGGCGCTGGTCAGCTC
TCTCAACCAGCAGGAACATGAGCAGGGAAACATGCATTACAGAGCCGCGGCTCCTCAAGGGATGTCGTC
CTCTAGCGATCAGGGCTCTGGCAGCACCTGCTGTAGCAGTCCAGAGTGGAGCAGTACGTCTGAGCACTGT
GCCCCCGCCTACAGCTCCACCCATGAGGATCTCCTGAATGACGACTCCACAGAGCAAA

Protein Sequence

MVGLCGDGRLLSNGVGMEDKPSPSSSLGLSMSPHQEQQHCPGQCLPWACKVCKRKSV
TMDRRKAATLREKRRLKKVNEAFEALKRSTLMNPNQRLPKVEILRSAIQYIERLQALVS
SLNQQEHEQGNMHYRAAAPQQGMSSSSDQGSGSTCCSSPEWSSTSEHCAPAYSSTHEDLLN
DDSTEQTNLRSLT

3) Heat shock protein HSP-70kDa

Nucleotide sequence

GAGATTGCTGAAGCCTATTCTGGGGCAGAAGGTGACAAATGCAGTTGTCACAGTTCCTGCCTATTTCAAT
GACTCCCAGAGGCAAGCGACTAAAGATGCTGGAGTAATCGCTGGACTGAATGTCCTGAGAATCATCAAC
GAGCCCACAGCTGCAGCTATTGCCTACGGCCTTGACAAAGGCAAAGCTTCAGAGCGCAACGTCCTGATCT
TTGACCTGGGCGGAGGCACCTTTGATGTGTCCATCCTGACCATTGAAGATGGCATCTTTGAGGTGAAGGC
CACAGCTGGAGACACTCATCTGGGTGGGGAGGACTTTGACAACCGCATGGTGAATCACTTTGTTGAAGAA
TTCAA

Protein Sequence

RLLKPILGQKVTNAVVTVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTAAAIAYGLDKG
KASERNVLIFDLGGGTFDVSILTIEDGIFEVKATAGDTHLGGEDFDNRMVNHFVEEF

Submission of data in NCBI world gene bank

Nucleotide sequences were submitted at NCBI to get their gene bank accession numbers.

- BankIt2127432 MyoD MH545701
- BankIt2127432 MyoG MH545702
- BankIt2127432 HSP70KDa MH545703

Quantitative Polymerase chain reaction (q-PCR) primer designing

Primers were designed for gene expression analysis of MyoD, MyoG, and HSP-70kDa in fish reared on diet supplemented with a graded level of cobalt (table 14).

Linearity test of primers

Linearity test was performed through serial dilutions of sample and blank (Milli-Q water) to check the validity of designed oligonucleotides for gene expression analysis studies through q-PCR. The reaction mixture and qPCR reaction conditions are shown in tables 15 and 16 respectively. Four serial dilutions (duplicate) of samples were prepared for each gene i.e., 1/2/5; 1/5; 1/10; 1/20 and blank. Graphs were made to validate the results of the linearity test. Results confirmed the linearity of designed primers.

Table 14. Primers designed for quantitative Polymerase chain reaction (q-PCR)

Gene	Accession No.	Length of PCR product	Sequence 5′ to 3′
β-actin	KU714644.1	99	GCTGTGCTGTCCCTGTATGC
			GGCGTAACCCTCGTAGATGG
MyoD	MH545701	107	GATTGCTGAAGCCTATTCTGG
			GCGCCTGCAGAGACTCAATG
Myogenin	MH545702	165	GGACAAACCGTCTCCATCTTC
			CCTCCTCTTCTCCCTCAAAGTG
HSP-70kDa	MH545703	83	GATTGCTGAAGCCTATTCTGG
			TTGCCTCTGGGAGTCATTG

Table 15. Quantitative Polymerase chain reaction (q-PCR) master mix

Ingredients	Quantity (µl)
SYBR® Green	20
Oligo – Forward	1.6
Oligo – Reverse	1.6
PCR Water	13.6
cDNA sample	3.2
Final volume	**40µl**

Table 16. Quantitative Polymerase chain reaction (q-PCR) reaction conditions

Temperature (ºC)		Time duration (minutes)
95		10
95		0.25
62	40 cycles	1
95		0.25
60		1

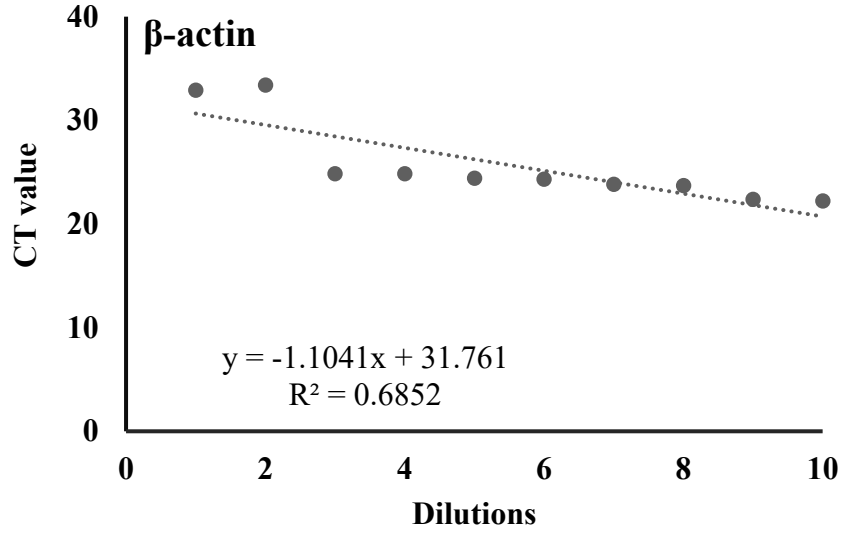

Figure 26. Linearity of β-actin primers designed for qPCR

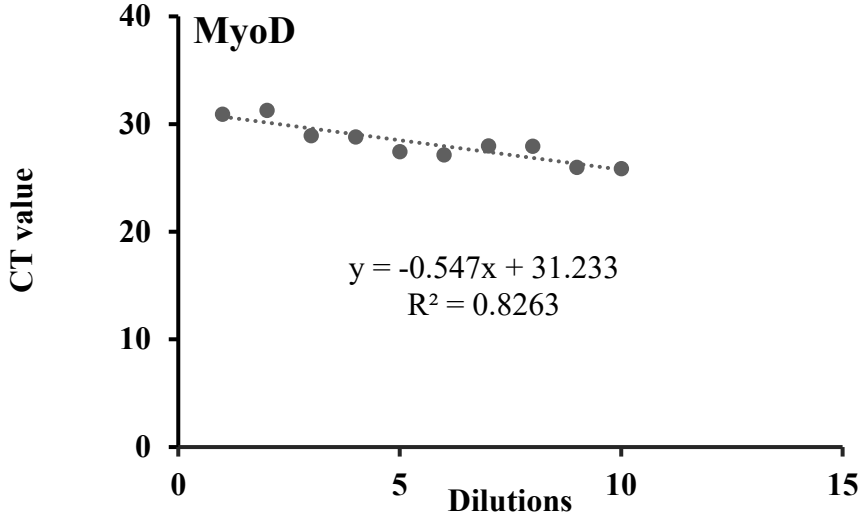

Figure 27. Linearity of MyoD primers designed for quantitative qPCR

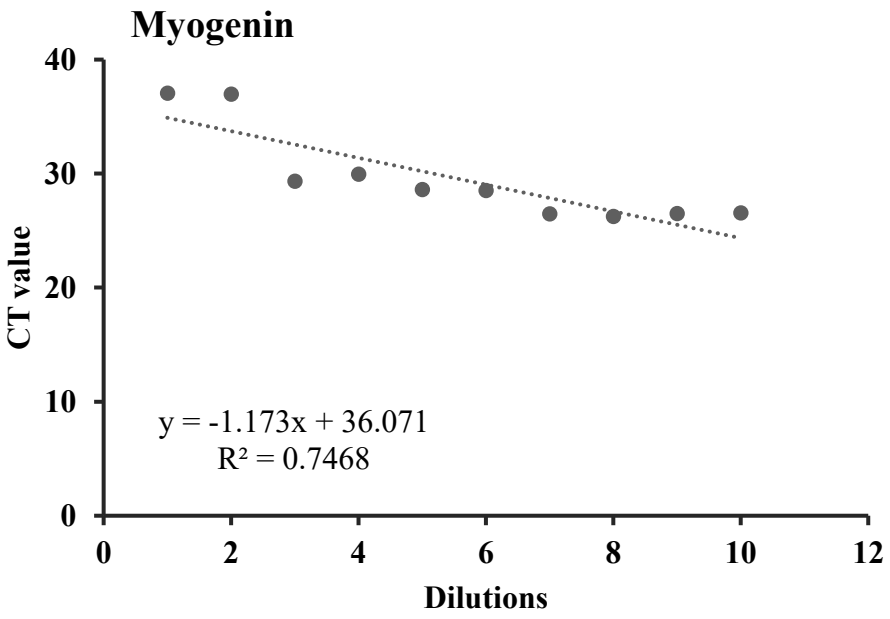

Figure 28. Linearity of myogenin (MyoG) primers designed for qPCR

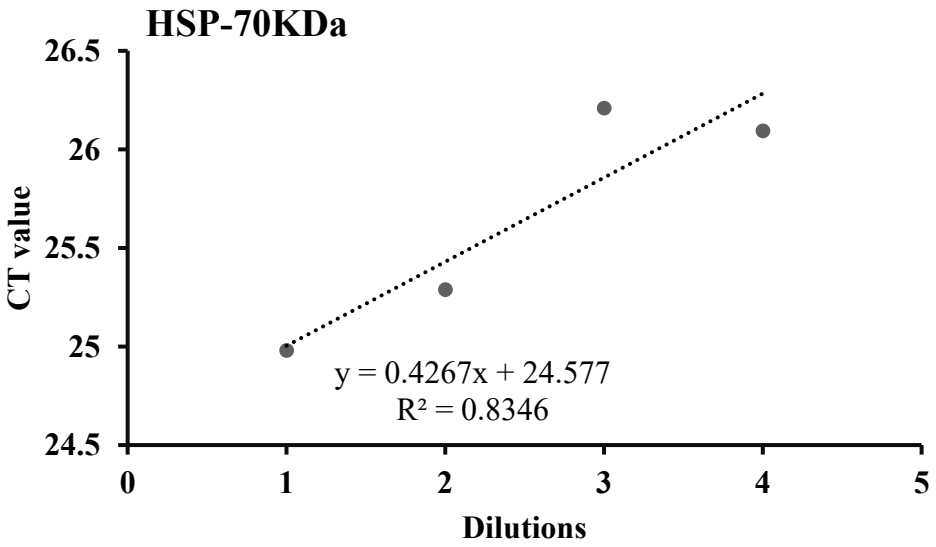

Figure 29. Linearity of HSP-70KDa primers designed for qPCR

Quantitative PCR (qPCR) for muscle growth and liver stress response genes

Step OnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used to quantify the myoblast determination protein 1 homolog MyoD and myogenin MyoG in the muscle and heat shock protein 70 KDa in the liver cDNA samples of advanced fry of *T. putitora* reared on diet supplemented with graded levels of cobalt chloride. Primers used for gene expression studies were designed after nucleotide sequencing of the MyoD, MyoG, and HSP-70kDa gene (Table 17). A β-actin primer having PCR product 99kb was used for qPCR to normalize the expression levels of the MyoD, MyoG, and HSP-70kDa gene.

Briefly, diluted cDNA was taken in small tubes (Applied Biosystems MicroAmp 8-Tube Strip with attached optical or domed caps) and mixed with SYBR® Green (Applied Biosystems, Foster City, CA, USA), primers (both forward and reverse), and water (qPCR reaction mix composition having a final volume of 20 µl is shown in Table 18). After that, these small tubes having a reaction mixture were centrifuged for 1 minute at 1500rpm (214×g; high speed centrifuge, SCILOGEX LLC). Then tubes having reaction mixture were placed in Real-Time PCR System having pre-set temperature protocol (Table 19). To ensure the amplification of products, the dissociation curve was run and the efficiency of qPCR reaction for each selected gene was checked by generating the standard curves via serial dilutions of a control cDNA sample. The mRNA expression of all three selected genes was normalized to the expression level of *T. putitora* β- actin. Afterward standard ΔΔCT method (Pfaffl 2001) was adopted to calculate the variation in expression of MyoD, MyoG, and HSP-70kDa in samples.

Table 17. Primers designed for quantitative Polymerase chain reaction (q-PCR)

Gene	Accession No.	Length of PCR product	Sequence 5´ to 3´
β-actin	KU714644.1	99	GCTGTGCTGTCCCTGTATGC
			GGCGTAACCCTCGTAGATGG
MyoD	MH545701	107	GATTGCTGAAGCCTATTCTGG
			GCGCCTGCAGAGACTCAATG
Myogenin	MH545702	165	GGACAAACCGTCTCCATCTTC
			CCTCCTCTTCTCCCTCAAAGTG
HSP-70kDa	MH545703	83	GATTGCTGAAGCCTATTCTGG
			TTGCCTCTGGGAGTCATTG

Table 18. Quantitative polymerase chain qPCR reaction composition

Ingredient	Quantity (µl)
SYBR® Green	10
Forward primer	0.8
Reverse primer	0.8
Water	6.8
cDNA sample	1.6
Total	20µl

Table 19. Quantitative polymerase chain reaction (qPCR) condition (temperature protocol)

Temperature (ºC)		Time duration (minutes)
95		10
95		0.25
62	40 cycles	1
95		0.25
60		1

CHAPTER 3

Comparative effects of different forms of dietary cobalt on growth performance, hemato-immunological indices, intestinal enzymes activities, and muscle composition of *T. putitora*

This part of the study involved the synthesis of inorganic Cobalt chloride nanoparticles (Co-NPs) and formation of organic Cobalt methionine complex (Co-Met) and then conduction of feeding trials for the comparative evaluation of their efficiency.

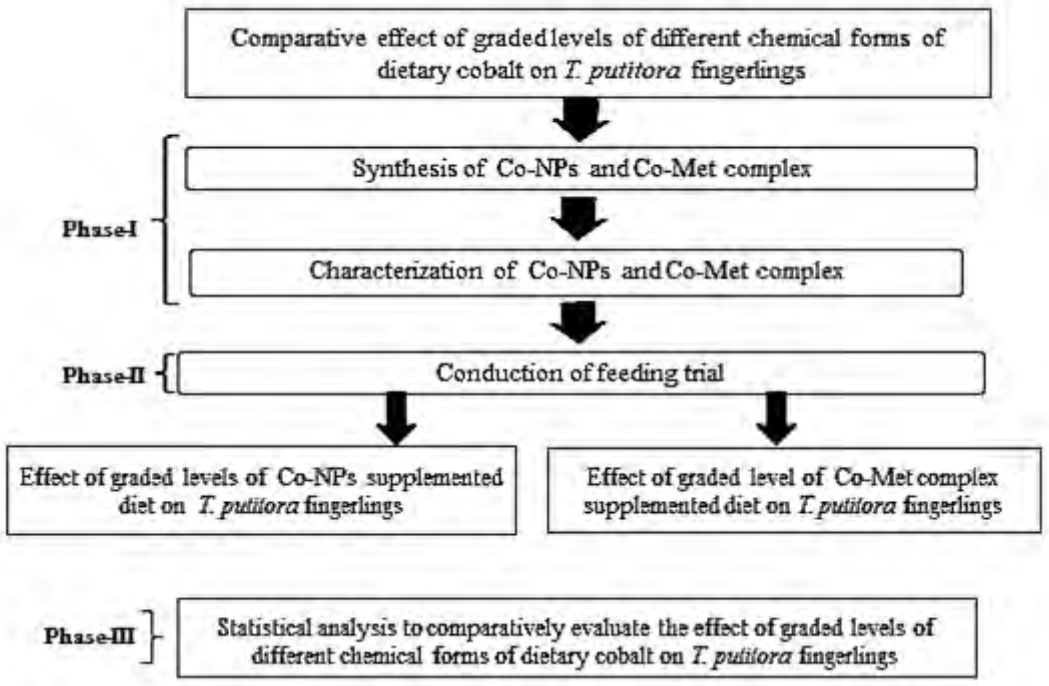

Figure 30. Experimental design to study the comparative effects of different forms of dietary cobalt on growth performance, intestinal enzymes activity, hemato-immunological indices, and muscle composition of *T. putitora*

Phase I

Preparation of cobalt chloride nanoparticles (Co-NPs)

The mechanical method was used for the conversion of crude cobalt chloride salt in its nano form. Initially, cobalt chloride hexahydrate ($CoCl_2.6H_2O$) was taken in a china dish and its moisture was removed by heating at 80ºC for 20 minutes on a hot plate. After that, de-hydrated cobalt chloride was placed in a ball mill grinder and the lid was sealed to restrict the entrance of moisture into the chamber. The ball mill had a barrel with 24 small and 9 large metallic balls, which collided with each other at high speed and ground the sample. Here, the collision of balls with the sample for about 12 hours resulted in the mechanical conversion of cobalt chloride (crude) into its nano form.

Synthesis of Cobalt methionine chelated complex (Co-Met)

Cobalt methionine chelated complex was prepared by the protocol reported by Stănilă et al. (2011). Briefly, 2mM of methionine, i.e., 0.286g was dissolved in 20 ml of distilled water in a glass conical flask under magnetic stirring (Magnetic Stirrer; ATO-HS-19). After that, 0.33 ml of 30% NaOH was added to the flask. In the final step, 1mM of the cobalt chloride (0.237g) was dissolved in 2 ml of distilled water and added dropwise to the above reaction mixture under constant stirring. Instantaneously, pink color precipitates were formed in the flask which was filtered, washed several times with distilled water, and then dried in air.

Characterization Co-NPs and Co-Met complex

The synthesized Co-NPs and Co-Met complex were characterized for their size, surface morphology, and composition.

Physical properties

For the determination of the physical morphology of powdered Co-NPs and Co-Met complex, X-rays diffraction spectroscopy XRD (Shimadzu-700 diffractometer; Cu-kα radiation; wavelength= 1.5406Å) was done at the Nanoscience and Technical Division, National Centre for Physics, Islamabad. It is a non-destructive and analytical technique for the identification of different solid and crystalline substances which is based on constructive interference of monochromatic X-rays on inter-planer surfaces of the crystalline sample.

Size, shape, and surface morphology

For the determination of size, shape, and surface morphology of synthesized Co-NPs and Co-Met complex, scanning electron microscopy was conducted. Briefly, particles were first dried by using vacuum and then covered with a layer of gold (10mm thickness) by using Q150 Ea- Quorum Sputter coater and then examined by using MIRA-3 Scanning electron microscope (Tescan FE, Brno, Czech Republic) at Institute of Space Technology, Islamabad

Elemental composition of prepared particles

Energy dispersive spectroscopy (EDS) is a chemical microanalysis technique used in conjunction with scanning electron microscopy (SEM). The elemental composition of the samples was determined by using the EDS detector attached to the MIRA-3 Scanning electron microscope (Tescan FE, Brno, Czech Republic)

Phase II:

Purchase, transportation, and acclimatization of fish

Mahseer (*Tor putitora*) fingerlings (weight range=6.36±0.54g) were procured from the Mahseer Fish Seed Hatchery, Garyala, District Attock and transported live in well-aerated plastic bags to the Aquaculture Research Station at Quaid-i-Azam University, Islamabad and after tempering, they were shifted to circular fiberglass tank (500 Gal.) having a flow-through system and acclimatized there for a week before the initiation of feeding experiment. During acclimatization, fingerlings were provided a 40% crude protein basal diet.

Diet preparation

The feed was prepared by adopting the same method mentioned previously (experiment 1; section Materials and methods). Briefly, three groups of feed, i.e., without Co supplement, supplemented with increasing level of Co-NPs and Co-Met complex respectively were formulated (Table 1). The dosage levels of Co-NPs and Co-Met complex were the same as used for the determination of optimal dietary cobalt supplementation level in advanced fry of *T. putitora* (Experiment. 1). After formulation, the respective diets were prepared by mixing dried ingredients with the help of a small scale local made feed mixer. Afterward, the dough was prepared by adding oil and a small amount of water, then spaghetti was prepared by passing the dough through a small meat grinder. The prepared feed was oven-dried at pre-set 60ºC. Subsequently, a handheld pestle motor was used to grind the dried feed to make crumbled feed pellets and saved in zip lock bags. Fresh feed was prepared after every 15 days.

Phase II

Experimental Design

Two sets of completely randomized 90 days feeding experiments, one for Co-NPs and the other for Co-Met complex were designed and conducted in triplicate.

Experiment A

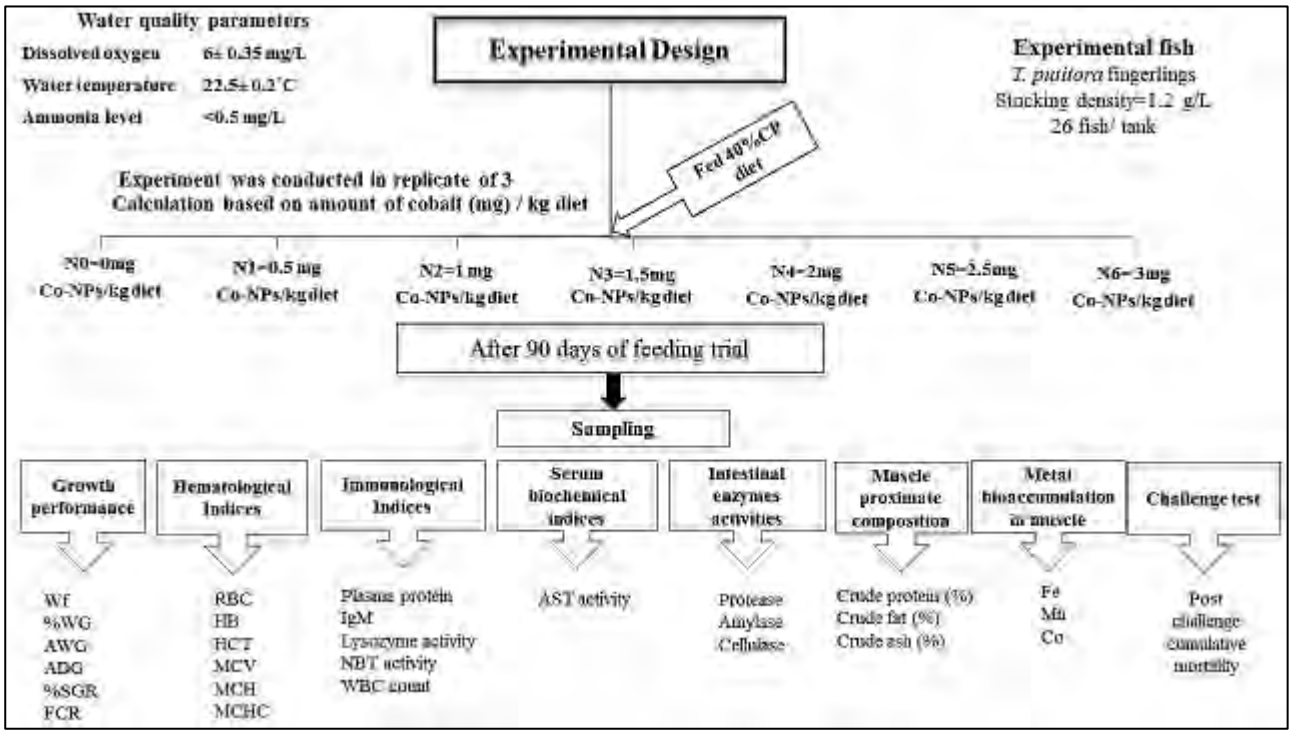

Figure 31. Experimental design to evaluate the effect of graded levels of Co-NPs supplemented diet on *T.putitora* fingerlings

For the feeding trial, the acclimatized fingerlings of mahseer (average body weight= 6.36± 0.54g) were netted from a circular tank and stocked in twenty-one (21) rectangular fish rearing

fiberglass tanks (capacity; 50 Gal.) at a stocking density of 1.2 g/L (26 fish/ tank). After shifting, fish were again acclimated to their respective tanks for 3 days. The tanks had a well-fitted aeration system to maintain a dissolved oxygen level in water up to 6.0 mg/L and water heaters to keep the water temperature at an optimal level, i.e., 22.5°C. The tanks were divided into seven groups having three replicates in each group and 26 fish in each tank.

Feeding practices

During the feeding trial, fingerlings were fed a basal diet without Co supplement to the control group (N0), while experimental groups N1→N6 were fed diets containing an increasing level of Co-NPs (0.5-3mg/kg with 0.5mg/kg increment).

At the start of the experiment, fish were fed at the rate of 4% of body weight twice a day, i.e., 9:00 am, and 4:00 pm. The feeding rate was adjusted fortnightly and 2% body weight by the end of experiment. Daily, the unconsumed feed was removed after 2 hours of feeding while the fish fecal matter was also collected after 24 hours of feeding by siphoning, filtered, and collected separately for later calculation of FCR. After siphoning, the volume of water was adjusted by adding fresh water to the tanks. During the experiment, water quality parameters like temperature (ºC), pH, and dissolved oxygen (mg/L) were checked by using Multi-parameter Hanna HI 9829-01102 (Woonsocket, USA), while dissolved ammonia was checked weekly by using the ammonia Kit (H13824. ROMANIA). The feeding trial proceeded for 90 days, during which temperature and DO levels fluctuated slightly, i.e., temperature ± 0.2˚C and DO level ± 0.35 mg/L, while total ammonia remained <0.5 mg/L. The feeding trial was conducted under controlled conditions, therefore all experimental tanks did not show any noticeable difference in water quality parameters. All experimental procedures were conducted after the approval of the Animal Welfare Committee of Quaid-i-Azam University (BEC-FBS-67-QAU-2017).

Growth performance

At the end of the feeding trial, i.e., after 90 days, fish were starved for 24 hours before sampling. On the day of sampling, fish from each tank were collectively captured by using a scoop net and weighed on a top-loading balance (SHIMADZU-ELB3000, Japan), and counted for determining growth performance. The standard formulas reported previously were adopted for determining growth performance and feed conversion ratio (Amir et al., 2018; Younus et al., 2020)

Sample collection

For hematological analysis, fish were anesthetized with buffered MS222 (0.1mg/L buffered with Na_2CO_3) and blood from each group was collected by the procedure reported earlier (Ahmed et al., 2020). Briefly, blood from each group of fish was drawn from the caudal vein by using 1-2 ml of syringes connected to a 22-gauge needle (Becton, Dickinson and company ©2006) and stored in purple top EDTA tubes (VACUETTE® Liuyang Sanli Medical Technology Development Co. Ltd.).To obtain enough samples for hematological analysis, the blood of 2 fish from each tank was collected in the same EDTA tube (pool of 2 fish =1 sample, i.e., 3 sample/tank, or 9 samples/ treatment.

Similarly, by adopting the same procedure, the blood of 18 fingerlings/treatment was drawn and stored in heparinized Eppendorf tubes for obtaining plasma. To get enough samples, the blood of 2 fish from each tank was collected in the same collecting tube (3 samples/tank: 9 samples/ treatment). For serum, the blood of 18 fish/treatment (pool of 2 fish: 3samples/tank: 9 sample/ treatment) was drawn from a caudal vein by using 1-2 ml of syringes connected to a 22-gauge needle (Becton, Dickinson and company ©2006) and collected in an Eppendorf tube (without any coagulant). The blood was allowed to clot for 60 minutes at room temperature for obtaining serum. The fresh heparinized blood was used for respiratory burst activity (2 samples/tank; 6samples/treatment).

After drawing blood, fish were aseptically dissected on an ice bag and the alimentary canal was removed. For intestinal enzyme analysis, the guts of 3 fish from the same tank were pooled (9 sample/treatment), frozen in liquid nitrogen, and stored at -20˚C. Subsequently, the 6fish / tank was stored for the analysis of proximate composition. To get enough muscle samples, tissues of 3 fish were pooled (6 samples/treatment). The muscle of 6 fish/treatment was also collected for metal bioaccumulation analysis.

Collection of blood plasma

Plasma was obtained by centrifuging heparinized blood samples at 3000 rpm (SCILOGEX LLC) for 15 minutes. The obtained transparent plasma of each sample was stored in separate collecting tubes and saved at 4ºC.

Collection of blood serum

The serum was collected by centrifuging the clotted blood at 2,000 (SCILOGEX LLC) rpm for 10 minutes. After centrifugation, the supernatant (serum) was collected in a separate Eppendroff tube and saved at 4˚C

Hematological Indices

Hematological indices, i.e., erythrocytes (RBCs), hemoglobin (Hb), HCT % (hematocrit value), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), and (MCHC corpuscular hemoglobin concentration) were determined by using a hematology analyzer (Sysmex XS 800i, Japan).

Aspartate aminotransferase activity AST activity

Aspartate aminotransferase activity (AST) was determined with the help of an AST/GOT kit (AMEDA Laborodiagronstiks GmBH Austria).

Briefly, 100µl of serum was taken in a clean Eppendorf tube and mixed with 910µl of reagent A and 90µl of reagent B. After adding both reagents, the mixture was gently mixed and incubated at 37ºC for 1 minute. Subsequently, the absorbance of the mixture was measured with the help of a UV-visible spectrophotometer (Model; Agilent 8453, Japan) by fixing wavelength at 340 nm. The absorbance was noted after every 1 min for 3 times. The AST activity was expressed in U/L

Immunological Indices

Total plasma protein

Total plasma protein was determined by adopting Lowry's method (Lowry et al., 1951). Bovine serum albumin (BSA) was used as a standard for the determination of protein concentration of plasma. At first, the BSA stock solution was prepared by dissolving 5mg of BSA in 5ml of distilled water. Then, a standard curve was plotted from the protein concentration of BSA, i.e., 5%, 10%, 15%, 20%, 25%, and 30%.

The following solutions were prepared before the initiation of protein estimation.

Solution 1:

a) 2% Sodium carbonate (w/v: 2g in 100 ml distilled water)

b) 0.1 N Sodium hydroxide (w/v: 4g in 1000 ml H_2O)

After that, 50ml of solution (a) was mixed with 50ml of solution (b) to make solution 1.

Solution 2

(c) 1.56% copper sulfate (w/v: 1.56g in 100 ml H_2O)

(d) 2.37% of Sodium-Potassium Tartrate (w/v: 2.37g in 100 ml of water)

After that, 10ml of solution (c) was mixed with 10ml of solution (d) to make solution 2

Solution 3

Folin Phenol: water (v/v: 1:1)

Solution 4 (Alkaline Solution)

It was prepared by mixing 2ml of solution 2 and 100 ml of solution 1

Briefly, 0.1ml BSA dilution was taken in a clean test tube, and 1ml of alkaline solution 4 was added and incubated at room temperature for 10 minutes. After incubation, 1ml of solution 2 was added to it and vortexed. Then the mixture was incubated at room temperature for 30minutes and afterword absorbance was measured by using a UV-visible spectrophotometer (Agilent 8453, Japan) adjusted at 600nm. A standard calibration curve was made by plotting absorbance at Y-axis and BSA dilutions on X-axis.

The protein content of plasma was determined by following the same protocol. The absorbance of unknown samples (plasma) was noted and their concentrations were determined with the help of a standard curve.

Plasma Immunoglobulin (IgM)

Plasma immunoglobulin was determined by adopting Anderson et al. (1995) protocol. Briefly, 100µl of plasma was mixed with 0.1 ml of 12% polyethylene glycol solution and incubated under constant shaking (New Brunswick™ Innova® 43, 230, CAT# M1320-0002) for 120 minutes at room temperature. After incubation, the mixture was centrifuged at 7000 rpm (SCILOGEX LLC) for 10

minutes and the supernatant was collected in a separate Eppendorf tube. The protein content of the supernatant was calculated by following Lowry's method as mentioned above. Subsequently, total immunoglobulin IgM was calculated by using the following formula:

Plasma IgM (mg/ml) = Total plasma protein – protein content of supernatant

Lysozyme activity

Lysozyme activity of serum was determined by adopting Siwicki (1995) method, using hen egg-white lysozyme (lyophilized powder, Sigma-Aldrich, > 40,000 units per mg of protein) as standard. Briefly, 100µl serum was taken in a test tube and mixed with 900 µl of 0.75mg/l of *Micrococcus lysodeikticus* suspension in phosphate-buffered saline; pH 6.2 (Sigma-Aldrich, USA). The tube was shaken well to mix the serum with bacteria. Afterward, absorbance was measured at 450 nm with the help of a UV- visible spectrophotometer (Agilent 8453, USA) after one-minute intervals for 10 minutes.

WBC's counting

White blood cell count (WBCs) of 9 fingerlings/treatment was determined by adopting a method reported by Khan et al. (2017). Briefly, WBC's were counted by mixing 400 µL of commercially available WBCs solution with 20µl of blood and counted by using a Neubauer hemocytometer under the light microscope (100X)

Respiratory burst activity

The respiratory burst activity was determined by adopting Anderson and Siwicki (1995) nitro blue tetrazolium (NBT) assay as reported earlier (Younus et al., 2020). It measures by the reduction of NBT to formazan as a measure of the production of reactive oxygen species in blood. Briefly, 0.1ml of heparinized blood was taken in clean Eppendroff and mixed with 0.1ml of 0.2% NBT

(Sigma, USA) solution and incubated for 30 minutes at 25 ºC. After incubation, 0.05ml of the above solution was taken in a glass tube and 1 ml of N-N di-ethyl methyl formamide solution was added to it and then centrifuged at 3000 rpm (SCILOGEX LLC) for 5 minutes and supernatant of each sample was collected in separate test tubes. The absorbance of the supernatant was measured at 540 nm by UV- visible spectrophotometer (Agilent 8453, USA). To standardize the results, distilled water was used as blank.

Challenge test

Challenge test was conducted by adopting the same protocol as reported earlier (Younus et al., 2020) and pathogenic strain of *Aeromonas hydrophila* isolated from an infected *L. rohita* at National Veterinary Laboratory Islamabad. Briefly, at the end of the feeding trial, 15 fish/treatment were used for conducting the challenge test. Each group of fish was placed in a separate tank, well equipped with a water heater and air stones for maintaining temperature (22.5ºC) and DO level at 6 mg/L. Fish were acclimatized in their respective tanks for 3 days before the start of the challenge test and injected with freshly cultured *Aeromonas hydrophila*. Briefly, each fish was injected with a bacterial suspension intraperitoneally (IP) at the rate of 100 CFU/g of fish. Everyday fish were observed for disease symptoms and their mortality was recorded. Two days after the exposure of fish with pathogenic bacteria, they were fed till satiation daily with their respective diets. The post-challenge cumulative mortality curve was plotted in graph pad prism by observing the mortalities in the control and experimental groups of fish during 20 days.

Intestinal enzyme activities

For intestinal enzyme analysis, enzyme solution and phosphate buffer were prepared.

Preparation of 0.1 M phosphate buffer

Briefly, 2.27g of Monosodium phosphate (NaH_2PO_4) and 22.38g of sodium phosphate dibasic heptahydrate ($Na_2HPO_4.7H_2O$) were dissolved in 100ml of distilled water to make 0.1 M phosphate buffer; pH=7.5.

For the quantitative assay, 1g pooled intestine was taken in a clean tube and homogenized in 10ml of chilled phosphate buffer saline with a handheld electric homogenizer (VWR power 200). After homogenization, samples were centrifuged at 10,000 rpm (SCILOGEX LLC) for 10 minutes at 4 ºC. Clear supernatant 'enzyme solution' was collected in collecting tubes and stored at -20ºC.

Protein estimation

Intestinal protein content in the samples of each group was determined by adopting Lowry's method as described above. BSA dilutions were used for the preparation of a standard curve and estimation of protein in each sample

Protease Activity

Preparation of assay solutions

All analytical grade chemicals (Sigma –Aldrich) were purchased from a local supplier

- 0.65% (w/v) of casein solution. It was prepared by dissolving 0.65g of technical grade casein (Sigma-Aldrich) in100 ml of 50mM of KH_2PO_4 buffer (pH 7.5) and then stored at 37ºC for 5 minutes.

- 110 mM Trichloroacetic Acid (v/v). The solution was prepared by diluting 9ml of Trichloroacetic acid (6.1 N stock solution; Sigma-Aldrich, CAS# 76-03-09) in 500ml of deionized water.
- 0.5M Folin & Ciocalteu's Phenol Reagent (v/v): It was prepared by mixing 10 ml of Folin & Ciocalteu's Phenol in 40ml of deionized water.
- 500mM solution of Na_2CO_3: It was prepared by dissolving 35.29g of sodium carbonate in 100 ml deionized water.

Briefly, 1ml of the above-prepared enzyme solution was taken in a clean glass tube and 5ml of casein solution was added and incubated at 37ºC for 10 minutes. After that, the reaction was stopped by adding 5ml of the trichloroacetic acid solution and incubated at 37ºC for 30 minutes. After incubation, the solution was cooled at room temperature for 30 minutes and then filtered by using Whatman filter paper. In the next step, 2ml of the above obtained filtered solution was taken in a clean glass test tube and 1ml of prepared folin & Ciocalteu's Phenol reagent was added. In the final step, 5ml of a sodium carbonate solution was added to the test tube and the solution was incubated at 37ºC for 30 minutes. After incubation, the absorbance of the reaction mixture was measured at 660nm using a UV-visible spectrophotometer (Agilent, 8453, USA).

Amylase Activity

One amylase unit is the amount of enzyme in 1ml of the filtrate which can release 1µg of reducing sugar (glucose) per minute.

Solution preparation for assay

Starch solution (w/v): Briefly, 1g of powdered starch (Sigma-Aldrich; CAS#9005-84-9) was dissolved in 100 ml of distilled water in a glass beaker.

DNS- reagent (w/v): it was prepared by taking 1g of 3, 5-dinitro salicylic acid and dissolved in 20ml of 2.0 M NaOH solution under constant magnetic stirring (Magnetic Stirrer; ATO-HS-19). Then, 30g sodium potassium tartrate was slowly added to the reaction mixture. In the final step, distilled water was added to the mixture to make the final volume of 100ml. Prepared dark orange-colored DNS reagent was stored at room temperature.

Briefly, 0.5ml of frozen intestinal enzyme solution was taken in a clean test tube and placed at room temperature for 5 minutes. Then, 500µl of the starch solution was added to it and kept at room temperature for 3 minutes. In the next step, 1ml of DNS reagent was added and incubated for 5 minutes in boiling water. After incubation, the solution was cooled at room temperature, and 100ml of reagent grade water was added to it. The absorbance of the solution was measured at 450nm with the help of a UV-visible spectrophotometer (Agilent 8543, USA).

Cellulase Activity

Cellulase enzyme activity was measured by the usage of Carboxymethyl- cellulose (CMC) as a substrate and estimation of released glucose.

Solution preparation for assay

Citrate Phosphate buffer: It was prepared by dissolving 0.307g of citric acid monohydrate (Sigma-Aldrich, CAS# C1909) and 2.932g of sodium phosphate (Sigma-Aldrich, CAS#S9763) in 100ml of distilled water under magnetic stirring (Magnetic hot plate Stirrer; ATO-HS-19). The final desired pH=5 of a solution was adjusted with HCl or NaOH

1% Carboxy-methyl-cellulose solution (w/v): It was prepared by taking 1g of CMC and dissolved in 100ml of distilled water under magnetic stirring at 40 ºC.

40% sodium potassium tartrate (w/v): 40g of sodium-potassium tartrate (Sigma-Aldrich, CAS #217255) was dissolved in 100ml of water.

Briefly, 1ml of enzyme solution was taken in a clean glass test tube and mixed with 1ml of CMC solution and 1ml of citrate phosphate buffer and incubated for 30 minutes in a water bath at 50ºC. After incubation, 3ml of DNS reagent was added and again heated for 15 minutes in a water bath. In the final step, 1ml of sodium-potassium tartrate solution was added and cooled the solution at room temperature. The absorbance of the solution was measured at 540nm using a UV-visible spectrophotometer (Agilent 8543, USA).

Muscle proximate composition

Proximate composition of the muscle of different groups of fish was determined by adopting a standard AOAC (2000) protocols at the ISO-certified laboratory (ISO/IEC 17025), Poultry Research Institute, Islamabad. The same protocol was followed for proximate composition as mentioned in experiment 1(Material and methods section). Briefly, muscle %crude protein and % fat contents were determined via the Kjeldahl and Soxhlet extraction technique respectively, while the crude %ash content was determined by heating the sample in a muffle furnace at 550°C.

Metal bioaccumulation

For metal bioaccumulation analysis, the same protocol as mentioned before in Experiment 1 (Material and methods section) was adopted.

Briefly, 1g of muscle sample (6 samples/treatment) was taken in a flask and digested on a hot-plate in the presence of nitric acid (5ml) and perchloric acid (1ml). After digestion, the obtained clear solution was cooled at room temperature and filtered by using filter paper. Afterward, 50ml distilled water was added to the filtrate and Atomic absorption spectroscopy (AA240FS, Palo Alto, CA, USA)

was conducted to determine iron, manganese, and cobalt concentration in the muscle of control and experimental groups of fish.

Experiment B

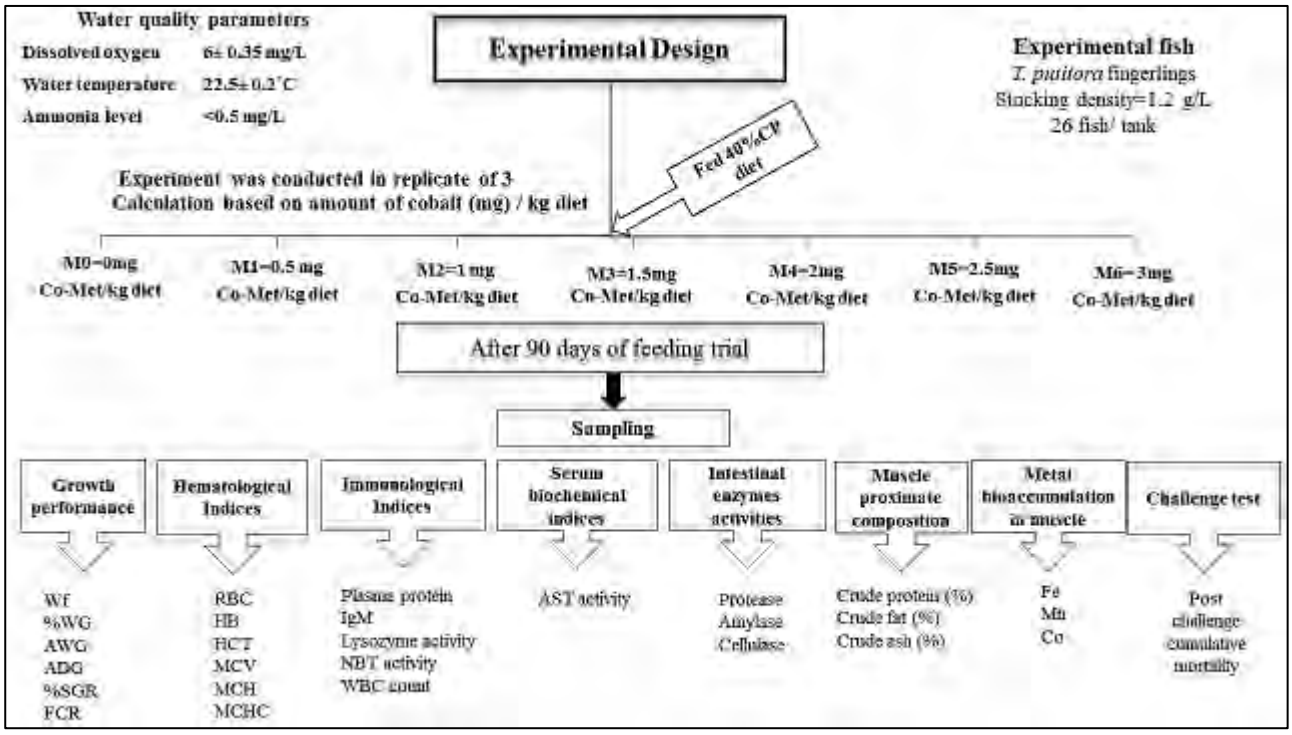

Figure 32. Experimental design to evaluate the effect of graded levels of Co-Met complex supplemented diet on *T.putitora* fingerlings

A similar set of experiment was designed by replacing the Co-NPs supplementation with the Co-Met complex. Briefly, fish were stocked in a set of twenty-one (21) rectangular fish rearing fiberglass tanks at the stocking density of 1.2g/L (26 fish/tank). Feeding trial was also conducted in controlled conditions i.e., DO up to 6.0 mg/L and water temperature 22.5°C.

Feeding practices

During the feeding trial, fingerlings were fed a basal diet without Co supplement to the control group (M0), while experimental groups M1→M6 were fed diets containing an increasing level of Co-Met (0.5-3mg/kg with 0.5mg/kg increment).

At the start of the experiment, fish were fed at the rate of 4% of body weight twice a day, i.e. 9:00 am, and 4:00 pm. The feeding rate was adjusted fortnightly and 2% by the end of experiment. Daily, the unconsumed feed was removed after 2 hours of feeding while the fish fecal matter was also collected after 24 hours of feeding by siphoning, filtered, and collected separately for later calculation of FCR. After siphoning, water volume was adjusted by adding fresh water to the tanks. During the experiment, water quality parameters like temperature (ºC), pH, and dissolved oxygen (mg/L) were checked by using Multi-parameter Hanna HI 9829-01102 (Woonsocket, USA) while dissolved ammonia was checked weekly by using the ammonia Kit (H13824. ROMANIA). The feeding trial proceeded for 90 days, during which temperature and DO levels fluctuated slightly, i.e., temperature±0.2˚C and DO level±0.35 mg/L, while total ammonia remained <0.5 mg/L. No noticeable difference in water quality parameters were observed in all experimental tanks as the feeding trial was conducted under controlled conditions. All experimental procedures were conducted after the approval of the Animal Welfare Committee of Quaid-i-Azam University (BEC-FBS-67-QAU-2017).

Growth performance

At the end of the feeding trial, i.e., after 90 days, fish were starved for 24 hours before sampling. On the day of sampling, fish from each tank were collectively captured by using a scoop net and weighed on a top-loading balance (SHIMADZU-ELB3000, Japan), and counted for determining growth performance. The standard formulas reported previously were adopted for determining growth performance and feed conversion ratio (Amir et al., 2018)

Sample collection

Blood for hematological analysis, and for obtaining serum and plasma was collected by adopting the same protocol as described previously.

After drawing blood, fish were aseptically dissected on an ice bag and the alimentary canal was removed. For intestinal enzyme analysis, the guts of 3 fish from the same tank were pooled (9 sample/treatment), frozen in liquid nitrogen, and stored at -20˚C. Subsequently, 6fish /tank were stored for the analysis of proximate composition. To get enough muscle samples, tissues of 3 fish were pooled (6 samples/treatment). The muscle sample of 6 fish/treatment was also collected for metal bioaccumulation analysis.

Samples analysis

Hematological indices, AST activity, immunological indices, intestinal enzyme activities, muscle proximate composition, challenge test, and bioaccumulation of Fe, Mn, and Co in the muscle of fish were determined adopting the protocol mentioned previously.

Data analysis

Statistical package program SPSS (version 20) was used to analyze the obtained results to find out the statistical differences among experimental groups in response to Co-NPs and Co-Met

complex supplemented diets at different dosage levels. Data were analyzed by adopting a One-way ANOVA followed by LSD test to determine the significant differences ($P < 0.05$) among different groups of *T. putitora* for growth performance, hematological indices, immunological indices, intestinal enzymes, proximate composition, and bioaccumulation level of iron, cobalt, and manganese in the muscle.

Phase III:

Comparative effect of graded levels of different chemical forms of dietary cobalt on *T.putitora* fingerlings

In the III phase of study, we conducted a statistical analysis to study the comparative effect the graded levels of different chemical forms of dietary cobalt on *T.putitora* fingerlings. Since both sets of feeding trials i.e., Experiment A (dietary Co-NPs) and Experiment B (dietary Co-Met complex) were conducted by stocking similar-sized fingerlings under similar controlled conditions, so results obtained from both experiments were analyzed by adopting two-way ANOVA using the statistical package program SPSS (version 20). We analyzed the effect of different chemical forms of cobalt, at different supplementation levels in the diet and the interaction of two variables i.e., chemical form and dosage levels on growth, hematological indices, immunological indices, intestinal enzyme activities, proximate muscle composition, and bioaccumulation of Fe, Mn, and Co in the muscle of fish.

CHAPTER 4

Evaluation of the practical application of organic and inorganic forms of cobalt supplemented diets on *T.putitora*

The practical application of dietary cobalt–methionine complex Co-Met (organic) and nano-scale cobalt chloride Co-NPs (inorganic) as feed supplements was evaluated by conducting 90 days feeding trial in earthen ponds under semi-intensive culture conditions. A completely randomize feeding experiment in triplicate was designed based on of results obtained in experiment 2. Two supplementation levels of cobalt, i.e., 1.5mg and 3mg Co/kg of diet as Co-NPs and Co-Met complex were selected. Dry feed ingredients were obtained from local suppliers and 35% crude protein feed was formulated (Table 20). Experimental groups were as follows:

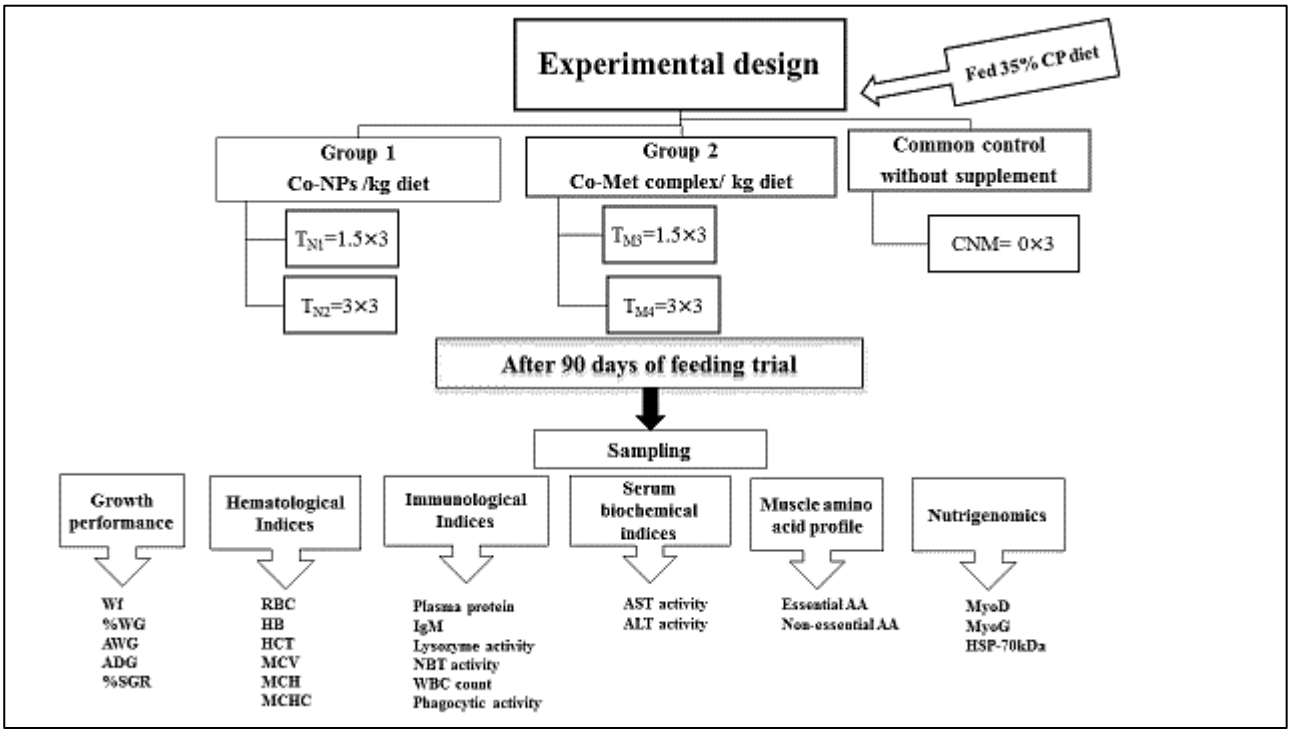

Figure 33. Experimental design to study the effect of Co-Met complex (organic) and nano-scale cobalt chloride Co-NPs (inorganic) supplemented diets on *T.putitora* cultured in earthen ponds

Table 20. Formulation of 35 % crude protein feed for *Tor putitora*.

Ingredients	Quantity(g/kg)
White fish meal	400
Soybean meal	133.3
Sunflower meal	133.3
Gluten	133.3
Rice polish	90
Wheat bran	90
CMC[a]	10
Vitamin premix[b]	10
Proximate composition (%)	
Gross energy (k calories)	2751.37
Crude protein	34.68
Crude fat	11.23
Ash	9.16

[a] Carboxy-methyl-cellulose; [b] Vitamin premix contains vitamins, amino acids and minerals premix/100g

Control and experimental diets, i.e., control (CMM) devoid of Co supplement, TN1 and TN2 diets supplemented with 1.5 and 3 mg of Co as Co-NPs per kg diet respectively while TM3 and TM4 diets supplemented with similar levels of Co but as Co-Met complex, were prepared by methods described in detail in the previous section (Section, Material and method; feed formulation mentioned in table 20). Briefly finely ground feed ingredients along with Co-NPs and Co-Met supplement in the specific ratio were thoroughly mixed in feed mixer. Then the dough was prepared by adding oil and

water and then passed through the fish feed pellet machine (HDC-P120; Wuhan HDC Technology Co., Ltd. China) to make pellets. Fresh feed was prepared after every 15 days.

Fish collection and management

T. putitora (average size range= 13-17g) were purchased from Mahseer Hatchery Garyala Attock Pakistan. They were transported live in well-aerated plastic bags to the Fisheries and Aquaculture research station Quaid-i-Azam University, Islamabad and after tempering, shifted randomly to circular fiberglass tanks (capacity each 500Gal.) having a flow-through system and acclimatized there for a week before initiation of feeding experiment. During acclimatization, fish were provided a 35% crude protein basal diet.

Preparation of earthen ponds

Rectangular fish ponds (average size 0.012ha or 120m2; 1.75m depth), present adjacent to each other at Fisheries and Aquaculture research station were prepared for the feeding trial. The ponds were prepared by following the method reported earlier (Amir et al., 2019). Briefly unwanted aquatic weeds were removed from the bottom and sides of the ponds and they were sun-dried for 7 days. Before filling the water, each pond was prepared by spreading agriculture lime (calcium carbonate) at a rate of 125 kg/ha and cow dung at the rate of 333.33kg/ha to the bottom of the dried pond. The lime was applied for disinfection and stabilization of pH, while animal manure was used to fertilize the pond and enhance pond productivity. Afterward, all ponds were filled with water, initially at a level of 1.1 meters. Then inorganic fertilizers (Urea; 0.5 kg/100 m^2 and Di-ammonium phosphate (DAP); 0.25 kg/100 m2) were applied to produce a live feed. After 7 days of application of fertilizer, the water level was raised to 1.5m and fish were stocked.

Experimental design

A completely randomized feeding experiment was designed in triplicate in earthen ponds during March 2018-May 2018. Healthy fish of *T. putitora* (average weight= 15±2.1g) with no sign of infection or disease were randomly caught from circular tanks and shifted to 15 earthen ponds at a stocking density of 45 fish/pond) (1500/acre) and acclimatized there for 3 days. Afterward, the feeding trial was started and each group of fish was provided their respective diets at 2% body weight once a day (9:00 am) for 90 days. Water quality parameters such as dissolved oxygen, temperature, pH were daily checked by using a multiparameter (Hanna 9828, Woonsocket, USA) while the ammonia level was checked once a week. During the experiment, water quality parameters showed fluctuation but remained within a range suitable for the rearing of *T. putitora* (Table 20). Since all earthen ponds were adjacent to each other, so no noticeable change in water quality parameters among them was observed. Throughout the feeding experiment, pond fertility was checked fortnightly by using Secchi disc and maintained by adding inorganic and organic fertilizers while water quality and level were managed by daily adding freshwater at dawn and dusk.

Table 21. Average ranges of water quality parameters throughout the experiment

Parameters	Range
Water temperature (ºC)	20.1-22.5
Dissolved oxygen (mg/l)	5-6.7
pH	6.9-7.2
Dissolved ammonia (mg/l)	<0.5

Growth measurements

At the end of the feeding trial, i.e., 90 days, fish were starved a day before sampling. On the day of sampling, one by one outlet of each pond was opened, and the water was drained in the fish collecting basin for sampling. Fish from each earthen pond were captured separately, weighed on a top-loading balance (SHIMADZU-ELB3000, Japan), and counted for evaluating the growth performance by using standard formulas reported previously (Younus et al., 2020; Amir et al., 2018).

Sample Collection

For sampling, fish from each pond were anesthetized with buffered MS222 (0.1mg/L buffered with Na_2CO_3). After that, for hematological analysis, the blood of 10 fish/tank (30fish/treatment) was drawn from the caudal vein by using a 2ml syringe (24G, Becton, Dickinson and company ©2006) and stored in the purple top EDTA tubes (VACUETTE® Liuyang Sanli Medical Technology Development Co. Ltd.) (Duman et al., 2019).

Similarly, by adopting the same procedure, the blood of the other 10 fish/pond was drawn and stored in heparinized Eppendorf tubes for obtaining plasma, while the blood of 10 more fish/tank (30 fish/treatment) was drawn from the caudal vein by using a non-heparinized syringe and allowed to clot at room temperature for obtaining serum. Afterward, the fish of each group were dissected aseptically, and internal visceral organs were removed. Muscle tissues of 10 fish/pond (30fish/treatment) were collected and stored at 4ºC for proximate analysis while for muscle amino acid profile, muscle tissues of 5 fish/pond were pooled (3 sample/treatment) and stored at -20°C till further use. Moreover, for nutrigenomics studies, anesthetized fish from each treatment (6 fish/treatment) i.e., common control group (CNM), TN1, TN2, TM3, and TM4 were dissected at low temperature (on ice pad) and their muscle and liver were immediately collected and preserved in RNA later™ (Thermo scientific CAT# AM7020).

Collection of blood plasma

Plasma was obtained by centrifuging heparinized blood samples at 3000 rpm (SCILOGEX LLC) for 15 minutes. The obtained transparent plasma of each sample was stored in a separate collecting tube and saved at 4ºC.

Collection of blood serum

The serum from whole blood was separated by centrifuging the clotted blood at 2,000 rpm (SCILOGEX LLC) for 10 minutes. The supernatant (serum) was collected in a separate Eppendorf tube and saved at 4˚C

Hematological indices

Hematological indices, i.e. erythrocytes (RBCs), hemoglobin (Hb), HCT % (hematocrit value) and MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin) and (MCHC corpuscular hemoglobin concentration) were determined by using a hematology analyzer (Japan, Sysmex XS 800i) as described in Experiment 2.materials and methods section

Aspartate aminotransferase activity AST activity

Serum aspartate aminotransferase (AST) activity of 10 fish/pond (30fish/treatment) was determined with the help of AST/GOT kit (AMEDA Laborodiagronstiks GmBH Austria) as mentioned in experiment 2 (Material and Method section).

Alanine aminotransferase activity (ALT)

Serum alanine aminotransferase (ALT) activity of 10 fish/pond (30fish/treatment) was determined with the help of-052 kit (Sigma-Aldrich).

ALT activity (Nanomole/min/ml)=Amount of enzyme that generates 1.0 nanomole of pyruvate/minute.

Before the initiation of the ALT assay, all reagents were placed at room temperature (as they are supplied frozen and kept at low temperature). Subsequently, 5 ml of the master mix was prepared by mixing salt Assay buffer (4.3ml) with fluorescent peroxidase substrate (100µL), ALT substrate (500µL) and, ALT enzyme mix (100µL).

Briefly, after the preparation of the reaction mixture, 10 µL of each plasma sample was mixed with ALT buffer (10 µL) and then added to the 96-welled microplate. Afterward, the master mix (100µL) was added to each well and mixed well by placing the microplate on a horizontal shaker (Thomas scientific; Model # 8289S63). Afterward, absorbance was measured initially at 570 nm, then further absorbance readings were taken by incubating microplate at 37°C and taking reading after every 5 minutes for 20 minutes. The change in absorption was measured by using the following formula:

ΔA570 = A570 (Final) - A570 (Initial)

Immunological indices

Immunological indices, i.e., white blood cell count, plasma protein, plasma immunoglobulin level, lysozyme activity, and respiratory burst activity (from fresh blood; 10 fish/pond (30fish/treatment) were determined by adopting the protocol mentioned above.

Phagocytic activity

The Phagocytic activity of heparinized blood of control and experimental groups of fish was determined by following the procedure reported by Anderson and Siwicki (1995) and reported in our publication (Younus et al., 2020).

Briefly, 100µl of blood was placed in a micro-well plate. Afterward killed *S. aureus* (100µL) was added to it and then the plate was placed at room temperature for half an hour. Later on, after

incubation, glass slides were prepared by taking 5µL of the incubated solution, and a smear was prepared. The smear was air-dried, then fixed in 95% ethanol for five minutes, and then air-dried again. In the last step, the smear was fixed with a Giemsa stain.

Glass slides were then visualized under the light microscope and phagocytic cells that had ingested bacteria were counted. Overall hundred phagocytes/slide was observed to determine their phagocytic activity.

$$\text{Phagocytic activity} = \frac{\text{Total No. of phagocytes with engulfed bacteria}}{\text{Total No. of phagocytes observed}} \times 100$$

Muscle proximate composition

Proximate composition of muscle tissues of different groups of fish was determined by adopting a standard AOAC (2000) protocols at the ISO-certified laboratory (ISO/IEC 17025), Poultry Research Institute, Islamabad. Briefly, crude protein was determined by the Kjeldahl technique while Soxhlet extraction was used for the determination of crude fat content. The crude ash content of muscle tissues was determined by a heating sample in a muffle furnace at 550°C. The detailed protocols are mentioned in experiment 1 (Section, Materials, and methods).

MyoD, MyoG and HSP-70kDa mRNA levels

RNA was extracted from the liver and muscle samples and then cDNA was prepared by adopting the protocol mentioned in experiment 1; material and method section). Similarly, the mRNA levels of MyoD and MyoG in the muscle and HSP-70KDa in the liver of each group of fish was determined by the method described before (Experiment 1; Section, Materials, and methods)

Muscle amino acid profile

High-performance liquid chromatography (HPLC: CBM-20A, Shimadzu, Japan) amino acid-based analyzer available at the Medical Botanical Center of PCSIR (Pakistan Council of Scientific Research) Complex, Peshawar was used for the amino acid profile

Sample preparation

For amino acid profile, 50g frozen muscle tissues from each group of fish were thawed, dried in a vacuum oven (Fisher brand™ Isotemp™ Model 282A Vacuum Oven), and then weighed on an analytical balance (SHIMADZU-ELB3000, Japan). After drying, muscle tissues were powdered using glass pestle and motor and shifted in a clean dried glass tube. Subsequently, 5ml of HCl (0.1%) was added, vortexed for 2-3 minutes, and then centrifuged for 15 minutes at 3500 rpm (SCILOGEX LLC). The supernatant was collected in a fresh sterile Eppendorf and filtered by passing through a 0.45µm membrane filter (Shimadzu, Japan, CAT# 670-12540-01). The obtained supernatant was then used for amino acid profile analysis.

Preparation of reaction solutions

Preparation of Borate buffer solution

It was prepared by dissolving 40.7g of sodium carbonate (Na_2CO_3) and 13.57g of Boric acid (H_3BO_3) in 600 ml of deionized water. The solution was mixed thoroughly to make it clear.

Reaction solution A

The reaction solution A was prepared by dissolving 0.2ml of Sodium hypochlorite solution in 500 ml of borate buffer. The solution was mixed thoroughly and filtered by passing through 0.45µm membrane filter paper (Shimadzu, Japan, CAT# 670-12540-01).

Reaction solution B

The reaction solution B was prepared by dissolving 0.4g of ortho-phthalaldehyde-OPA $C_6H_4(CHO)_2$ in 7ml of ethanol (CH_3CH_2OH). In the next step, 450ml of previously prepared borate buffer solution was taken in a glass flask and the OPA - ethanol solution was mixed in it. Subsequently, 0.5g of N-Acetyl-L-cysteine ($C_5H_9NO_3S$) was added to the above solution and mixed well by manual shaking, and made a homogenous solution. In the final step, impurities were removed by filtering the reaction mixture through 0.45µm membrane filter paper.

Preparation of mobile phase 'A'

For mobile phase A, 19.6g of Tri-ammonium citrate ($C_6H_{17}N_3O_7$) was dissolved in 600ml of double-distilled water in a volumetric flask. Then, 70ml of ethanol (CH_3CH_2OH) and 7.8ml of Perchloric acid HClO4 (20mM) was added to the above solution and mixed the solution by slightly shaking the flask. Afterward, double-deionized water was added to make the final volume up to 1L. The pH (3.2) of the mobile phase was adjusted with 20mM Perchloric acid.

Preparation of mobile phase 'B'

For mobile phase B, 12.4g of boric acid and 58.8g of *citrate* tribasic was dissolved in800ml of double-distilled water. Afterward, 25ml of sodium hydroxide (4.0N) was added to the above solution and then water was added to make the final volume of 1L. Here, NaOH (4.0N) was used to adjust the pH of the reaction mixture at 10. In the final step, the reaction mixture was filtered by passing through filter paper (pore size, 0.45µm: Shimadzu, Japan, CAT# 670-12540-01).

Preparation of mobile phase 'C'

For mobile phase C, 0.2N NaOH solution was prepared by dissolving 8g NaOH in 1L of H_2O. The solution was then filtered by using membrane filter paper having a pore size of 0.45 µm (Shimadzu, Japan, CAT# 670-12540-01).

High-pressure liquid chromatography (HPLC)

Amino acids were analyzed by using post-column derivation with ortho-phthalaldehyde. Briefly, 20µl of a sample (filtered supernatant) was injected in high-pressure liquid chromatography (Shimadzu Japan) having sodium-based column (Shim-pack ISC-07/S1504; 4.00mm internal diameter×1.5cm) and Spectrofluoro-photometric detector (Excitation350nm, emission 450nm; (RF-20A Fluorescence Detector, Shimadzu Japan). The flow rate 0.3ml/minute and temperature 55ºC were adjusted and amino acids were separated by using mobile phase A and B. After separation, amino acids were spelled out from the column. In the final step, the post-column derivatization was initiated, and after that complex was passed through the cuvette to detect the concentration of different amino acids in the sample.

Data Analysis

Results were analyzed by using statistical package program SPSS (version 20) by using one way ANOVA followed by LSD to find out the effect of organic and inorganic forms of dietary cobalt at different dosage level on growth performance, hemato-immunological indices, proximate muscle composition, amino acid profile and mRNA levels of MyoD, MyoG in muscle HSP-70KDa in the liver of different groups (CNM, TN1, TN2, TM3, and TM4) of *T. putitora.*

RESULTS

CHAPTER 1

Effect of cobalt supplemented diet on early rearing of advanced fry of *Tor putitora*

Growth performance

Cobalt chloride supplemented diets showed a significant effect on the growth performance of advanced fry of *Tor putitora* (Table 22). One way ANOVA showed significant difference in final body weight (n=3, $F_{6,14}$=166.07; PSE=0.03; P<0.001), %WG (n=3, $F_{6,14}$=816.08; PSE=1.047; P<0.001), %SGR (n=3, $F_{6,14}$=9.915; PSE= 0.55; P<0.001) and AWG (n=3, $F_{6,14}$=3031.31; PSE=0.007; P<0.001) among all diet groups of fish. The positive correlation between the dietary cobalt concentration and the percentage weight gain, i.e., R^2=0.889 (Fig.34) was observed up to 2mg Co/kg diet (group D). However, further increase in cobalt supplementation negatively affected the growth performance parameter (R^2=0.98) (Fig. 35).

Additionally, FCR also showed significant differences (n=3, $F_{6, 14}$=6005.59; PSE=0.023; P<0.001) among all diet groups. The pair-wise comparison showed the highest FCR in the F group while group D showed the lowest value (Table 22).

Body proximate composition

The effect of graded levels of cobalt chloride enriched diets on whole-body proximate composition of advanced fry of *T.putitora* is shown in Table 23. One way ANOVA demonstrated significant difference in whole body % crude protein (n=9, $F_{6,56}$=199.75; PSE=0.182; P<0.001), % crude fat (n=9, $F_{6,56}$=987.07; PSE=0.264; P<0.001) and % crude ash (n=9, $F_{6,56}$=864.25; PSE=0.210; P<0.001) among all diet groups of fish. The positive correlation (R^2=0.99) between the dietary cobalt concentration and the whole-body crude protein was observed up to 2mg Co/kg diet (group D) (Fig. 36). However, further increase in the cobalt supplementation level negatively affected the whole-body crude protein contents. In contrast to protein, crude fat and ash contents showed an increasing trend with an increase in dietary cobalt concentration (crude fat, R^2=0.86, and ash R^2=0.85). The post hoc LSD test indicated the highest values of crude fat (Fig. 37) and ash contents (Fig. 38) in the F group of fish.

Muscle metal bioaccumulation

Bioaccumulation of metal in the muscle of advanced fry of *T. putitora* reared on graded levels of dietary cobalt is shown in Table 24. One way ANOVA showed a significant difference in the muscle bioaccumulation of iron (n=3, $F_{6,14}$=11543.4; PSE=0.001; P<0.001), manganese (n=3, $F_{6,14}$=10.774; PSE=0.0018; P<0.001), and cobalt (n=3, $F_{6,14}$=132.09; PSE=0.0001; P<0.001) among all diet groups of fish. Furthermore, the dietary Co supplementation level indicated a positive correlation (R^2 = 9526) with its bio-accumulation (Fig.41), while negative correlation with the bio-accumulation of Fe (R^2 = 0.99) and Mn (R^2 = 9069) (Fig. 39 and 40 respectively) in the muscle of fish.

Table.22. Effect of graded levels of dietary cobalt supplementation on the growth performance of advanced fry of *T. putitora*

	Diet groups							Statistical Analysis		
	Control	**A**	**B**	**C**	**D**	**E**	**F**	**PSE[a]**	**P-value**	**F- value**
W_i	1.35^{a}	1.34^{a}	1.34^{a}	1.35^{a}	1.32^{a}	1.34^{a}	1.35^{a}	0.011	0.498	0.95
W_f (g)	2.39^{d}	2.41^{cd}	2.46^{c}	2.73^{b}	2.98^{a}	2.24^{e}	2.20^{e}	0.03	0.001	166.07
WG (%)	75.73^{e}	78.44^{d}	83.61^{c}	103.98^{b}	123.47^{a}	67.77^{f}	64.46^{g}	1.047	0.001	816.08
SGR	0.63^{bc}	0.64^{bc}	0.68^{b}	0.79^{a}	0.81^{a}	0.58^{c}	0.55^{c}	0.044	0.001	9.915
AWG (g)	1.03^{e}	1.06^{d}	1.12^{c}	1.39^{b}	1.65^{a}	0.91^{f}	0.86^{g}	0.007	0.001	3031.31
FCR	5.61^{c}	5.52^{d}	5.13^{e}	4.06^{f}	3.32^{g}	6.36^{b}	6.19^{a}	0.022	0.001	6005.59

Data is expressed as average value (n=3). Values with different letters within the same rows are significantly different ($P<0.05$). Statistical analysis was done by adopting one way ANOVA followed by LSD.

a=Pooled standard error mean; wf= average final weight of fish; %WG= percentage weight gain; %SGR=Specific growth rate (% body weight/day); AWG=Average weight gain, FCR= Feed conversion ratio

A= 0.5 mg cobalt/kg diet; B= 1.0 mg cobalt/kg diet; C= 1.5 mg cobalt/kg diet; D= 2.0 mg cobalt/kg diet; E= 2.5 mg cobalt/kg diet; F= 3.0 mg cobalt/kg diet.

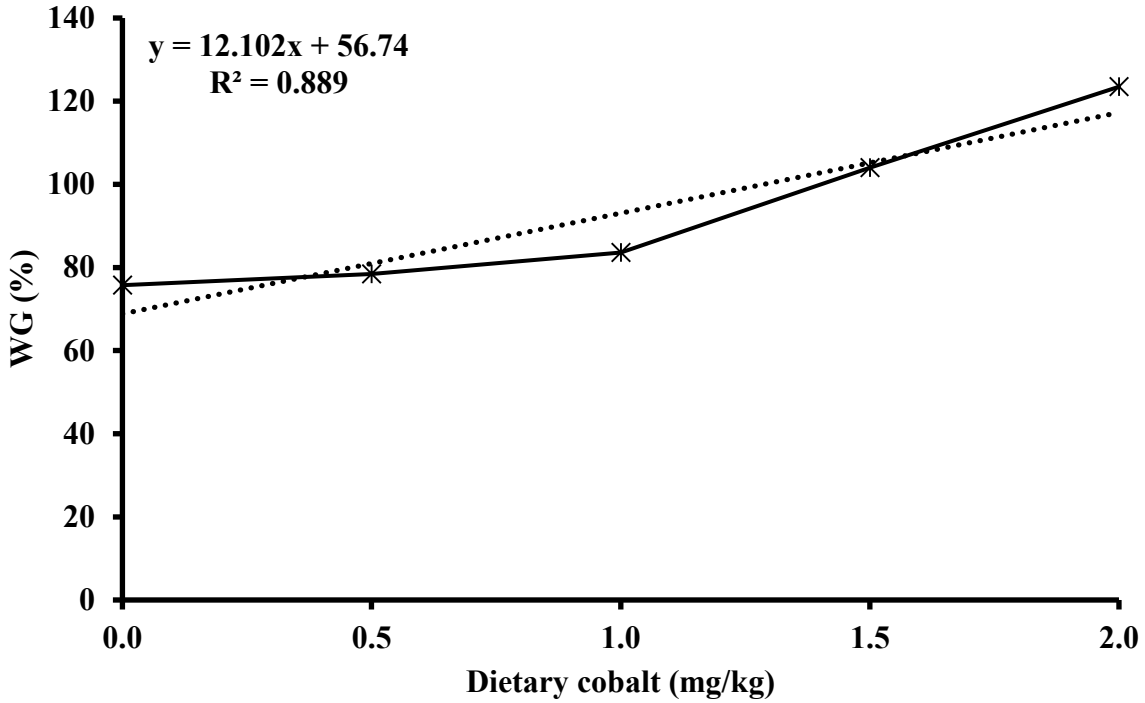

Figure 34. Linear regression between dietary cobalt supplementation and %weight gain of advanced fry of *T. putitora*

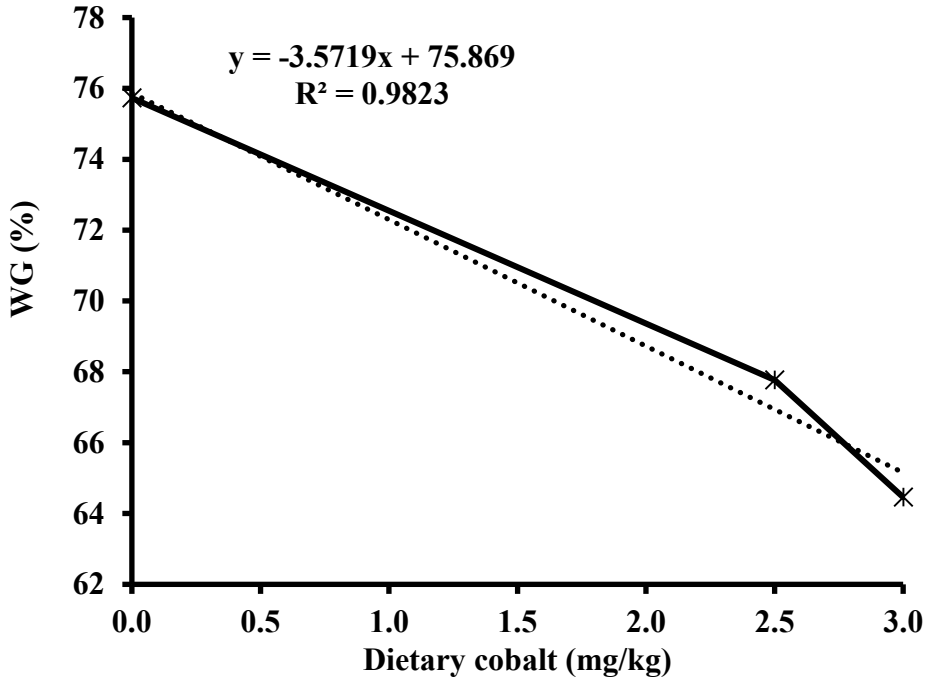

Figure 35. Inverse relationship between dietary cobalt supplementation above 2mg/kg diet and %WG of advanced fry of *T. Putitora*

Table 23. Whole body proximate composition of *T. putitora* advanced fry after feeding graded levels of cobalt chloride supplemented diets for 90 days

	Diet Groups							Statistical Analysis		
	Control	**A**	**B**	**C**	**D**	**E**	**F**	**SEM[a]**	**P-value**	**F-value**
Crude protein (%)	16.31^{e}	16.90^{d}	18.14^{c}	18.98^{b}	20.02^{a}	17.01^{d}	15.09^{f}	0.182	0.001	199.75
Crude fat (%)	1.53^{f}	1.61^{e}	1.63^{e}	1.82^{d}	2.13^{c}	2.48^{b}	3.14^{a}	0.264	0.001	987.07
Crude ash (%)	1.56^{f}	1.63^{e}	1.65^{e}	1.71^{d}	2.06^{c}	2.26^{b}	2.75^{a}	0.210	0.001	864.25

Data is expressed as average value (n=9). Values with different letters within the rows are significantly different (P<0.05). Statistical analysis was done by adopting one way ANOVA followed by LSD.

a=Pooled standard error mean

A= 0.5 mg cobalt/kg diet; B= 1.0 mg cobalt/kg diet; C= 1.5 mg cobalt/kg diet; D= 2.0 mg cobalt/kg diet; E= 2.5 mg cobalt/kg diet; F= 3.0 mg cobalt/kg diet.

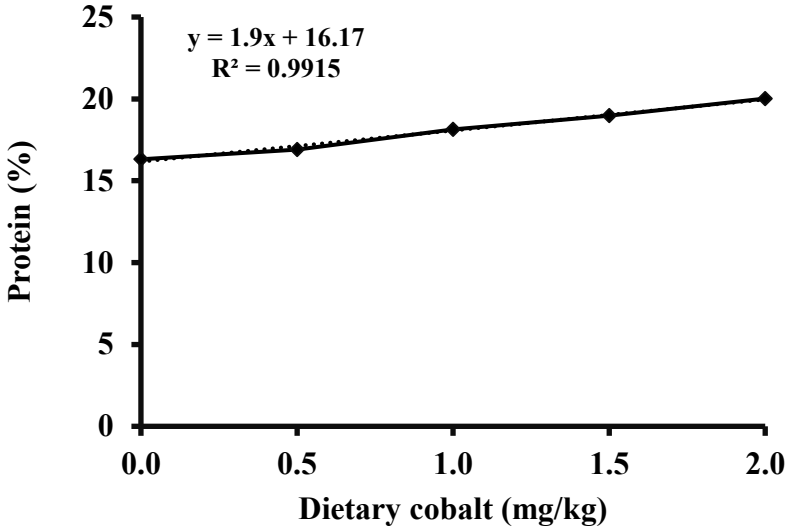

Figure 36. Linear regression between dietary cobalt chlroide supplementation up to 2mg Co/kg diet and whole body crude protein (%) of advanced fry of *T.putitora*

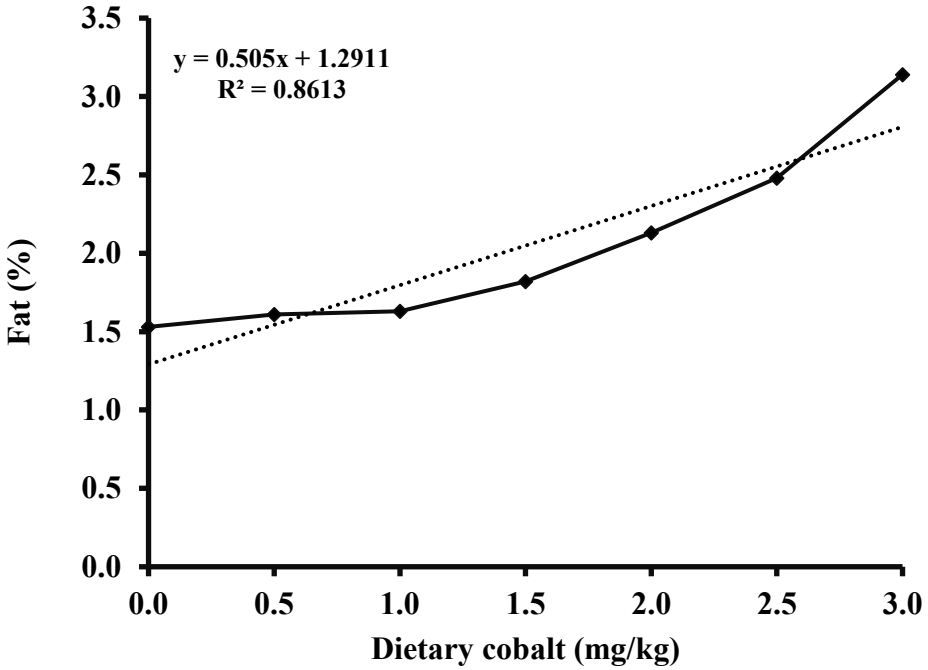

Figure 37. Linear regression between dietary cobalt chlroide supplementation and whole body crude fat (%) of advanced fry of of *T.putitora*

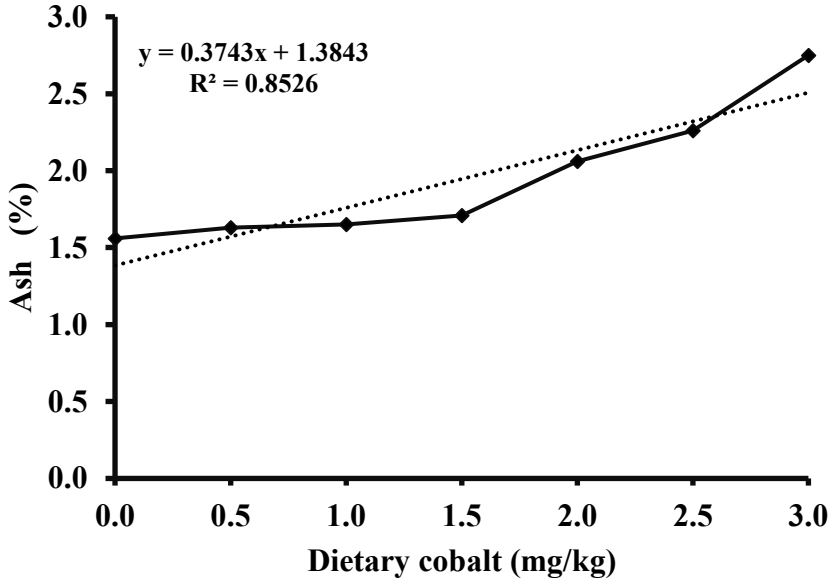

Figure 38. Linear regression between dietary cobalt chlroide supplementation and whole body crude ash (%) of advanced fry of *T.putitora*

Table 24. Bioaccumulation of metals in the muscle of advanced fry *T.putitora*, after feeding graded levels of cobalt chloride supplemented diets for 90 days

	Diet Groups							Statistical Analysis		
Metal concentration (µg/g)	**Control**	**A**	**B**	**C**	**D**	**E**	**F**	**SEM**	**F-value**	**P-value**
Iron	0.0303^{a}	0.0281^{b}	0.0250^{c}	0.0211^{d}	0.0163^{e}	0.0133^{f}	0.0096^{g}	0.001	11543.4	0.0001
Manganese	0.0042^{a}	0.0044^{a}	0.0041^{a}	0.0038^{b}	0.0034^{c}	0.0034^{c}	0.0031^{d}	0.0018	10.774	0.0001
Cobalt	0.0107^{d}	0.0108^{d}	0.0111^{c}	0.0113^{c}	0.0121^{b}	0.0122^{b}	0.0126^{a}	0.0001	132.09	0.0001

Data is expressed as average value (n=3). Values with different letters within the rows are significantly different ($P<0.05$). Statistical analysis was done by adopting one way ANOVA followed by LSD. a=Pooled standard error mean

A= 0.5 mg cobalt/kg diet; B= 1.0 mg cobalt/kg diet; C= 1.5 mg cobalt/kg diet; D= 2.0 mg cobalt/kg diet; E= 2.5 mg cobalt/kg diet; F= 3.0 mg cobalt/kg diet.

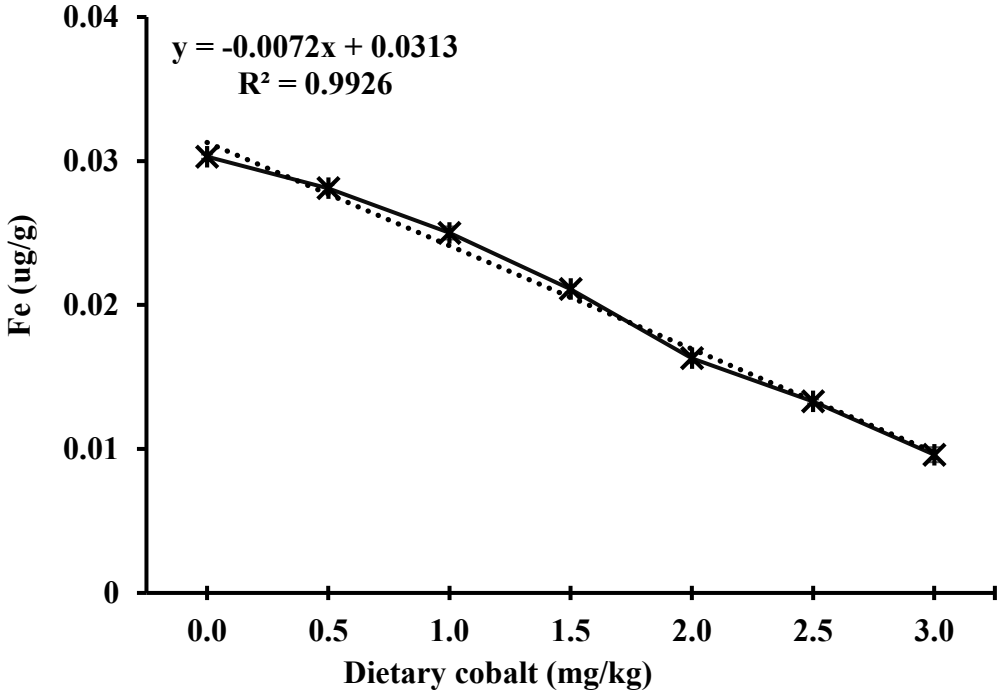

Figure 39. Linear regression between dietary cobalt chloride supplementation and bioaccumulation of Fe in the muscle of advanced fry of *T.putitora*

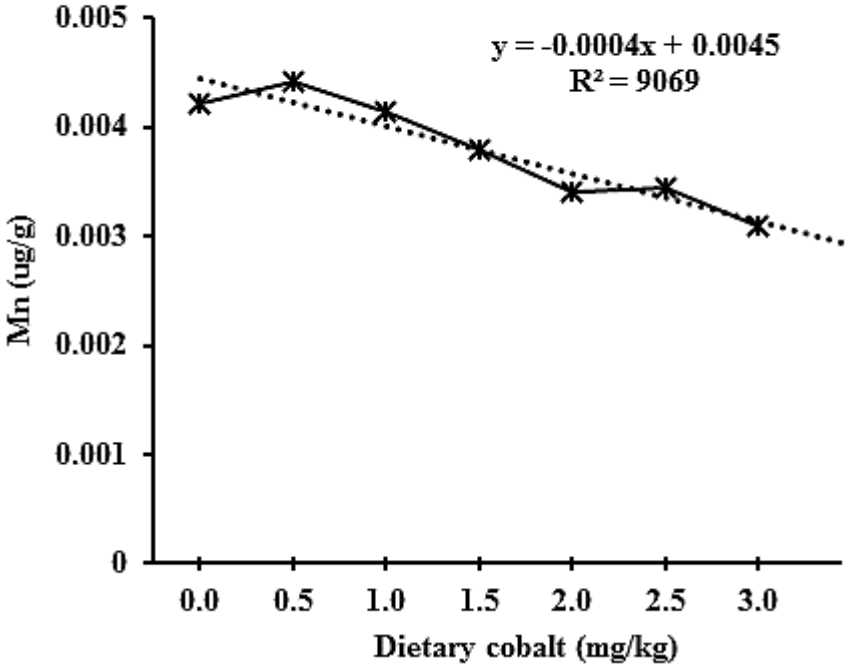

Figure 40. Linear regression between dietary cobalt chloride supplementation and bioaccumulation of Mn in the muscle of advanced fry of *T.putitora*

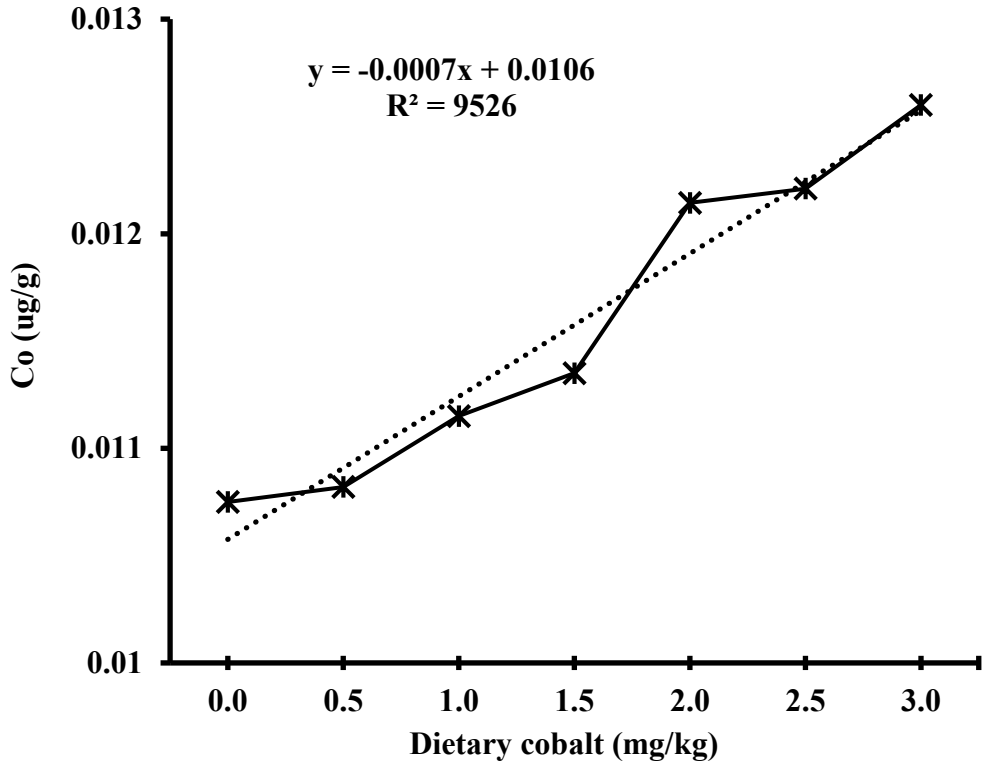

Figure 41. Linear regression between dietary cobalt chloride supplementation and Co bioaccumulation in the muscle of advanced fry of *T.putitora*

CHAPTER 2

Effect of cobalt supplemented diet on the expression of myoblast determination protein 1 homolog (MyoD) myogenin (MyoG) in muscle and HSP-70KDa in liver of *Tor putitora*

Expression of MyoD, MyoG, and HSP-70KDa

The cloning of MyoD, MyoG, and HSP-70KDa had given us the nucleotide sequence of these genes. Their cDNA sequences were then used to design primers to study their quantitative expression levels in muscle and liver of advanced fry of *T.putitora*. Results indicated significantly higher mRNA levels of MyoD and MyoG in the muscle of fish reared on a diet supplemented with 2mg/kg cobalt (group D) as compared to other groups of fish. However, at a higher supplementation level, i.e., 3mg/Kg diet (group F) showed the down-regulation of both genes (Fig. 42 and 43 respectively). Moreover, HSP-70KDa mRNA expression showed an increasing trend with an increase in the concentration of the dietary Co and, the highest expression was observed in a group of fish fed 3mg Co/kg diet (Fig.44).

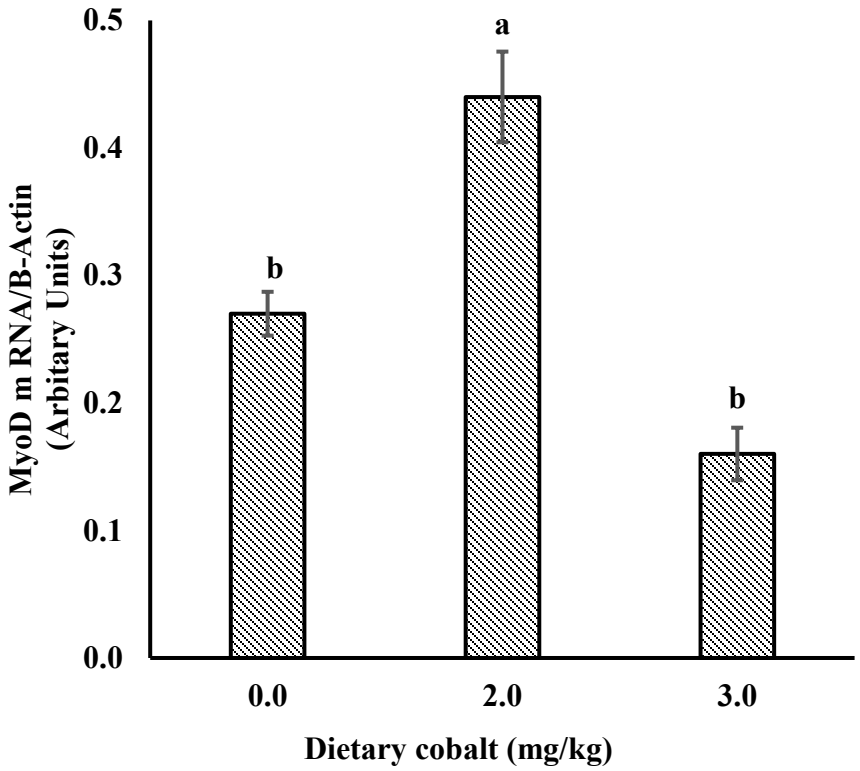

Figure 42. Expression of myoblast determination protein 1 homolog MyoD gene in the muscle of *T.putitora* advanced fry reared on graded level of dietary cobalt

Data is expressed as average value (n=4). Bars with different letters are significantly different ($P<0.05$). Statistical analysis was done by adopting One-way ANOVA followed by LSD.

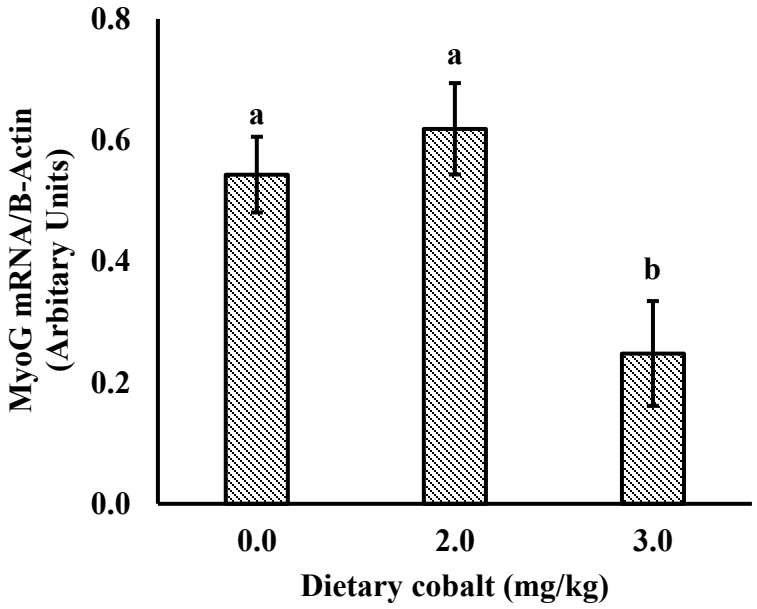

Figure 43. Expression of myogenin gene MyoG in the muscle of *T.putitora* advanced fry reared on graded level of dietary cobalt

Data is expressed as average value (n=4). Bars with different letters are significantly different ($P<0.05$). Statistical analysis was done by adopting One-way ANOVA followed by LSD.

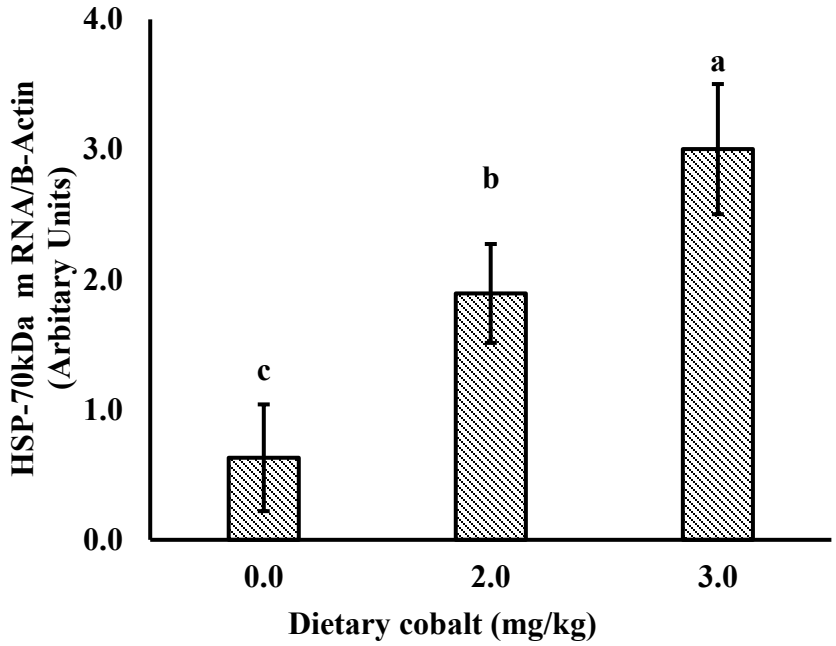

Figure 44. Expression of Heat shock protein 70 KDa in the liver of *T.putitora* advanced fry reared on graded level of dietary cobalt

Data is expressed as average value (n=4). Bars with different letters are significantly different ($P<0.05$). Statistical analysis was done by adopting One-way ANOVA followed by LSD.

CHAPTER 3

Comparative effects of different forms of dietary cobalt on growth performance, hemato-immunological indices, intestinal enzymes activities, and muscle composition of *T. putitora*

Phase I

Characterization Co-NPs and Co-Met complex

Physical properties

XRD spectrums of Co-NPs and Co-Met complex are shown in figures 45 and 46 respectively. XRD spectrum of Co-NPs showed peaks indicating the crystalline nature of Co-NPs with particle size <20nm. However, the XRD spectrum of the Co-Met complex did not show any peak which indicated the amorphous nature of the complex.

Size, shape and surface morphology

Scanning electron microscope images of Co-NPs indicated their spherical shape and smooth surface (Fig.47). The Co-Met complex appeared as a honeycomb-like structure with an average size of 82.69 nm (Fig.48).

Elemental composition of prepared particles

The EDS spectrums of Co-NPs and Co-Met complex are shown in figures 49 A and B respectively. Elemental analysis indicated 21% oxygen, 40.33 % chloride and 37% cobalt in Co-NPs (Fig. 50A). The Co-Met complex contained 31% carbon, 1.32% nitrogen, 48.53 % oxygen, 2.06% cobalt, and 0.31% chlorine (Fig. 50B).

Phase II

Experiment A

Effect of graded levels of Co-NPs supplemented diet on *T.putitora*

Growth performance

Dietary Co-NPs supplementation showed significant effect on the growth performance of the fish (Table 25).One-way ANOVA indicated significant differences in final body weight (n=3, F_6,14=40.48;PSE=0.065; P<0.001), %WG (n=3,$F_{6,14}$=50.31; PSE=0.921; P<0.001), %SGR (n=3, $F_{6,14}$=40.56;PSE=0.006;P<0.001), AWG (n=3,$F_{6,14}$=45.92;PSE=0.061;P<0.001) and ADG (n=3, $F_{6,14}$=55.53; PSE=0.006; P<0.001) among all diet groups of fish. Furthermore, pair-wise comparison showed the highest values of growth parameters (%WG, %SGR, AWG and ADG) in a N3 group followed by the N2 diet group. A positive correlation between the dietary Co-NPs and the percentage weight gain (i.e., R^2 = 0.985) was observed up to 1.5 mg Co/kg diet (group N3). However, further increase in Co-NPs supplementation negatively affected the %WG (R^2 = 0.946). Additionally, FCR also showed significant difference (n=3, $F_{6, 14}$=88.834; PSE=0.0021; P<0.001) among diet groups of fish. The pair-wise comparison showed the highest FCR in a N6 group (3mg/kg) while N3 group (1.5 mg/kg) showed the lowest value.

Hematological indices

Dietary Co-NPs supplementation showed significant effect on hematological indices of *T. putitora* fingerlings (Table 26). One-way ANOVA demonstrated significant differences in RBC count (n=9, $F_{6,56}$ 434.22; PSE= 0.052; P<0.001), % HCT (n=9, $F_{6,56}$ 127.25; PSE= 0.374; P<0.001)

and levels of Hb (n=9, $F_{6,56}$ 59.758; PSE= 0.182; P<0.001), MCV (n=9, $F_{6,56}$ 191.72; PSE= 0.077; P<0.001), MCH (n=9, $F_{6,56}$ 666.55; PSE= 0.093; P<0.001) and MCHC (n=9, $F_{6,56}$ 845.28; PSE= 0.283; P<0.001) among all diet groups of fish. The pair-wise comparison showed the highest values of hematological indices in a N3 group as compared to other diet groups. The Co-NPs supplementation up to 1.5mg /kg diet showed positive correlation with hematological indices of fish, i.e., RBC count (R^2=0.843) Hb (R^2=0.895), %HCT (R^2=0.885), MCV (R^2=0.708), MCH (R^2=0.992), MCHC (R^2=0.946). However, further increase in supplementation showed inverse correlation between dietary Co-NPs and RBC count (R^2=1), Hb (R^2=0.999), HCT (R^2=0.95), MCV (R^2=0.92), MCH (R^2=0.998), MCHC (R^2=0.91). All hematological parameters showed the lowest values in a N6 group of fish.

Furthermore, AST activity also showed significant difference (n=9, $F_{6,56}$=136.12; PSE= 0.0494; P=0.001) among all diet groups of fish. Initially, dietary Co-NPs up to 1.5 mg /kg diet did not show any significant effect on the AST activity however, further increase in Co-NPs supplementation showed the increasing trend (R^2=0.906) with the highest activity in a N6 group as compared to other diet groups (Fig. 51).

Immunological Indices

Dietary Co-NPs supplementation considerably affected the immunological indices of *T.putitora* fingerlings (Table 27). One-way ANOVA showed significant differences in plasma protein (n=9, $F_{6,56}$=110.89; PSE=0.190; P<0.001), IgM level (n=9, $F_{6,56}$=49.69; PSE=0.131; P<0.001), serum lysozyme activity (n=9, $F_{6,56}$=39.561; PSE=0.073; P<0.001), WBCs count (n=9,

$F_{6,56}$ =11106.33; PSE=0.484; P<0.001) and NBT activity (n=6,$F_{6,35}$=426.46; PSE=0.0044; P<0.001) among all diet groups of *T.putitora*.

The pair-wise comparison showed the highest values of immunological indices in a N3 group as compared to other diet groups. The positive correlation was observed between dietary Co-NPs up to 1.5mg /kg supplementation level and immunological parameters of fish, i.e., plasma protein (R^2=0.983), IgM level (R^2=0.950), lysozyme activity (R^2=0.881), WBC count (R^2=0.861) and NBT activity (R^2=0.937). However, further increase in Co-NPs supplementation negatively affected the fish health with the lowest values of immunological indices were observed in a N6 group of fish. Moreover, the N6 group of fish showed the highest mortality rate after challenge with *A. hydrophila,* i.e., 73.3% while the lowest mortality (33%) was observed in a N3 group of fish (Fig. 53).

Intestinal enzyme activities

Dietary Co-NPs supplementation showed a significant effect on the intestinal protease and amylase activities of *T. putitora* fingerlings (Table 28). One-way ANOVA showed significant differences in protease (n=9, $F_{6,56}$=114.05; PSE= 0.0049; P<0.001) and amylase activity (n=9, $F_{6,56}$=2.392; PSE=0.0198; P=0.04) among all diet groups of fish. Furthermore, pair-wise comparison indicated the highest protease and amylase activity in a N3 group of fish. However, the cellulase activity did not show any significant difference (ANOVA: n=9, $F_{6,56}$=1.748; PSE=0.002; P=0.117) among all Co-NPs supplemented diet groups. The pair-wise comparison indicated no effect of Co-NPs supplemented diet up to 1.5mg Co/kg (N3) on cellulase activity of

fish. However further increase in dietary Co-NPs negatively affected the intestinal cellulase activity with the lowest activity being observed in a N6 group of fish.

Muscle proximate composition of fish

Dietary Co-NPs supplementation demonstrated a significant effect on the muscle proximate composition of fish (Table 29). One-way ANOVA showed significant differences in muscle % crude protein (n=6, $F_{6,35}$=118.34; PSE=0.41; P<0.001), % crude fat (n=6, $F_{6,35}$=3.961; PSE=0.35; P=0.004) and % crude ash (n=6, $F_{6,35}$=87.68; PSE=0.261; P<0.001) among all diet groups of fish. The pair-wise comparison showed highest muscle (%) crude protein and (%) fat in a N3 group of fish. However, the highest muscle crude ash (%) was observed in a N6 group of fish.

Muscle metal bioaccumulation

Bioaccumulation of metal in the muscle of *T. putitora* fingerlings reared on graded level of dietary Co-NPs is shown in Table 30. One way ANOVA showed a significant difference in the muscle bioaccumulation of Fe (n=6, $F_{6,35}$=161.145; PSE= 0.0085; P<0.001), Mn (n=6, $F_{6,35}$=149.97; PSE= 0.0009; P<0.001), and Co (n=6, $F_{6,35}$= 24142; PSE= 0.0002; P<0.001) among all diet groups of fish. Furthermore dietary Co-NPs supplementation level demonstrated a positive correlation (R^2=1) with Co bio-accumulation, while a negative correlation with the bio-accumulation of Fe (R^2=0.975) and Mn (R^2=0.893) in the muscle of fish

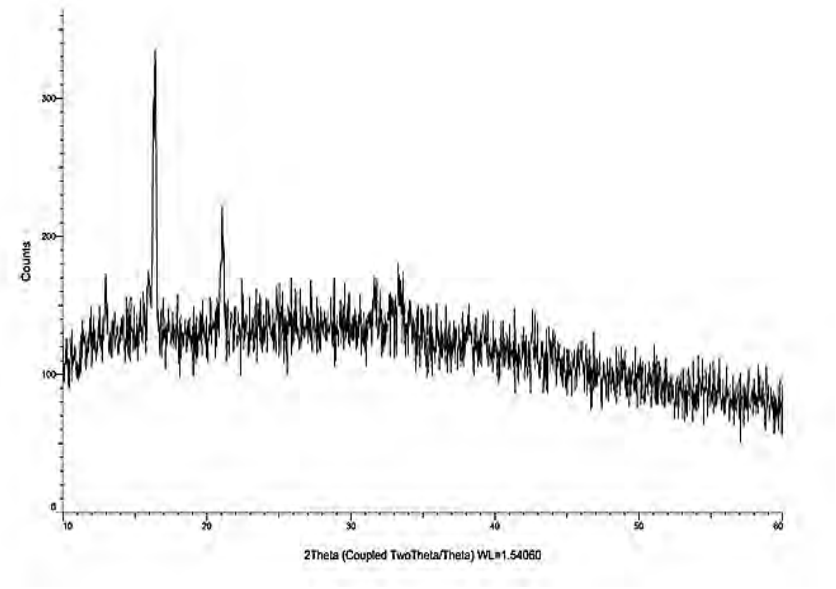

Figure 45. X-ray diffraction pattern of Co-NPs

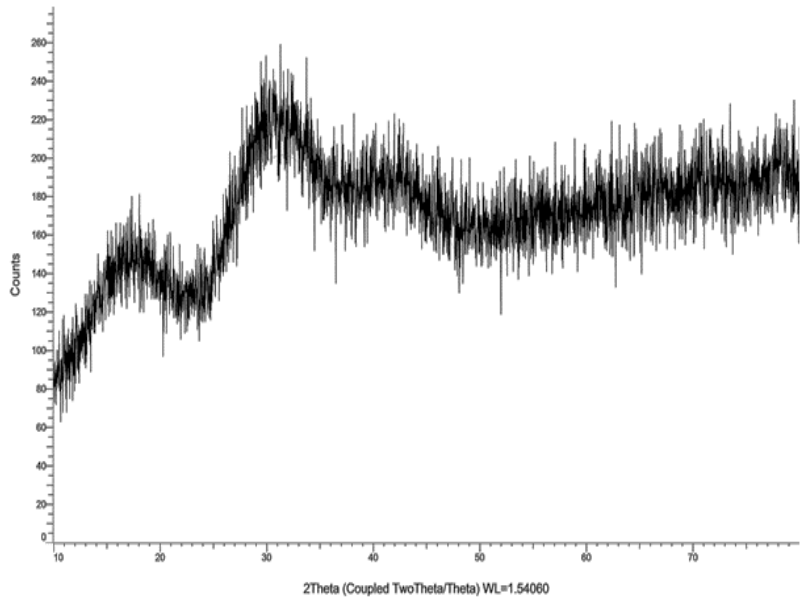

Figure 46. X-ray diffraction pattern of Co-Met complex

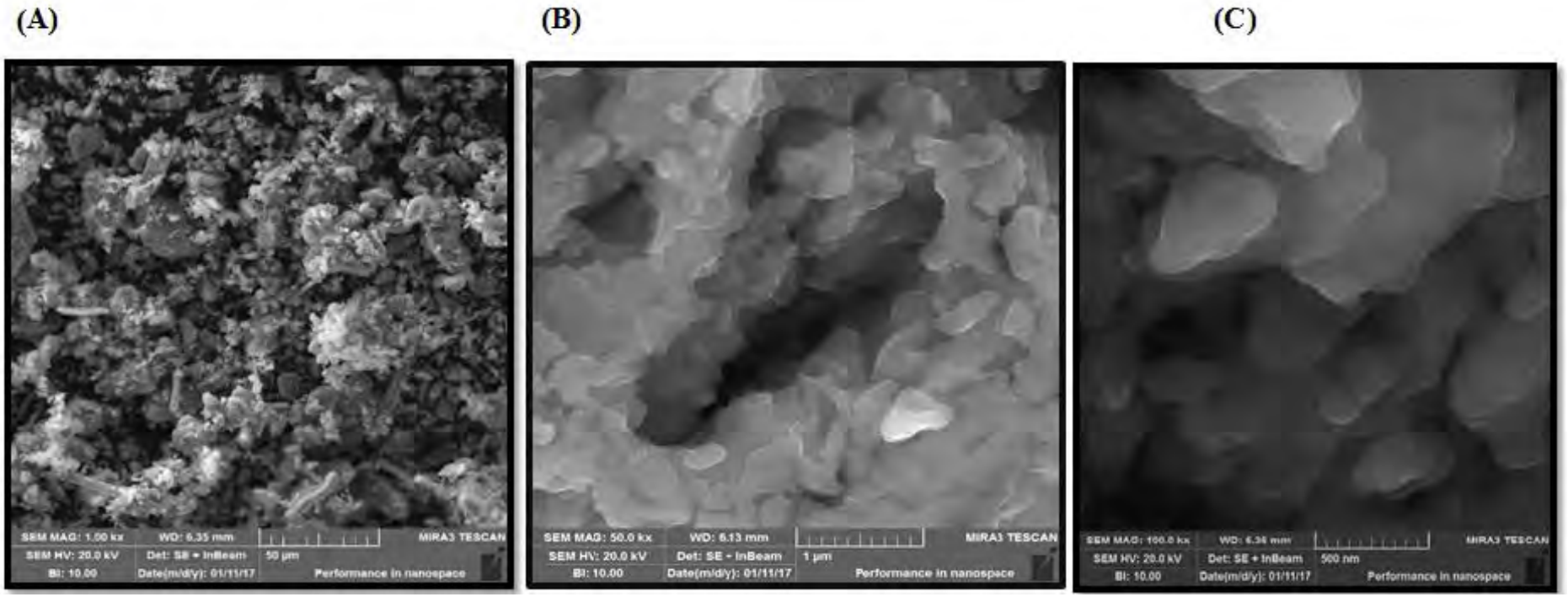

Figure 47. Scanning electron microscope image of Co-NPs; (a) 50µm, (b) 1µm, (c) 500nm

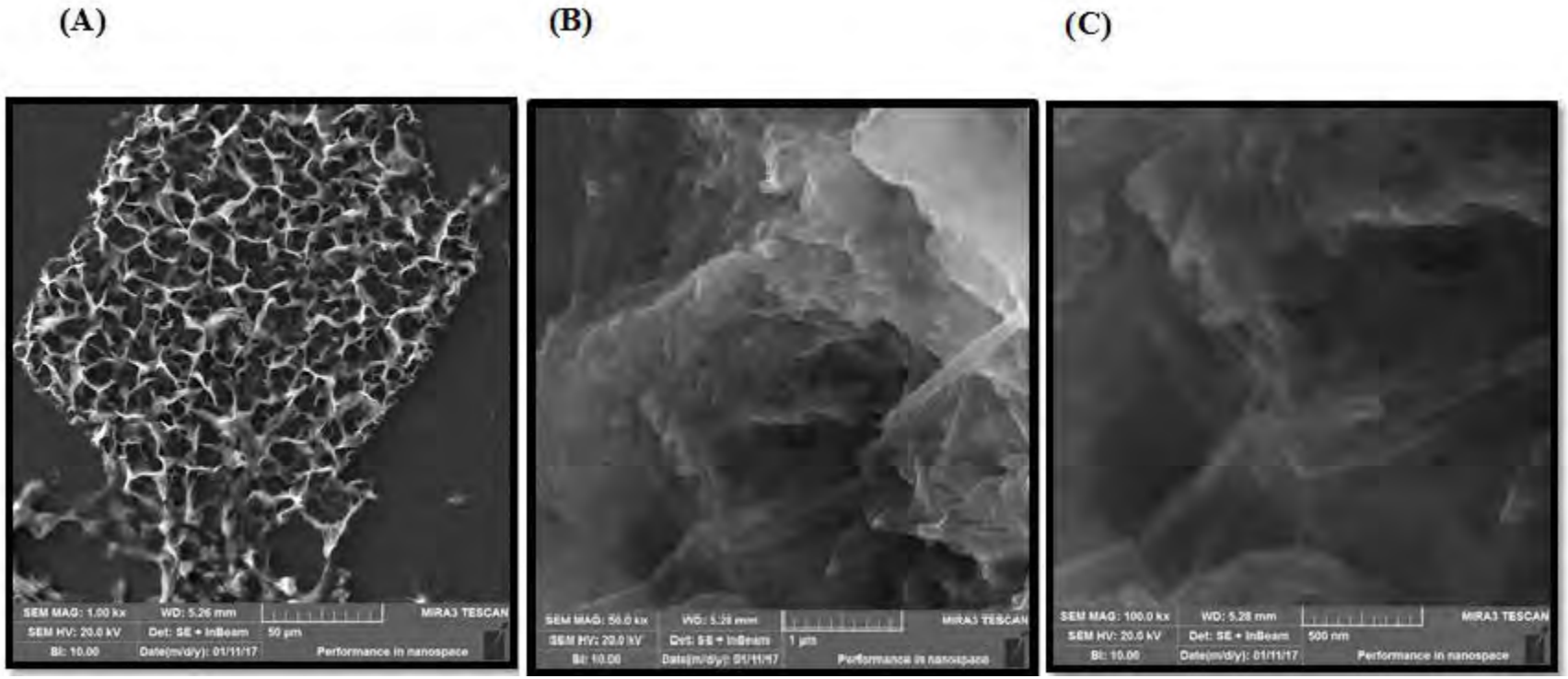

Figure 48. Scanning electron microscope image of Co-Met complex; (a) 50µm, (b) 1µm, (c) 500nm

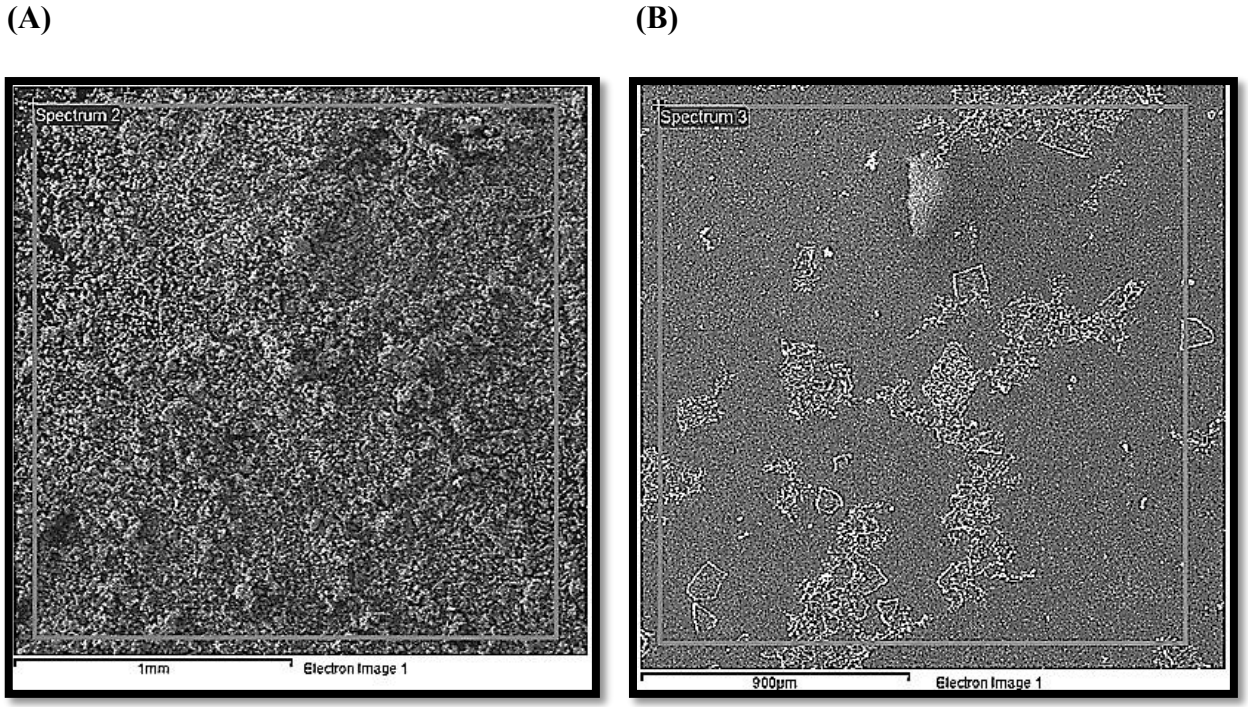

Figure 49. Energy Dispersal spectrum of (A) Co-NPs and (B) Co-Met complex

(A) **(B)**

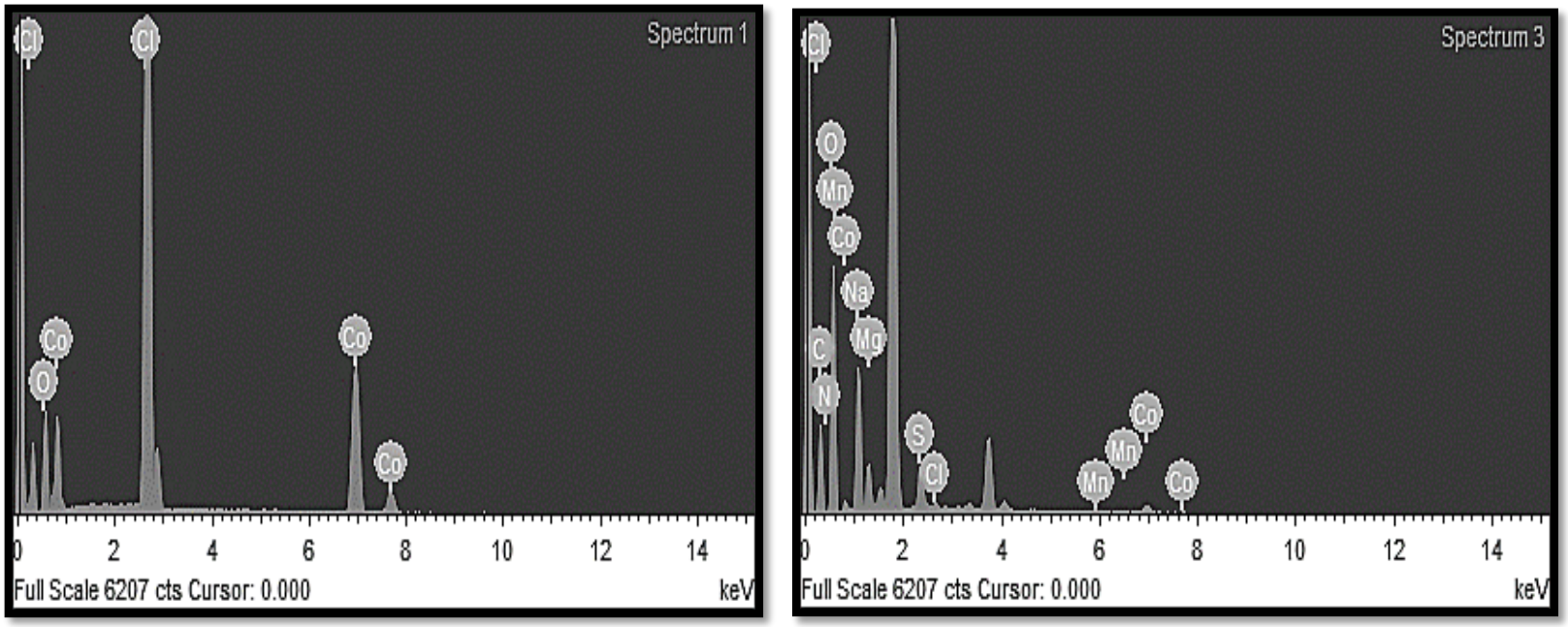

Figure 50. EDS spectrum showing elemental composition of (A) Co-NPs and (B) Co-Met complex

Table 25. Growth performance of *T.putitora* fingerlings after feeding graded levels of Co-NPs supplemented diets for 90 days

	Diet groups							Statistical Analysis		
	N0	**N1**	**N2**	**N3**	**N4**	**N5**	**N6**	**PSE [a]**	**P-value**	**F- value**
W_i (g)	6.36^{a}	6.359^{a}	6.356^{a}	6.364^{a}	6.354^{a}	6.351^{a}	6.37^{a}	0.007	0.270	1.434
W_f (g)	11.65^{c}	11.79^{b}	11.93^{ab}	12.01^{a}	11.46^{d}	11.42^{d}	11.2^{e}	0.065	0.001	40.48
WG (%)	83.16^{c}	85.38^{b}	87.52^{a}	88.78^{a}	80.15^{d}	79.5^{d}	76.05^{e}	0.921	0.001	50.31
AWG/fish (g)	5.29^{c}	5.43^{b}	5.57^{a}	5.65^{a}	5.12^{d}	5.06^{d}	4.84^{e}	0.061	0.001	45.92
ADG fish (g)	0.058^{c}	0.060^{b}	0.06^{a}	0.062^{a}	0.056^{d}	0.056^{d}	0.053^{e}	0.006	0.001	55.53
SGR(%body weight/day)	0.67^{c}	0.68^{b}	0.69^{a}	0.71^{a}	0.65^{d}	0.65^{d}	0.62^{e}	0.006	0.001	40.56
FCR	2.87^{c}	2.79^{d}	2.73^{de}	2.71^{e}	2.96^{b}	2.99^{b}	3.14^{a}	0.023	0.001	88.83

Data is expressed as average value (n=3). Values with different letters within the same rows are significantly different ($P<0.05$). Data was analyzed by adopting one-way ANOVA followed by LSD to determine the significant difference among diet groups of fish.

a=Pooled standard error mean; W_i= average initial weight of fish; W_f= average final weight of fish; %WG= percentage weight gain; AWG= average weight gain during 90 days/fish; ADG= average daily weight gain/fish;%SGR=specific growth rate (%body weight/day); FCR= feed conversion ratio

N0= Control group; N1= 0.5 mg Co-NPs/kg diet; N2= 1.0 mg Co-NPs /kg diet; N3= 1.5 mg Co-NPs/kg diet; N4= 2.0 mg Co-NPs/kg diet; N5= 2.5 mg Co-NPs/kg diet; N6= 3.0 mg Co-NPs/kg diet.

Table 26. Hematological indices of *T.putitora* fingerlings after feeding graded levels of Co-NPs supplemented diets for 90 days

	Diet groups							Statistical Analysis		
	N0	**N1**	**N2**	**N3**	**N4**	**N5**	**N6**	**PSE[a]**	**P-value**	**F-value**
RBC (10^6µ/L)	0.98^{c}	0.98^{c}	1.04^{b}	1.15^{a}	0.96^{d}	0.94^{e}	0.92^{f}	0.052	0.001	434.22
HB (g d/l)	6.25^{b}	6.33^{b}	6.54^{b}	6.99^{a}	5.68^{c}	5.01^{d}	4.43^{e}	0.182	0.001	59.758
HCT (%)	34.59^{c}	37.10^{b}	37.75^{ab}	38.47^{ab}	32.78^{d}	32.41^{d}	30.77^{e}	0.374	0.001	127.25
MCV (cm^3 erythrocyte)	151.54^{c}	170.65^{b}	173.59^{a}	173.69^{a}	138.78^{d}	134.74^{e}	110.79^{f}	0.077	0.001	191.72
MCH (pg)	30.60^{d}	30.96^{c}	31.56^{b}	32.06^{a}	29.66^{e}	28.55^{f}	27.36^{g}	0.093	0.001	666.55
MCHC (Hb/100 ml blood)	41.74^{d}	47.97^{c}	49.25^{b}	53.95^{a}	41.23^{d}	39.10^{e}	38.81^{e}	0.283	0.001	845.28

Data is expressed as average value (n=9). Values with different letters within the same rows are significantly different (P<0.05). Data was analyzed by adopting one-way ANOVA followed by LSD test to determine the significant difference among diet groups.

a=Pooled standard error mean; RBC= red blood cell count; HB= Hemoglobin level; HCT= hematocrit; MCV=mean corpuscular volume; MCH= mean corpuscular hemoglobin; MCHC= corpuscular hemoglobin concentration

N0= Control group; N1= 0.5 mg Co-NPs/kg diet; N2= 1.0 mg Co-NPs /kg diet; N3= 1.5 mg Co-NPs/kg diet; N4= 2.0 mg Co-NPs/kg diet; N5= 2.5 mg Co-NPs/kg diet; N6= 3.0 mg Co-NPs/kg diet.

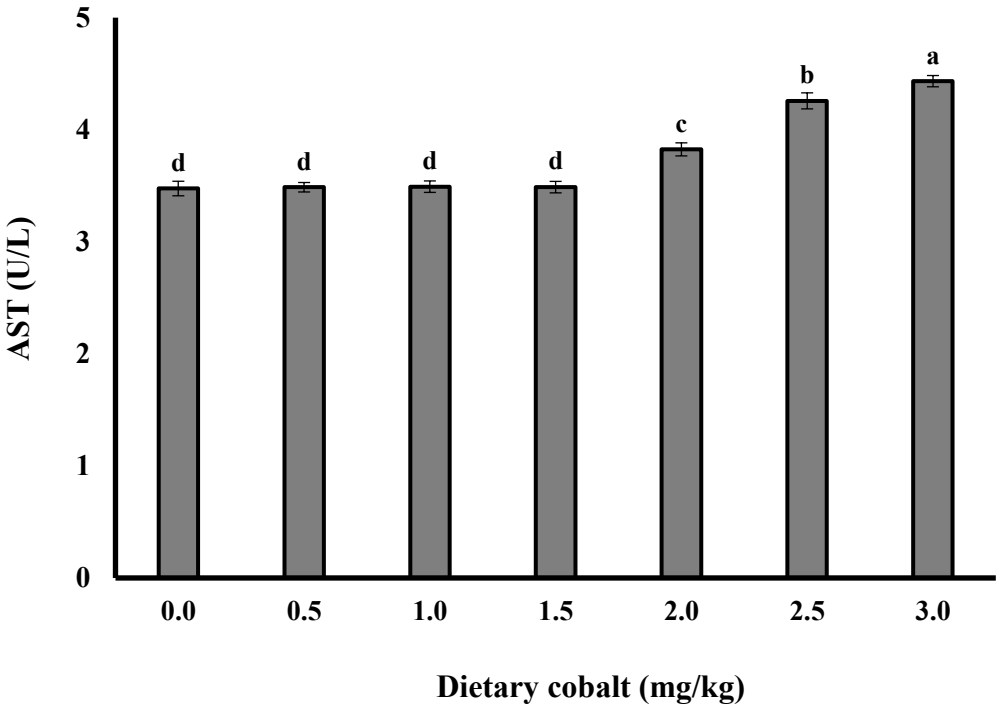

Figure 51. Serum aspartate aminotransferase activity (U/L) of *T.putitora* fingerlings after feeding graded levels of Co-NPs supplemented diets for 90 days

Bars shows the values as average± SE, n = 9. Different letters on bars shows significant difference. Data was analyzed by adopting one-way ANOVA followed by LSD test to determine the significant difference among diet groups.

Table 27. Immunological indices of *T.putitora* fingerlings after feeding graded levels of Co-NPs supplemented diets for 90 days

	Diet Groups							Statistical Analysis		
	N0	**N1**	**N2**	**N3**	**N4**	**N5**	**N6**	**SEM**	**P-value**	**F-value**
plasma protein (mg/ml)	11.93^{d}	13.13^{c}	13.68^{b}	14.75^{a}	11.65^{de}	11.37^{e}	10.80^{f}	0.190	0.001	110.89
IgM (mg/ml)	3.77^{c}	4.11^{b}	4.50^{a}	4.59^{a}	3.45^{d}	3.17^{e}	2.87^{f}	0.131	0.001	49.69
lysozyme activity (µg/ml)	1.92^{e}	2.52^{c}	2.68^{b}	2.84^{a}	2.37^{c}	2.20^{d}	2.15^{d}	0.073	0.001	39.561
WBC (10^3 µ/L)	165.11^{f}	169.50^{e}	169.87^{e}	171.61^{d}	220.97^{c}	238.90^{b}	245.52^{a}	0.484	0.001	11106.33

Data is expressed as average value (n=9). Values with different letters within the same rows are different significantly (P<0.05). Data was analyzed by adopting one-way ANOVA followed by LSD test to determine the significant difference among diet groups.

[a]=Pooled standard error mean

N0= Control group; N1= 0.5 mg Co-NPs/kg diet; N2= 1.0 mg Co-NPs /kg diet; N3= 1.5 mg Co-NPs/kg diet; N4= 2.0 mg Co-NPs/kg diet; N5= 2.5 mg Co-NPs/kg diet; N6= 3.0 mg Co-NPs/kg diet.

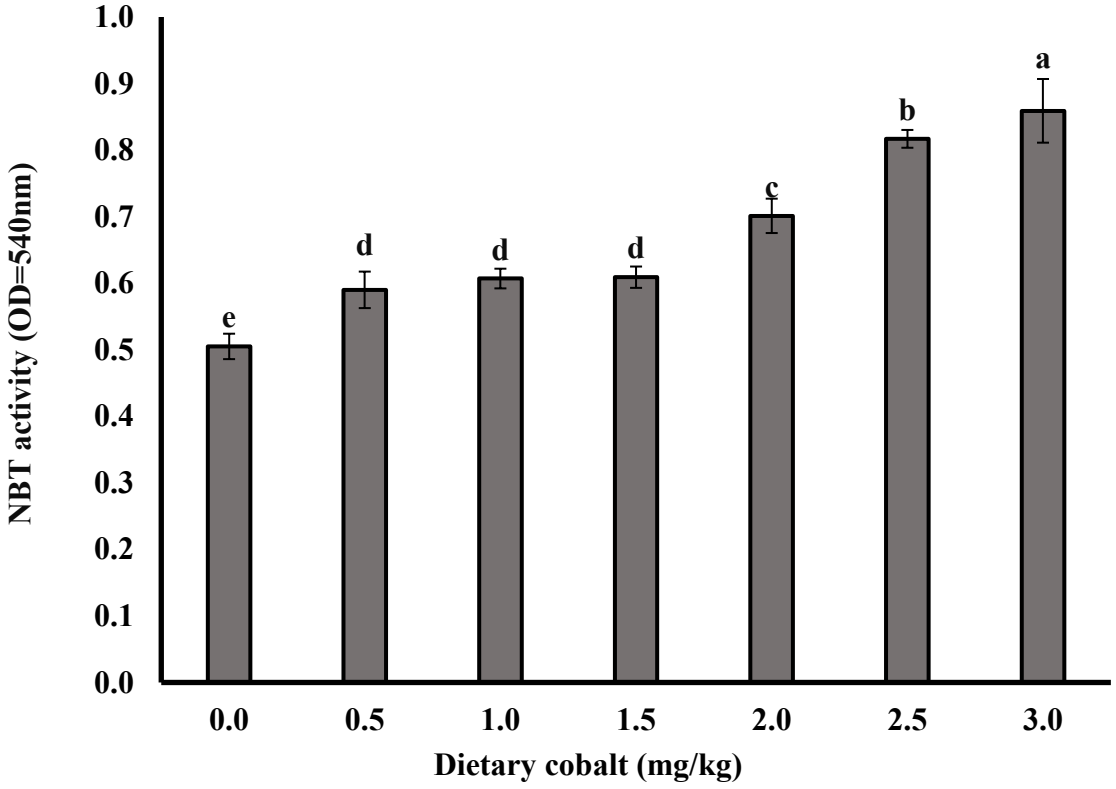

Figure 52. NBT (Nitro-blue tetrazolium) activity of *T.putitora* fingerlings after feeding graded levels of Co-NPs supplemented diets for 90 days

Bars shows the values as average ± SE, n = 6. Different letters on bars shows significant difference among Co-NPs fed diet groups. Data was analyzed by adopting one-way ANOVA followed by LSD test to determine the significant difference among diet groups.

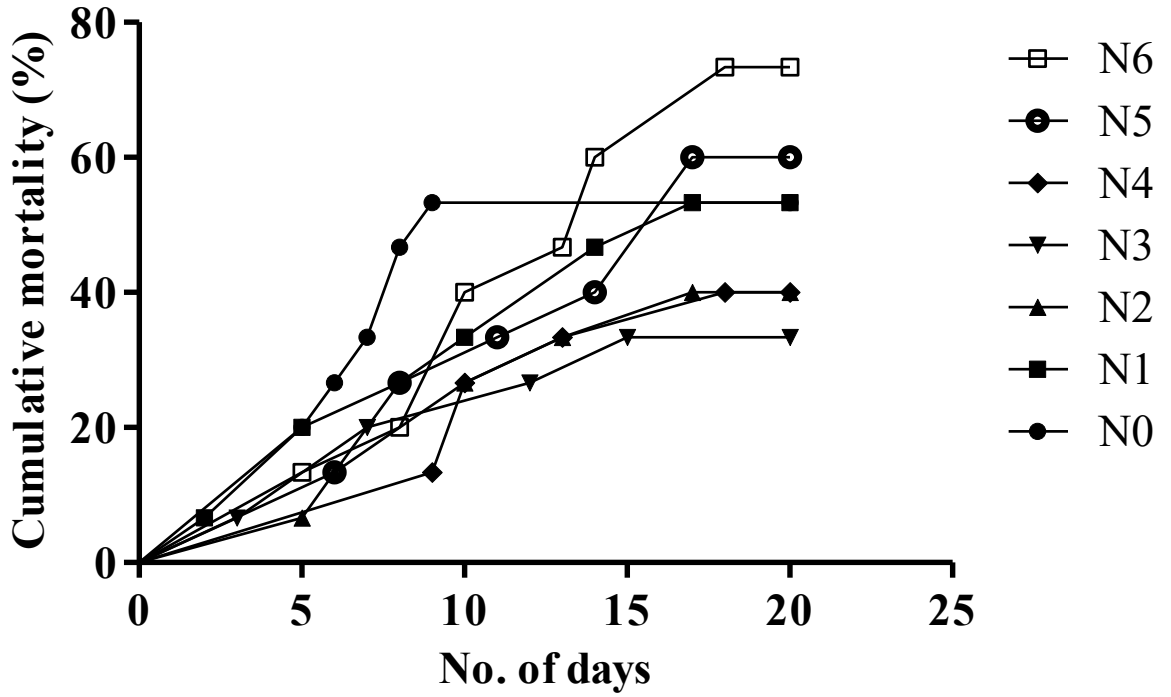

Figure 53. Cumulative mortality (%) after challenge with *A. hydrophila* in different groups of fish reared on Co-NPs supplemented diet

N0= Control group; N1= 0.5 mg Co-NPs/kg diet; N2= 1.0 mg Co-NPs /kg diet; N3= 1.5 mg Co-NPs/kg diet; N4= 2.0 mg Co-NPs/kg diet; N5= 2.5 mg Co-NPs/kg diet; N6= 3 mg Co-NPs/kg diet.

Table 28. Intestinal enzyme activities of *T.putitora* fingerlings after feeding graded levels of Co-NPs supplemented diets for 90 days

	Diet Groups							Statistical Analysis		
	N0	**N1**	**N2**	**N3**	**N4**	**N5**	**N6**	**SEM**	**P-value**	**F-value**
Amylase (U/mg)	0.076^{ab}	0.067^{abc}	0.100^{a}	0.079^{ab}	0.090^{ab}	0.056^{bc}	0.036^{c}	0.0198	0.04	2.392
Protease (U/mg)	0.153^{c}	0.160^{bc}	0.165^{b}	0.183^{a}	0.107^{d}	0.098^{de}	0.091^{e}	0.0049	0.0001	114.05
Cellulase (U/mg)	0.181^{a}	0.183^{a}	0.182^{a}	0.177^{a}	0.165^{b}	0.169^{b}	0.154^{c}	0.0206	0.117	1.748

Data is expressed as average value (n=9). Values with different letters within the same rows are significantly different ($P<0.05$). Data was analyzed by adopting one-way ANOVA followed by LSD test to determine the significant difference among diet groups.

[a]=Pooled standard error mean

N0= Control group; N1= 0.5 mg Co-NPs/kg diet; N2= 1.0 mg Co-NPs /kg diet; N3= 1.5 mg Co-NPs/kg diet; N4= 2.0 mg Co-NPs/kg diet; N5= 2.5 mg Co-NPs/kg diet; N6= 3.0 mg Co-NPs/kg diet

Table 29. Muscle proximate composition of *T.putitora* fingerlings after feeding graded levels of Co-NPs supplemented diets for 90 days

	Diet Groups							Statistical Analysis		
	N0	**N1**	**N2**	**N3**	**N4**	**N5**	**N6**	**PSE[a]**	**P-value**	**F-value**
Crude protein (%)	65.20^{c}	67.16^{b}	68.79^{a}	68.83^{a}	63.95^{d}	61.94^{e}	60.87^{f}	0.41	0.001	118.3
Crude fat (%)	6.015^{a}	6.11^{bc}	6.79^{ab}	7.16^{a}	6.78^{ab}	6.02^{c}	5.94^{c}	0.35	0.004	3.961
Crude ash (%)	9.566^{e}	11.34^{d}	11.47^{d}	11.82^{cd}	12.15^{c}	12.72^{b}	15.39^{a}	0.26	0.001	87.68

Data is expressed as average value (n=6). Values with different letters within the same rows are significantly different (P<0.05). Data was analyzed by adopting one-way ANOVA followed by LSD test to determine the significant difference among diet groups.

[a]=Pooled standard error mean

N0= Control group; N1= 0.5 mg Co-NPs/kg diet; N2= 1.0 mg Co-NPs /kg diet; N3= 1.5 mg Co-NPs/kg diet; N4= 2.0 mg Co-NPs/kg diet; N5= 2.5 mg Co-NPs/kg diet; N6= 3.0 mg Co-NPs/kg diet.

Table 30. Bioaccumulation of metals in the muscle of *T. putitora* fingerlings after feeding graded levels of Co-NPs supplemented diets for 90 days

Metal concentration	Diet Groups							Statistical Analysis		
(µg/g of tissue)	N0	N1	N2	N3	N4	N5	N6	SEM	P-value	F-value
Iron	0.064[a]	0.062[b]	0.058[c]	0.057[c]	0.052[d]	0.048[e]	0.043[f]	0.0085	0.001	161.145
Manganese	0.003[a]	0.003[a]	0.002[b]	0.002[c]	0.002[c]	0.001[d]	0.001[e]	0.0009	0.001	149.975
Cobalt	0.014[g]	0.015[f]	0.016[e]	0.017[d]	0.018[c]	0.019[b]	0.02[a]	0.0002	0.001	24142.7

Data is expressed as average value (n=6). Values with different letters within the same rows are significantly different (P<0.05). Data was analyzed by adopting one-way ANOVA followed by LSD test to determine the significant difference among diet groups of fish.

[a]=Pooled standard error mean

N0= Control group; N1= 0.5 mg Co-NPs/kg diet; N2= 1.0 mg Co-NPs /kg diet; N3= 1.5 mg Co-NPs/kg diet; N4= 2.0 mg Co-NPs/kg diet; N5= 2.5 mg Co-NPs/kg diet; N6= 3.0 mg Co-NPs/kg diet.

Phase II

Experiment B

Effect of graded levels of Co-Met complex supplemented diet on *T.putitora*

Growth performance

Dietary Co-Met complex supplementation showed significant effect on the growth performance of the fish (Table 31). One-way ANOVA indicated significant differences in final body weight (n=3, $F_{6,14}$=5043.39; PSE=0.183; P<0.001), %WG(n=3, $F_{6,14}$=2602.29;PSE=0.406; P<0.001), %SGR (n=3, $F_{6,14}$=28.077; PSE= 0.020; P<0.001), AWG(n=3, $F_{6,\ 14}$=4291.79;PSE= 0.02; P<0.Z001) and ADG (n=3, $F_{6,14}$ =2228.11; PSE= 0.003; P<0.001) among all diet groups of fish. Furthermore, pair-wise comparison showed the highest %WG in a M6 group of fish. Moreover, linear regression of data showed a significant increase in %WG (R^2=0.966), AWG (R^2=0.966), ADG (R^2=0.967) and %SGR (R^2 = 0.937) with an increasing Co-Met complex intake. In addition to that, significant difference was also observed in the feed conversion ratio (n=3, $F_{6,\ 14}$=614.53; PSE=0.021; P<0.001) among all diet groups of fish. Here, FCR showed an inverse relationship, i.e., FCR decreased with an increase in Co-Met complex intake (R^2 = 0.978).

Hematological indices

Dietary Co-Met complex supplementation showed significant effect on hematological indices of *T. putitora* fingerlings in a dose dependant manner (Table 32). One-way ANOVA showed significant differences in RBC count (n=9, $F_{6,56\ =}$590.65; PSE=0.179; P<0.001), Hb level (n=9, $F_{6,56}$ =533.13; PSE=0.122; P<0.001), %HCT (n=9, $F_{6,56}$=135.34; PSE=0.250; P<0.001),

MCV (n=9, $F_{6, 56}$=581.7; PSE=0.126; P<0.001), MCH (n=9, $F_{6,56}$=170.15; PSE=0.174; P=0.001) and MCHC (n=9, $F_{6, 56}$=151.55; PSE=0.467; P=0.001) among all Co-Met supplemented diet groups of fish. The pair-wise comparison showed the highest values of hematological indices in a M6 group followed by a M5 group of fish. Additionally, the positive correlation was observed between the dietary Co-Met complex supplementation level and the RBC count (R^2 = 0.964), Hb (R^2 = 0.983), %HCT (R^2 = 0.9911), MCH (R^2 = 0.935) and MCHC (R^2 = 0.93). Furthermore, significant difference was observed in serum AST activity of *T.putitora* fingerlings reared on diet supplemented with different levels of Co-Met complex (n=9, $F_{6,56}$=79.79; PSE=0.047; P<0.001).The pairwise comparison showed the lowest serum AST activity in a M6 group of fish (Fig.54). Moreover, an inverse relationship was observed between Co-Met complex supplementation level and serum AST activity (R^2 = 0.906).

Immunological Indices

Dietary Co-Met complex supplemented diets showed significant effect on the immunological indices of *T.putitora* fingerlings (Table 33). One-way ANOVA showed significant differences in plasma protein (n=9, $F_{6,56}$ =33.77; PSE=0.284; P<0.001), IgM level (n=9, $F_{6,56}$=34.44; PSE=0.194; P<0.001), lysozyme activity (n=9, $F_{6,56}$=22.93; PSE=0.211; P<0.001), NBT activity (n=6, $F_{6,35}$=76.63; PSE=0.035; P<0.001) and WBC count (n=9, $F_{6, 63}$ =8.119; PSE=0.345; P<0.001) among all diet groups of fish. The pair-wise comparison showed the highest values of immunological indices in a M6 group of fish as compared to other diet groups. Moreover, no mortality was observed in a M6 group of fish after challenged with pathogenic bacteria, *A. hydrophila* (Fig.56).

Intestinal enzyme activities

Dietary Co-Met complex supplementation also showed a significant effect on the intestinal enzyme activities of all diet groups of *T. putitora* fingerlings (Table 34).One-way ANOVA showed significant differences in the activity of amylase (n=9, $F_{6,56}$=158.41; PSE=0.0048; P<0. 0001), protease (n=9, $F_{6,56}$=75.97; PSE=0.0024; P<0.0001) and cellulase (n=9, $F_{6,56}$= 635.74; PSE= 0.0032; P<0001) among all diet groups of fish. Pairwise comparison showed the highest activity of intestinal enzymes in a M6 group of fish. The linear regression analysis data indicated an increase in the activities of intestinal enzymes amylase (R^2 = 0.985), protease (R^2 = 0.842), and cellulase (R^2 = 0.943) with an increase in the dietary Co-Met complex supplementation level.

Muscle proximate composition of fish

Dietary Co-Met complex supplemented diets also showed a significant effect on the proximate composition of muscle (Table 35). Statistical analysis done by using one-way ANOVA showed significant difference in muscle % crude protein (n=6, $F_{6,35}$=215.87; PSE=0.43; P<0.001) and % crude fat (n=6, $F_{6,35}$=132.66; PSE=0.31; P<0.001) and % crude ash (n=6, $F_{6,35}$=31.11; PSE=0.239; P<0.001) among all diet group of fish. Pairwise comparison showed the highest %crude protein and % fat in a M6 group of fish as compared to other diet groups.

Muscle metal bioaccumulation

Bioaccumulation of metal in the muscle tissues of *T. putitora* fingerlings reared on graded level of dietary Co-Met complex is shown in Table 36. Statistical analysis done by using one way ANOVA showed non-significant difference in the muscle bioaccumulation of Fe (n=6,

$F_{6,35}$=0.222; PSE=0.001; P=0.963), Mn (n=6, $F_{6,35}$= 2.15; PSE=0.001; P= 0.112) and Co (n=6, $F_{6,35}$= 3.545; PSE= 0.001; P=0.024) among all diet groups of fish.

Table.31. Growth performance of *T. putitora* fingerlings after feeding graded levels of Co-Met complex supplemented diets for 90 days

	Diet groups							Statistical Analysis		
	M0	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**	**PSE [a]**	**P-value**	**F- value**
W_i (g)	6.38	6.37	6.38	6.37	6.35	6.34	6.36	0.009	0.256	1.678
W_f (g)	11.68^{f}	12.25^{e}	12.53^{d}	13.21^{c}	13.61^{b}	14.01^{a}	14.02^{a}	0.183	0.001	5043.39
WG (%)	83.20^{f}	92.381^{e}	96.43^{d}	107.32^{c}	114.40^{b}	120.59^{a}	120.68^{a}	0.406	0.001	2602.29
AWG/fish (g)	5.30^{f}	5.88^{e}	6.15^{d}	6.84^{c}	7.26^{b}	7.66^{a}	7.66^{a}	0.020	0.001	4291.79
ADG fish (g)	0.059^{f}	0.065^{e}	0.068^{d}	0.076^{c}	0.080^{b}	0.085^{a}	0.085^{a}	0.003	0.001	2228.11
SGR (%body weight/day)	0.67^{b}	0.727^{c}	0.750^{c}	0.810^{b}	0.80^{b}	0.879^{a}	0.87^{a}	0.020	0.001	28.077
FCR	2.86^{c}	2.610^{b}	2.50^{c}	2.294^{d}	2.08^{e}	1.965^{f}	1.90^{f}	0.021	0.001	614.53

Data is expressed as average value (n=3). Values with different letters within the same rows are significantly different ($P<0.05$). Data was analyzed by adopting one-way ANOVA followed by LSD to determine the significant difference among diet groups of fish.

a=Pooled standard error mean; W_i= average initial weight of fish; W_f= average final weight of fish; %WG= percentage weight gain; AWG= Average weight gain during 90 days/fish; ADG= Average daily weight gain/fish; %SGR=Specific growth rate (%body weight/day); FCR= Feed conversion ratio

M0= Control group; M1= 0.5 mg Co-Met/kg diet; M2= 1.0 mg Co-Met /kg diet; M3= 1.5 mg Co-Met /kg diet; M4= 2.0 mg Co-Met/kg diet; M5= 2.5 mg Co-Met/kg diet; M6= 3.0 mg Co-Met/kg diet.

Table.32. Hematological indices of *T.putitora* fingerlings after feeding graded levels of Co-Met complex supplemented diets for 90 days

	Diet groups							Statistical Analysis		
	M0	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**	**PSE**	**P-value**	**F-value**
RBC (10^6µ/L)	0.96^{g}	1.05^{f}	1.11^{e}	1.15^{d}	1.18^{c}	1.27^{b}	1.39^{a}	0.179	0.001	590.65
HB (g d/l)	6.23^{g}	6.50^{f}	6.58^{e}	6.81^{d}	6.88^{c}	7.17^{b}	7.28^{a}	0.122	0.001	533.13
HCT (%)	34.53^{g}	35.39^{f}	36.75^{e}	37.74^{d}	38.57^{c}	39.15^{b}	40.35^{a}	0.250	0.001	135.34
MCV (cm^3 erythrocyte)	151.56^{g}	168.15^{f}	168.86^{e}	169.46^{d}	169.83^{c}	170.24^{b}	170.50^{a}	0.126	0.001	581.7
MCH (pg)	30.66^{g}	38.85^{f}	39.87^{e}	43.58^{d}	45.82^{c}	49.84^{b}	50.07^{a}	0.174	0.001	170.15
MCHC (Hb/100 ml blood)	41.70^{f}	42.40^{e}	43.57^{e}	45.56^{d}	47.21^{c}	48.55^{b}	53.35^{a}	0.467	0.001	151.55

Data is expressed as average value (n=9). Values with different letters within the same rows are significantly different ($P<0.05$). Data was analyzed by adopting one-way ANOVA followed by LSD test to determine the significant difference among all diet groups.

a=Pooled standard error mean; RBC= red blood cell count; HB= Hemoglobin level; HCT= hematocrit; MCV=mean corpuscular volume; MCH=mean corpuscular hemoglobin; MCHC= corpuscular hemoglobin concentration

M0= Control group; M1= 0.5 mg Co-Met/kg diet; M2= 1.0 mg Co-Met /kg diet; M3= 1.5 mg Co-Met /kg diet; M4= 2.0 mg Co-Met/kg diet; M5= 2.5 mg Co-Met/kg diet; M6= 3.0 mg Co-Met/kg diet.

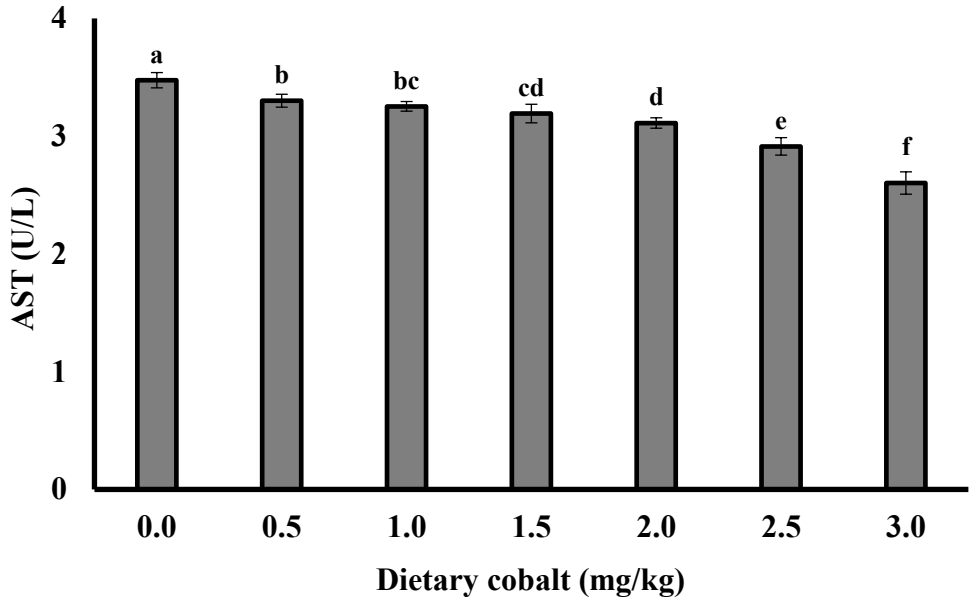

Figure 54. Serum aspartate aminotransferase activity (U/L) of *T.putitora* fingerlings after feeding graded levels of Co-Met complex supplemented diets for 90 days

Bars shows the values as average± SE, n = 9. Different letters on bars shows significant difference. Data was analyzed by adopting one-way ANOVA followed by LSD test to determine the significant difference among diet groups.

Table 33. Immunological indices of *T.putitora* fingerlings after feeding graded levels of Co-Met complex supplemented diets for 90 days

	Diet Groups							Statistical Analysis	
	M0	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**	**SEM**	**F-value**	**P-value**
plasma protein (mg/ml)	11.91^{d}	13.05^{c}	13.21^{c}	14.20^{b}	14.53^{b}	14.59^{b}	15.39^{a}	0.284	33.77	0.001
IgM (mg/ml)	3.75^{e}	4.02e	4.61^{d}	4.96^{cd}	5.31^{bc}	5.64^{ab}	5.91^{a}	0.194	34.44	0.001
lysozyme activity (µg/ml)	1.91^{c}	3.02^{b}	3.20^{b}	3.33^{b}	3.44^{b}	3.84^{a}	4.14^{a}	0.211	22.93	0.001
WBC (10^3 µ/L)	164.13^{a}	164.99^{a}	165.10^{a}	164.62^{ab}	164.11^{bc}	163.83^{cd}	163.35^{d}	0.345	8.119	0.001

Data is expressed as average value (n=9). Values with different letters within the same rows are significantly different (P<0.05). Data was analyzed by adopting one-way ANOVA followed by LSD test to determine the significant difference among diet groups.

[a]=Pooled standard error mean

M0= Control group; M1= 0.5 mg Co-Met/kg diet; M2= 1.0 mg Co-Met /kg diet; M3= 1.5 mg Co-Met /kg diet; M4= 2.0 mg Co-Met/kg diet; M5= 2.5 mg Co-Met/kg diet; M6= 3.0 mg Co-Met/kg diet.

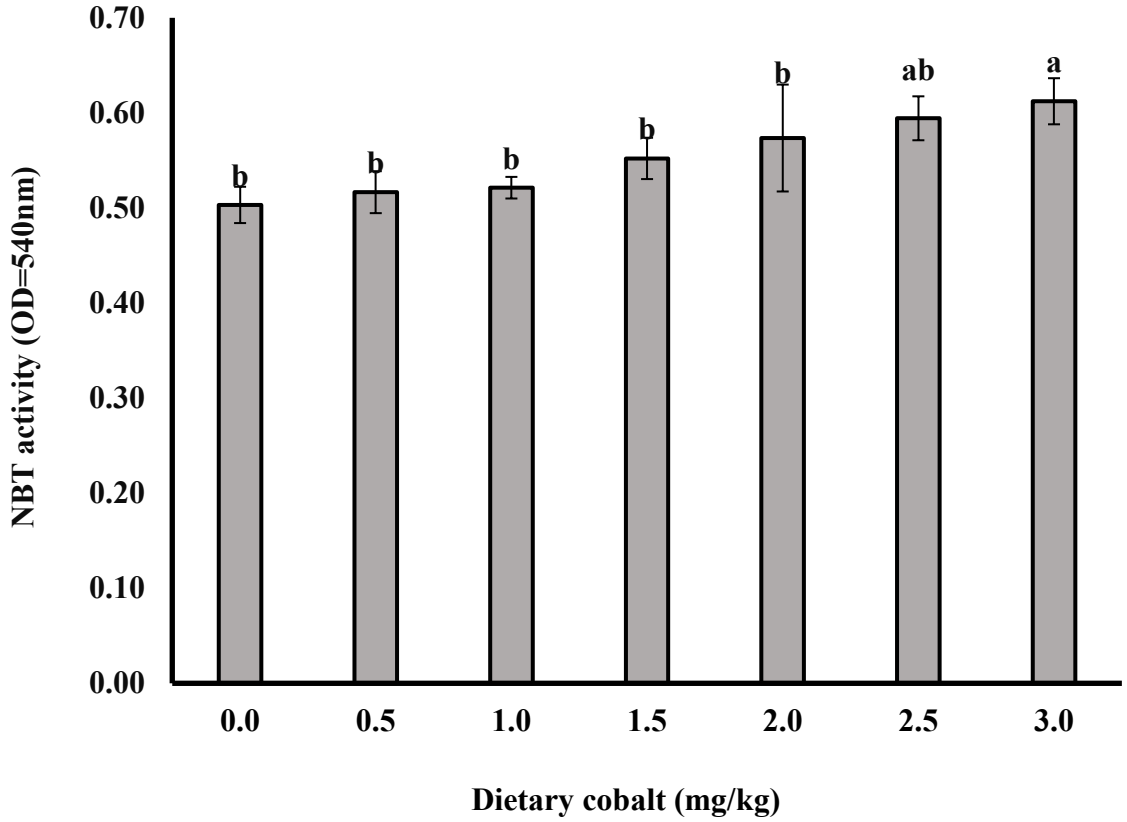

Figure 55. NBT activity of *T.putitora* fingerlings after feeding graded levels of Co-Met complex supplemented diets for 90 days

Bars shows the values as average ± SE, n = 6. Different letters on bars shows significant difference among Co-Met fed diet groups. Data was analyzed by adopting one-way ANOVA followed by LSD test to determine the significant difference among diet groups.

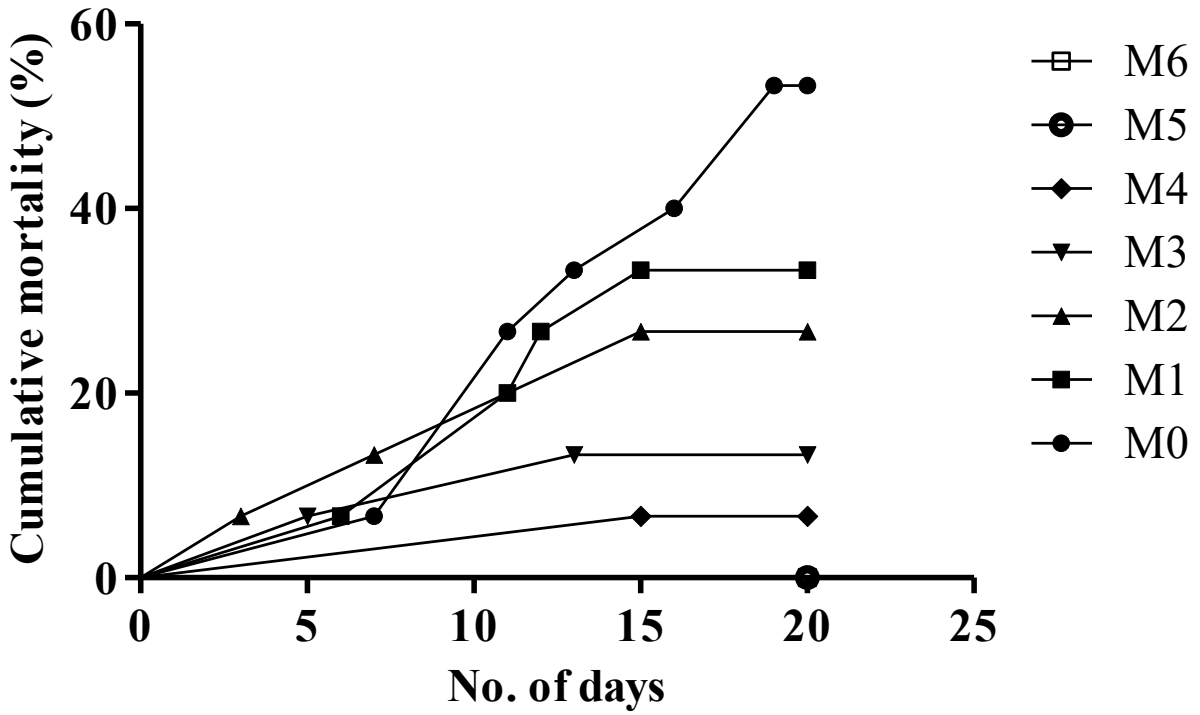

Figure 56. Cumulative mortality (%) after challenge with *A. hydrophila* in different groups of fish reared on Co-Met complex supplemented diet

M0= Control group; M1= 0.5 mg Co/kg diet; M2=1.0 mg Co/kg diet; M3= 1.5 mg Co/kg diet; M4=2.0 mg Co /kg diet; M5=2.5 mg Co /kg diet; M6=3.0 mg Co /kg diet.

Table 34. Intestinal enzyme activities of *T.putitora* fingerlings after feeding graded levels of Co-Met complex supplemented diets for 90 days

	Diet Groups							Statistical Analysis		
	M0	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**	**SEM[a]**	**F-value**	**P-value**
Amylase (U/ mg)	0.076^{g}	0.101^{f}	0.112^{e}	0.148^{d}	0.162^{c}	0.176^{b}	0.195^{a}	0.0048	158.41	0.001
Protease (U/mg)	0.153^{d}	0.153^{d}	0.168^{c}	0.168^{c}	0.173^{bc}	0.173^{b}	0.197^{a}	0.0024	75.97	0.001
Cellulase (U/mg)	0.181^{f}	0.242^{e}	0.250^{e}	0.272^{d}	0.288^{c}	0.307^{b}	0.356^{a}	0.0032	635.74	0.001

Data is expressed as average value (n=9). Values with different letters within the same rows are significantly different ($P<0.05$). Data was analyzed by adopting one-way ANOVA followed by LSD test to determine the significant difference among diet groups.

[a]=Pooled standard error mean

M0= Control group; M1= 0.5 mg Co-Met/kg diet; M2= 1.0 mg Co-Met /kg diet; M3= 1.5 mg Co-Met /kg diet; M4= 2.0 mg Co-Met/kg diet; M5= 2.5 mg Co-Met/kg diet; M6= 3.0 mg Co-Met/kg diet.

Table 35. Muscle proximate composition of *Tor putitora* fingerlings after feeding graded level of Co-Met complex supplemented diets for 90 days

	Diet Groups							Statistical Analysis		
	M0	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**	**PSE[a]**	**P-value**	**F-value**
Crude Protein (%)	65.20^{f}	67.76^{e}	71.03^{d}	71.90^{cd}	72.66^{c}	76.25^{b}	77.83^{a}	0.43	0.001	215.87
Crude Fat (%)	6.015^{d}	9.969^{c}	10.08^{c}	12.13^{b}	12.7^{ab}	12.89^{a}	13.28^{a}	0.31	0.05	132.66
Ash (%)	9.56^{d}	10.65^{c}	11.25^{b}	11.62^{b}	11.68^{b}	12.21^{a}	12.20^{a}	0.23	0.001	31.11

Data is expressed as average value (n=6). Values with different letters within the same rows are significantly different ($P<0.05$).Data was analyzed by adopting one-way ANOVA followed by LSD test to determine the significant difference among diet groups.

a=Pooled standard error mean

M0= Control group; M1= 0.5 mg Co-Met/kg diet; M2= 1.0 mg Co-Met /kg diet; M3= 1.5 mg Co-Met /kg diet; M4= 2.0 mg Co-Met/kg diet; M5= 2.5 mg Co-Met/kg diet; M6= 3.0 mg Co-Met/kg diet

Table 36. Bioaccumulation of metals in the muscle of *T. putitora* fingerlings after feeding graded levels of Co-Met complex supplemented diets for 90 days

Metal concentration (µg/g)	Diet Groups							Statistical Analysis		
	M0	M1	M2	M3	M4	M5	M6	SEM	F-value	P-value
Iron	0.0639^{a}	0.0639^{a}	0.0636^{a}	0.0636^{a}	0.0639^{a}	0.063^{a}	0.0644^{a}	0.0006	0.222	0.963
Manganese	0.0033^{a}	0.0031^{b}	0.003^{b}	0.0031^{b}	0.0031^{b}	0.003^{b}	0.0031^{b}	0.0001	2.15	0.112
Cobalt	0.0141^{c}	0.0141^{c}	0.0142^{bc}	0.0143^{abc}	0.0143^{bc}	0.014^{ab}	0.0145^{a}	0.001	3.545	0.024

Data is expressed as average value (n=6). Values with different letters within the same rows are significantly different (P<0.05). Data was analyzed by adopting one-way ANOVA followed by LSD test to determine the significant difference among diet groups

[a]=Pooled standard error mean

M0= Control group; M1= 0.5 mg Co-Met/kg diet; M2= 1.0 mg Co-Met /kg diet; M3= 1.5 mg Co-Met /kg diet; M4= 2.0 mg Co-Met/kg diet; M5= 2.5 mg Co-Met/kg diet; M6= 3.0 mg Co-Met/kg diet.

Comparative effect of graded levels of different chemical forms of dietary cobalt on *T.putitora* fingerlings

Growth performance

The comparative effect of graded levels of different chemical forms of dietary cobalt on the growth performance of mahseer is shown in figure 57. Statistical analysis was done by two-way ANOVA showed a significant effect of different chemical forms and dosage levels of dietary cobalt on growth performance, i.e., W_f (chemical form, $F_{1,40}$=5860.13, P<0.001; dosage level, $F_{13,28}$=259.03, P<0.001), %WG (chemical form, $F_{1,40}$=6716.24, P<0.001; dosage level, $F_{13,28}$=303.2, P<0.001), AWG (chemical form, $F_{1,40}$=6727.84, P<0.001; dosage level, $F_{13,\ 28}$=301.79,P<0.001) ADG (chemical form, $F_{1,40}$=6602.56, P<0.001; dosage level, $F_{13,28}$=300.91, P<0.001) and %SGR (chemical form, $F_{1,40}$=428.08, P<0.001; dosage level, $F_{13,28}$=18.5, P<0.001) of *T.putitora*. The FCR was also affected by chemical forms ($F_{1,\ 40}$=5427, P<0.001) and dosage level (dosage level, $F_{13,\ 28}$=214.8, P<0.001) of cobalt. Moreover, a significant interaction between two variables (Table 37) indicated the manner how chemical forms and dosage level of dietary cobalt influence the growth performance and FCR of fish. Pairwise comparison between different chemical forms of dietary cobalt at every dose level indicated a significantly higher positive effect of dietary Co-Met complex in contrast to Co-NPs supplemented diet on every growth performance parameters. Furthermore, post hoc pairwise comparison between dosage levels indicated that dietary Co-Met complex at 3mg/kg diet showed the highest %WG (120.68%) while Co-NPs showed its maximum effect (88.78%WG) at 1.5 mg/kg diet. Here further, increase in Co-NPs in diet negatively affected the growth performance of fish. Overall results indicated maximum %WG in a group of fish fed 3mg Co-Met/kg diet (37.48% higher WG as compared to control group), while at the same dosage level, Co-NPs showed a negative effect i.e., 7.11% reduced WG as compared to control group.

Hematological indices

The comparative effect of the graded level of different chemical forms of dietary cobalt on the hematological indices of fish is shown in figures 58→64. Statistical analysis done by two-way ANOVA showed a significant effect of different chemical forms and dosage levels on the hematological indices i.e., RBC count (chemical form, $F_{1,124}$= 4151.13, P<0.001; dosage level, $F_{13,112}$=378.71, P<0.001), HB level (chemical form, $F_{1,124}$= 4210.09, P<0.001; dosage level, $F_{13,112}$=338.73, P<0.001), HCT(chemical form, $F_{1,124}$= 491.04, P<0.001; dosage level, $F_{13,112}$=53.575, P<0.001), MCV (chemical form, $F_{1,124}$= 170579, P<0.001; dosage level, $F_{13,112}$=52752.8, P<0.001), MCH (chemical form, $F_{1,124}$=391013, P<0.001; dosage level, $F_{13,112}$=12438.3, P<0.001), MCHC (chemical form, $F_{1,124}$= 102.01, P<0.001; dosage level, $F_{13,112}$=168.55, P<0.001) of *T. putitora.* Furthermore, a significant interaction between two variables (chemical forms and dosage level of dietary cobalt) indicated how these two variables affected the hematological indices of *T.putitora* (Table 38). Pair-wise comparison between different chemical forms of dietary cobalt at every dosage level indicated the most significant positive effect of dietary Co-Met complex in contrast to Co-NPs supplemented diet on the hematological indices of fish. Furthermore, post hoc pairwise comparison between dosage levels indicated a dose-dependent most significant positive effect of dietary Co-Met complex in contrast to Co-NPs supplementation. However, dietary Co-NPs showed a positive effect up to 1.5 mg/kg diet while further increase negatively affected the hematological indices of fish (Table 26).

Two-way ANOVA also indicated the significant effect of different chemical forms ($F_{1,124}$=1873.25; P<0.001) and dosage level ($F_{13,\ 112}$=91.01; P<0.001) of dietary cobalt on the serum AST activity of fish. Moreover, a significant interaction between chemical forms and dosage level of dietary cobalt indicated how these two variables affected the serum AST activity of fish (Table 38).

Pair-wise comparison between different chemical forms of dietary cobalt at every dose level indicated a significantly higher serum AST activity in response to Co-NPs supplemented diet as compared to Co-Met complex supplemented diet on fish. Furthermore, post hoc pairwise comparison between dosage levels indicated the non-significant effect of Co-NPs supplemented diet (up to 1.5mg/kg) on serum AST activity of the fish. However further increase in Co-NPs supplementation in the diet resulted in a significant increase in serum AST activity with maximum activity in a N6 group of fish (3mg Co-NPs/kg diet). On the other hand, a decrease in serum AST activity was observed in fish with the increase of dietary Co-Met complex supplementation.

Immunological indices

The comparative effect of the graded level of different chemical forms of dietary cobalt on the immunological indices of mahseer is shown in figures 65→69. Statistical analysis done by two-way ANOVA showed a significant effect of different chemical forms and dosage levels of cobalt on the immunological indices i.e., plasma protein (chemical form, $F_{1,124}$= 225.6, P<0.001; Dosage level, $F_{13,112}$=38.35, P<0.001), IgM (chemical form, $F_{1,124}$= 311.76, P<0.001; dosage level, $F_{13,112}$=16.12, P<0.001), lysozyme activity (chemical form, $F_{1,124}$= 221.51, P<0.001; dosage level, $F_{13,112}$=28.8, P<0.001),WBC count (chemical form, $F_{1,124}$= 42741.1, P<0.001, dosage level, $F_{13,112}$=7091.51, P<0.001) and NBT activity (chemical form, $F_{1,124}$= 668.94, P<0.001; dosage level, $F_{13,112}$=61.63.51, P<0.001) of *T. putitora.* Furthermore, a significant interaction between two variables (chemical forms, and dosage level of dietary cobalt) indicated how these two variables affected the immunological indices of *T.putitora* (Table 39). Pair-wise comparison between different chemical forms of dietary cobalt at every dose level indicated the most significant positive effect of dietary Co-Met complex in contrast to Co-NPs supplemented diet on the immunological indices of fish. Furthermore, post hoc pairwise comparison between dosage levels indicated dose-dependent,

significant positive effect of dietary Co-Met complex in contrast to Co-NPs supplementation. However, dietary Co-NPs showed positive effect up to 1.5 mg/kg diet while further, increase negatively affected the immunological indices of fish (Table 27).

Intestinal enzymes activities

The comparative effect of the graded level of different chemical forms of dietary cobalt on the intestinal enzymes activities of fish is shown in figures 70→72. Statistical analysis done by two-way ANOVA showed a significant effect of different chemical forms and dosage levels of cobalt on the intestinal enzyme activities i.e. amylase (chemical form, $F_{1,124}$= 223.21, P<0.001; dosage level, $F_{13,112}$=4.396, P<0.001), protease (chemical form, $F_{1,124}$= 479.83, P<0.001; dosage level, $F_{13,112}$=55.38, P<0.001), and cellulase (chemical form, $F_{1,124}$= 880.51, P<0.001; dosage level, $F_{13,112}$=37.07, P<0.001) of *T. putitora.* Furthermore significant interaction between two variables (chemical forms, and dosage level of dietary cobalt) indicated how these two variables affected the intestinal enzyme activities of *T.putitora* (Table 40). Pair-wise comparison between different chemical forms of dietary cobalt at every dose level indicated the most significant positive effect of dietary Co-Met complex in contrast to Co-NPs on the intestinal enzyme activities of fish. Furthermore, post hoc pairwise comparison between dosage levels indicated a dose-dependent, comparatively most significant positive effect of dietary Co-Met complex in contrast to Co-NPs supplementation. However, dietary Co-NPs above 1.5 mg/kg inclusion level negatively affected the intestinal enzyme activities of fish (Table 28).

Muscle proximate composition

The comparative effect of the graded level of different chemical forms of dietary cobalt on the muscle proximate composition of *T. putitora* after 90 days feeding trial is shown in figures 73→75. Statistical analysis done by two-way ANOVA showed a significant effect of different chemical forms,

and dosage levels of cobalt on the muscle proximate composition i.e., %crude protein (chemical form, $F_{1,82}$= 1705.88, P<0.001; dosage level, $F_{13,70}$=70.1; P<0.001), %crude fat (chemical form, $F_{1,82}$= 1213.88, P<0.001; dosage level, $F_{13,70}$=60.17, P<0.001), and %crude ash (chemical form, $F_{1,82}$= 61.82, P<0.001; dosage level, $F_{13,70}$=106.17, P<0.001), of *T. putitora.* Furthermore, a significant interaction between two variables (chemical forms, and dosage level of dietary cobalt) indicated how these two variables affected the muscle proximate composition of *T.putitora* (Table 41). Pair-wise comparison between different chemical forms of dietary cobalt at every dosage level indicated the most significant positive effect of dietary Co-Met complex in contrast to Co-NPs supplemented diet on the muscle proximate composition of fish. Furthermore, post hoc pairwise comparison between dosage levels indicated most significant positive effect of dietary Co-Met complex in contrast to Co-NPs supplemented diet on the muscle crude protein and fat content of fish. Moreover, post hoc pairwise comparison between dosage level indicated that dietary Co-Met complex at 3mg/kg diet showed the highest muscle %crude protein (77.83%), and % fat (12.89%) contents while Co-NPs showed maximum effect (68.83% crude protein, and 7.16% crude fat) at i.e. 1.5 mg/kg diet and then showed decreasing trend.

Muscle metal bioaccumulation level

The comparative effect of the graded level of different chemical forms of dietary cobalt on the muscle metal bioaccumulation level of mahseer is shown in figures 76→78. Statistical analysis done by two-way ANOVA showed a significant effect of different chemical forms, and dosage levels of cobalt on the muscle metal bioaccumulation level i.e. Fe (chemical form, $F_{1,82}$= 990.42, P<0.001; dosage level, $F_{13,70}$=94.5, P<0.001;) figure 76, Mn (chemical form, $F_{1,82}$= 1086.6, P<0.001; dosage level, $F_{13,70}$=126.43, P<0.001) figure 77, and Co (chemical form, $F_{1,82}$= 10255.4, P<0.001; dosage level, $F_{13,70}$=1058.92, P<0.001) figure 78 of *T. putitora.* A significant interaction between two

variables (chemical forms, and dosage level of dietary cobalt) indicated how these two variables affected the muscle metal bioaccumulation level of *T.putitora* (Table 42). Post-hoc pairwise comparison between dosage levels indicated no effect of dietary Co-Met supplemented diet on the muscle Fe, Mn, and Co bioaccumulation level of fish. However, Co-NPs supplemented diet significantly reduced the muscle Fe, and Mn bioaccumulation level of fish. Moreover, an increase in muscle cobalt bioaccumulation level was observed with the increasing dosage level of Co-NPs in diet.

Table 37: Summary of Two way ANOVA showing the comparative effect of graded levels of different chemical forms of dietary cobalt on the growth performance of *T.putitora* fingerlings

	df	SS	MS	F- value	P-value
Wf (g)					
Chemical forms	1	20.63	20.63	5860.13	<0.001
Dosage	6	5.46	0.912	259.03	<0.001
Chemical forms × Dosage	6	11.26	1.87	533.17	<0.001
%WG					
Chemical forms	1	5109.09	5109.09	6716.24	<0.001
Dosage	6	1383.89	230.64	303.2	<0.001
Chemical forms × Dosage	6	2877.55	479.59	630.45	<0.001
AWG/fish (g)					
Chemical forms	1	20.66	20.66	6727.84	<0.001
Dosage	6	5.56	0.927	301.79	<0.001
Chemical forms × Dosage	6	11.43	1.906	620.42	<0.001
ADG/fish (g)					
Chemical forms	1	0.003	0.003	6602.56	<0.001
Dosage	6	0.001	0.00115	300.91	<0.001
Chemical forms × Dosage	6	0.001	0.000238	624.87	<0.001
%SGR					
Chemical forms	1	0.149	0.149	428.08	<0.001
Dosage	6	0.039	0.006	18.5	<0.001
Chemical forms × Dosage	6	0.085	0.014	40.58	<0.001
FCR					
Chemical forms	1	3.46	3.46	5427	<0.001
Dosage	6	0.823	0.137	214.8	<0.001
Chemical forms × Dosage	6	1.98	0.331	518.68	<0.001

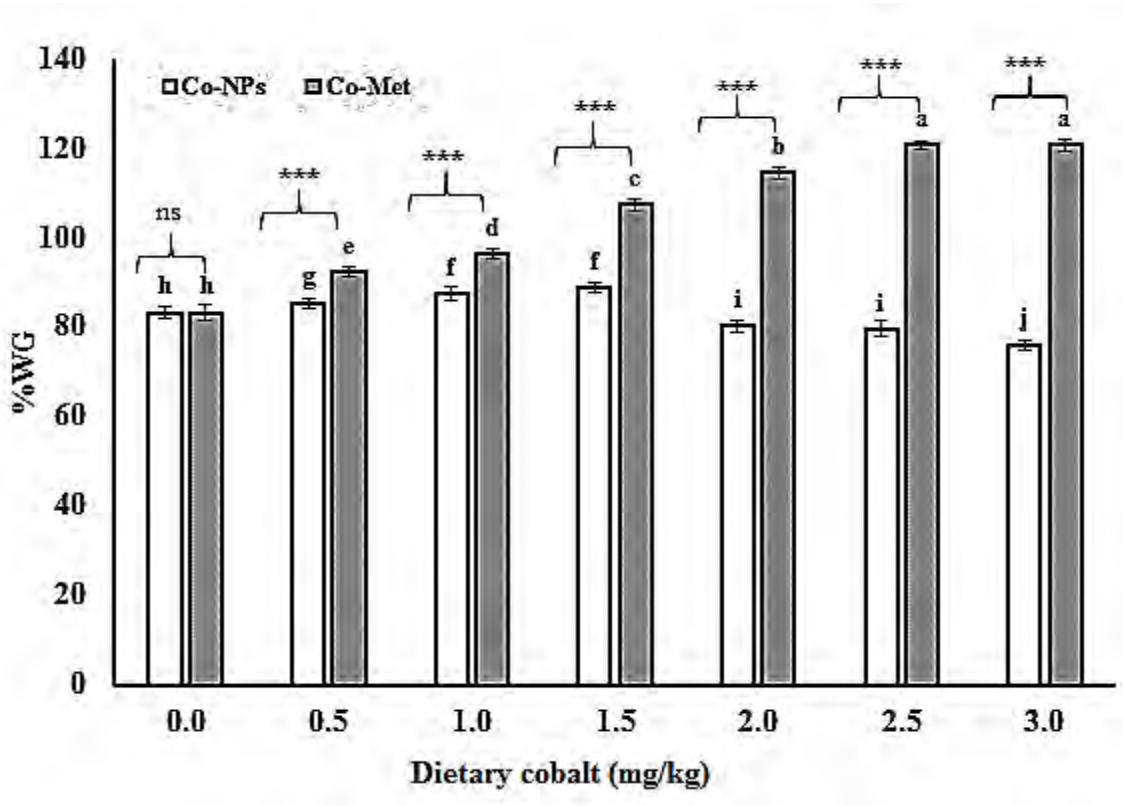

Figure 57. %WG of *T .putitora* fingerlings after feeding graded levels of Co-NPs, and Co-Met complex supplemented diet for 90 days

Bars show the values as average± SE (n=3). Different letters on bars show significant difference. Data was analyzed by adopting ANOVA followed by LSD test to compare the effect of different chemical forms, and dosage of dietary cobalt. Moreover, difference between the two diet groups i.e., Co-NPs, and Co-Met complex at specific dosage level was determined by using paired T-test at 95% confidence intervals.

ns= non-significant difference; *= P≤0.01 ; **= P≤0.001; ***= P≤0.001

Table 38. Summary of Two way ANOVA showing the comparative effect of graded levels of different chemical forms of dietary cobalt on the hematological indices of *T.putitora* fingerlings

	df	SS	MS	F- value	P-value
RBC (10^6µ/L)					
Chemical forms	1	0.852	0.852	4151.13	<0.001
Dosage	6	0.466	0.078	378.71	<0.001
Chemical forms × Dosage	6	0.873	0.146	708.78	<0.001
HB (g d/l)					
Chemical forms	1	25.046	25.046	4210.09	<0.001
Dosage	6	12.091	2.015	338.73	<0.001
Chemical forms × Dosage	6	38.034	6.506	1093.55	<0.001
HCT (%)					
Chemical forms	1	224.43	335.43	491.04	<0.001
Dosage	6	146.91	24.48	53.575	<0.001
Chemical forms × Dosage	6	564.42	94.07	205.62	<0.001
MCV (cm^3 erythrocyte)					
Chemical forms	1	8454.55	8454.55	170579	<0.001
Dosage	6	15687.77	2614.62	52752.8	<0.001
Chemical forms × Dosage	6	17784.01	2963.83	59798.3	<0.001
MCH (pg)					
Chemical forms	1	4963.02	4963.02	391013	<0.001
Dosage	6	947.255	157.87	12438.3	<0.001
Chemical forms × Dosage	6	1759.01	293.17	23097.4	<0.001
MCHC (Hb/100 ml blood)					
Chemical forms	1	68.546	68.546	102.01	<0.001
Dosage	6	679.52	113.25	168.55	<0.001
Chemical forms × Dosage	6	2048.188	341.36	508.05	<0.001
AST (U/L)					
Chemical forms	1	20.064	20.06	1873.25	<0.001
Dosage	6	5.84	0.975	91.01	<0.001
Chemical forms × Dosage	6	26.93	4.48	419.132	<0.001

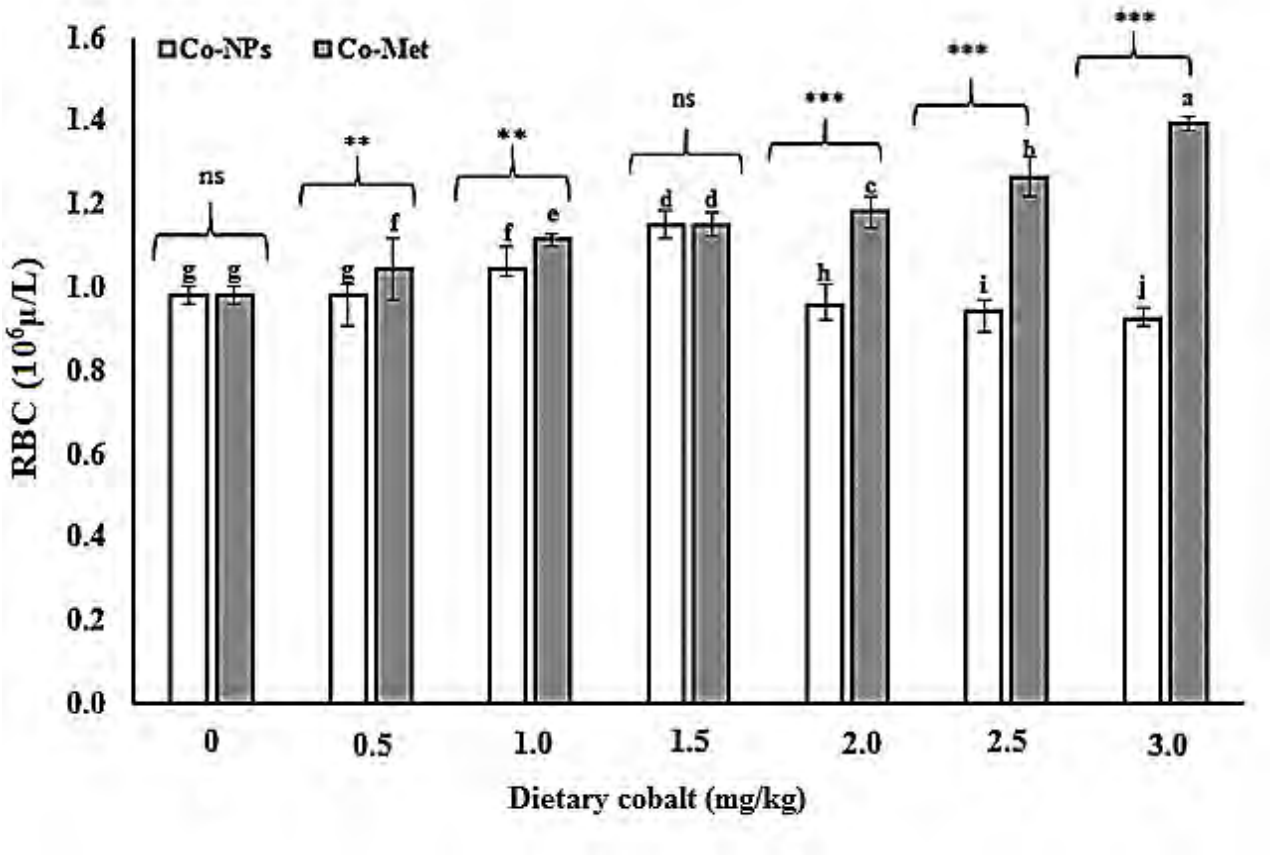

Figure 58. Red blood cells count RBCs (10^6µ/L) of *T.putitora* fingerlings after graded levels of Co-NPs, and Co-Met complex supplemented diet for 90 days

Bars show the values as average± SE (n=9). Different letters on bars show significant difference. Data was analyzed by adopting ANOVA followed by LSD test to compare the effect of different Chemical forms, and dosage of dietary cobalt.

Moreover, difference between the two diet groups i.e., Co-NPs, and Co-Met complex at specific dosage level was determined by using paired T-test at 95% confidence intervals.

ns= non-significant difference; *= P≤0.01 ; **= P≤0.001; ***= P≤0.001

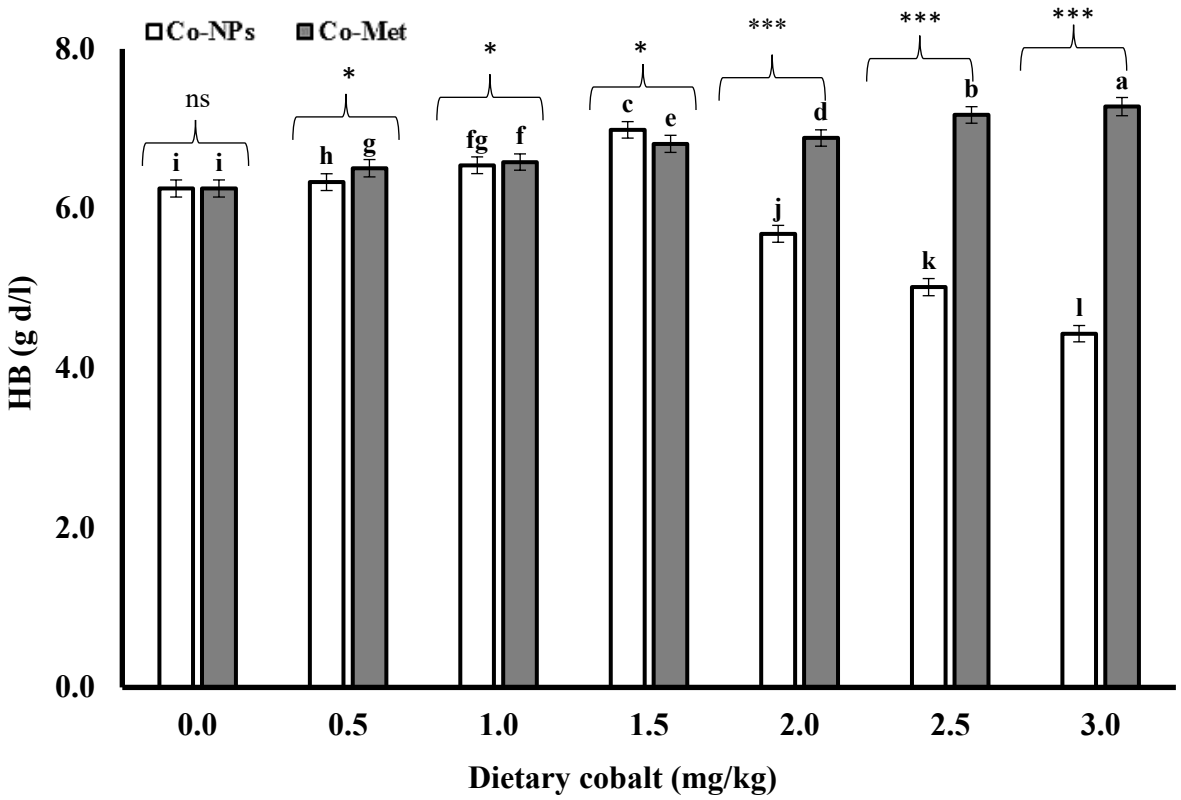

Figure 59. Hemoglobin level (g d/l) of *T.putitora* fingerlings after feeding graded levels of Co-NPs, and Co-Met complex supplemented diets for 90 days

Bars show the values as average± SE (n=9). Different letters on bars show significant difference. Data was analyzed by adopting ANOVA followed by LSD test to compare the effect of different Chemical forms, and dosage of dietary cobalt.

Moreover, difference between the two diet groups i.e., Co-NPs, and Co-Met complex at specific dosage level was determined by using paired T-test at 95% confidence intervals.

ns= non-significant difference; *= $P \leq 0.01$; **= $P \leq 0.001$; ***= $P \leq 0.001$

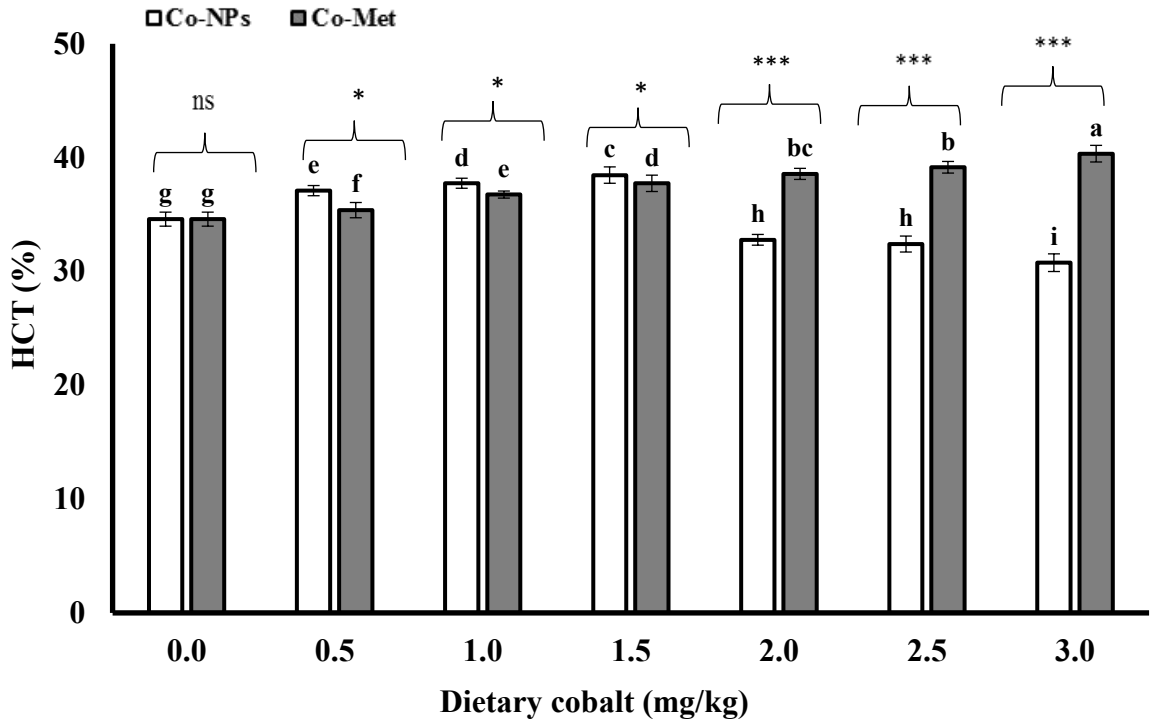

Figure 60. %HCT in blood of *T.putitora* fingerlings after graded levels of Co-NPs, and Co-Met complex supplemented diets for 90 days

Bars show the values as average± SE (n=9). Different letters on bars show significant difference. Data was analyzed by adopting ANOVA followed by LSD test to compare the effect of different Chemical forms, and dosage of dietary cobalt.

Moreover, difference between the two diet groups i.e., Co-NPs, and Co-Met complex at specific dosage level was determined by using paired T-test at 95% confidence intervals.

ns= non-significant difference; *= P≤0.01 ; **= P≤0.001; ***= P≤0.001

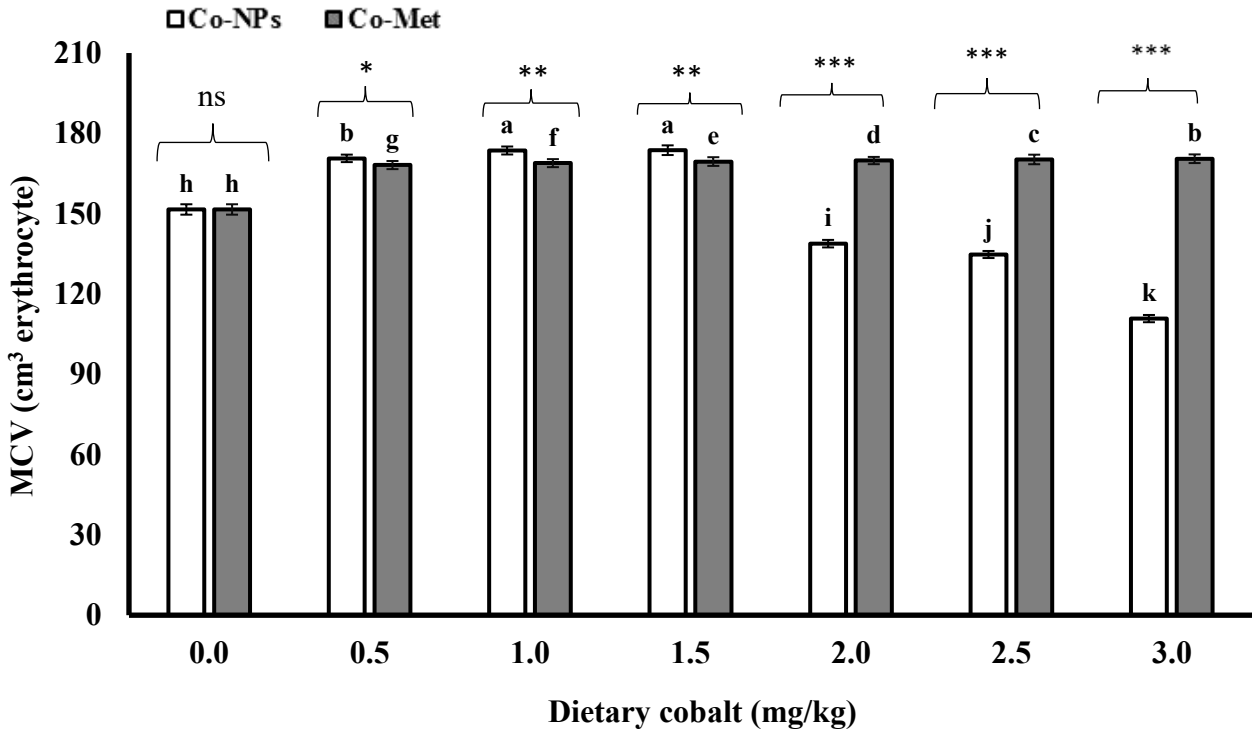

Figure 61. Mean corpuscular volume (cm^3 erythrocyte) of *T.putitora* fingerlings after feeding graded levels of Co-NPs, and Co-Met complex supplemented diets for 90 days

Bars show the values as average± SE (n=9). Different letters on bars show significant difference. Data was analyzed by adopting ANOVA followed by LSD test to compare the effect of different Chemical forms, and dosage of dietary cobalt.

Moreover, difference between the two diet groups i.e., Co-NPs, and Co-Met complex at specific dosage level was determined by using paired T-test at 95% confidence intervals.

ns= non-significant difference; *= $P \leq 0.01$; **= $P \leq 0.001$; ***= $P \leq 0.001$

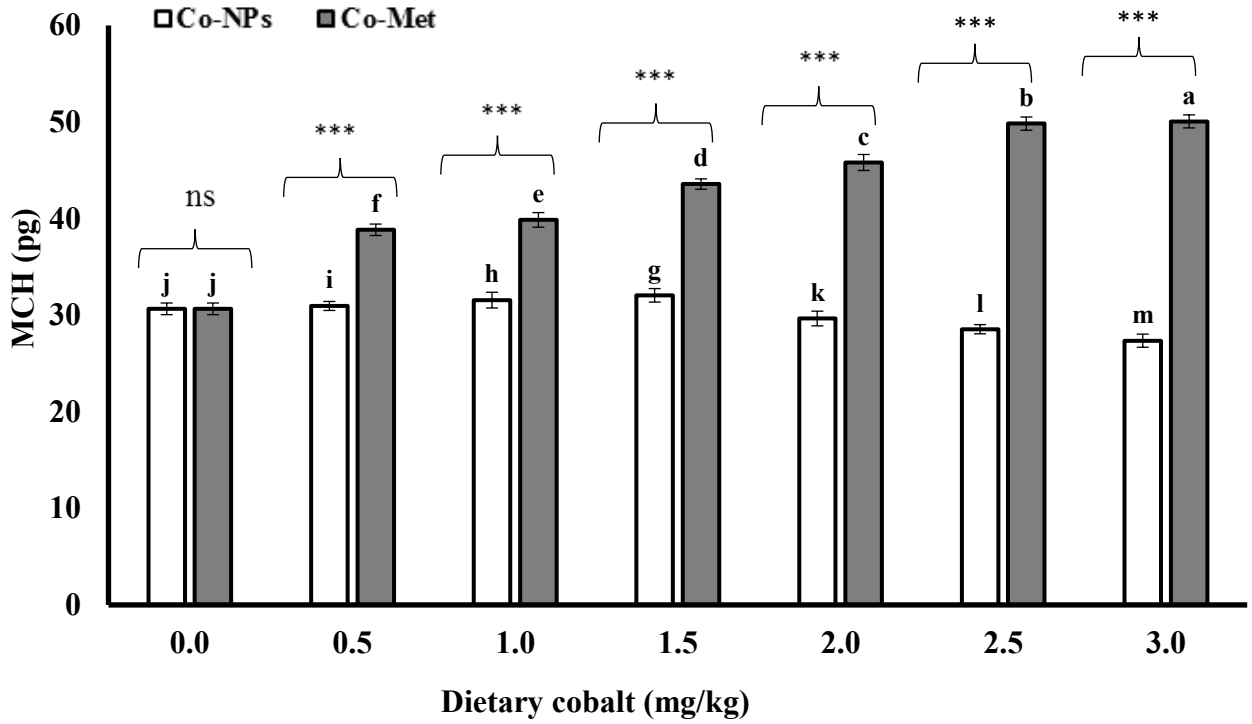

Figure 62. Mean corpuscular hemoglobin (pg) of *T.putitora* fingerlings after feeding graded levels of Co-NPs, and Co-Met complex supplemented diets for 90 days

Bars show the values as average± SE (n=9). Different letters on bars show significant difference. Data was analyzed by adopting ANOVA followed by LSD test to compare the effect of different Chemical forms, and dosage of dietary cobalt.

Moreover, difference between the two diet groups i.e., Co-NPs, and Co-Met complex at specific dosage level was determined by using paired T-test at 95% confidence intervals.

ns= non-significant difference; *= $P \leq 0.01$; **= $P \leq 0.001$; ***= $P \leq 0.001$

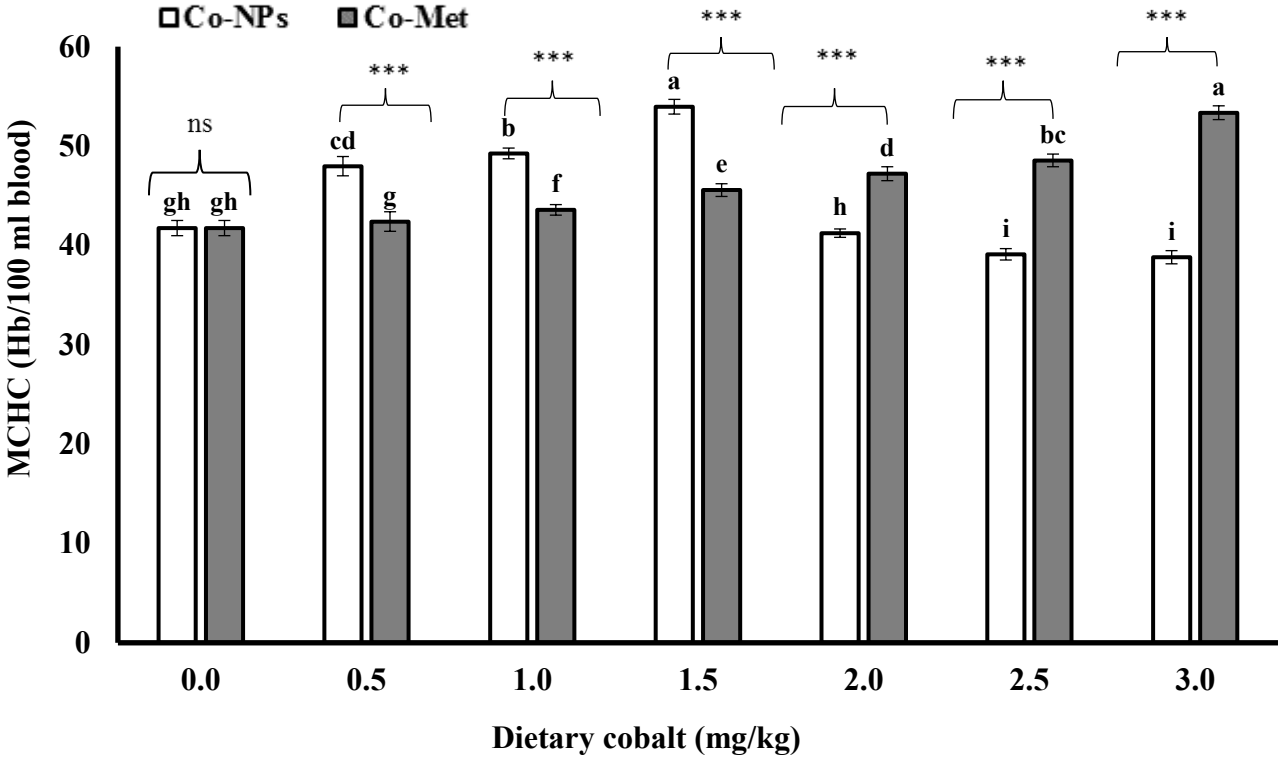

Figure 63. Mean corpuscular Hemoglobin dosage (Hb/100 ml blood) of *T.putitora* fingerlings after feeding graded levels of Co-NPs, and Co-Met complex supplemented diets for 90 days

Bars show the values as average± SE (n=9). Different letters on bars show significant difference. Data was analyzed by adopting ANOVA followed by LSD test to compare the effect of different Chemical forms, and dosage of dietary cobalt.

Moreover, difference between the two diet groups i.e., Co-NPs, and Co-Met complex at specific dosage level was determined by using paired T-test at 95% confidence intervals.

ns= non-significant difference; *= P≤0.01 ; **= P≤0.001; ***= P≤0.001

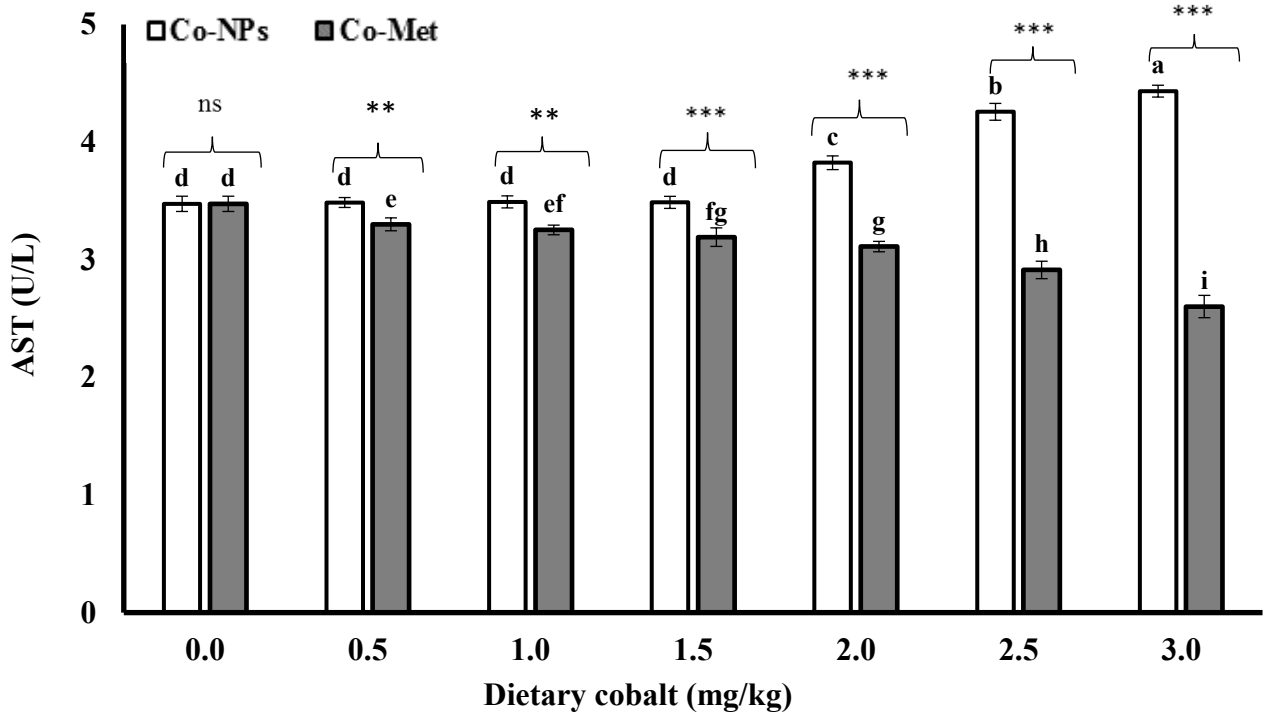

Figure 64. Serum aspartate aminotransferase activity AST (U/L) of *T.putitora* fingerlings after feeding graded levels of Co-NPs, and Co-Met complex supplemented diets for 90 days

Bars show the values as average± SE (n=9). Different letters on bars show significant difference. Data was analyzed by adopting ANOVA followed by LSD test to compare the effect of different Chemical forms, and dosage of dietary cobalt.

Moreover, difference between the two diet groups i.e., Co-NPs, and Co-Met complex at specific dosage level was determined by using paired T-test at 95% confidence intervals.

ns= non-significant difference; *= P≤0.01 ; **= P≤0.001; ***= P≤0.001

Table 39. Summary of Two way ANOVA showing the comparative effect of graded levels of different chemical forms of dietary cobalt on the immunological indices of *T.putitora* fingerlings

	df	SS	MS	F- value	P-value
Plasma protein(mg/ml)					
Chemical forms	1	59.443	59.44	225.6	<0.001
Dosage	6	60.63	10.1	38.35	<0.001
Chemical forms × Dosage	6	121.44	20.24	76.82	<0.001
IgM (mg/ml)					
Chemical forms	1	38.762	38.76	311.76	<0.001
Dosage	6	12.03	2.005	16.12	<0.001
Chemical forms × Dosage	6	46.54	7.76	62.42	<0.001
Lysozyme activity (µg/ml)					
Chemical forms	1	24.893	24.89	221.51	<0.001
Dosage	6	12.42	3.23	28.8	<0.001
Chemical forms × Dosage	6	13.58	2.32	20.65	<0.001
WBC count (10^3 µ/L)					
Chemical forms	1	34116	34116	42741.1	<0.001
Dosage	6	33962.8	5660.46	7091.51	<0.001
Chemical forms × Dosage	6	36589.8	6098.31	7640.06	<0.001
NBT activity (OD=540nm)					
Chemical forms	1	1.902	1.902	668.94	<0.001
Dosage	6	1.051	0.175	61.63	<0.001
Chemical forms × Dosage	6	1.753	0.292	102.79	<0.001

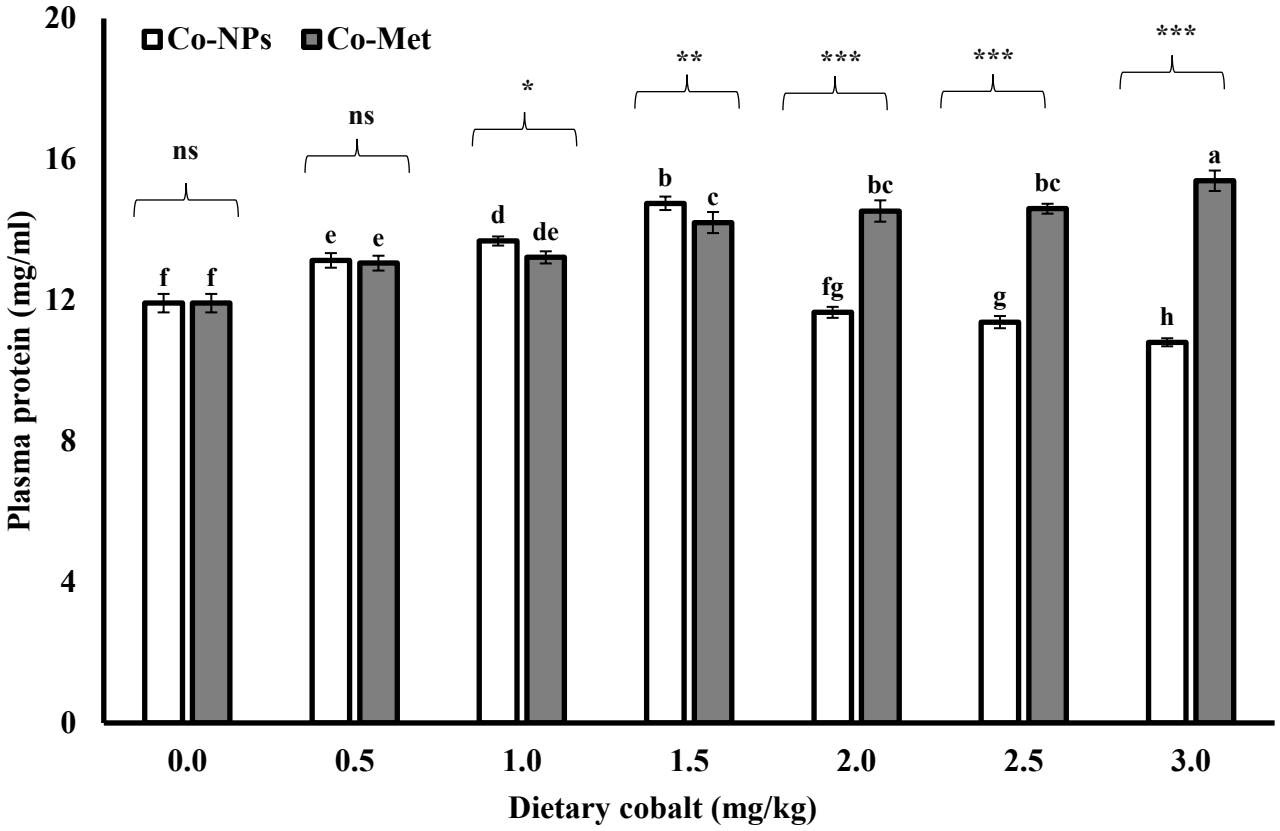

Figure 65. Plasma protein level of *T.putitora* fingerlings after feeding graded levels of Co-NPs, and Co-Met complex supplemented diets for 90 days

Bars show the values as average± SE (n=9). Different letters on bars show significant difference. Data was analyzed by adopting ANOVA followed by LSD test to compare the effect of different Chemical forms, and dosage of dietary cobalt.

Moreover, difference between the two diet groups i.e., Co-NPs, and Co-Met complex at specific dosage level was determined by using paired T-test at 95% confidence intervals.

ns= non-significant difference; *= P≤0.01 ; **= P≤0.001; ***= P≤0.001

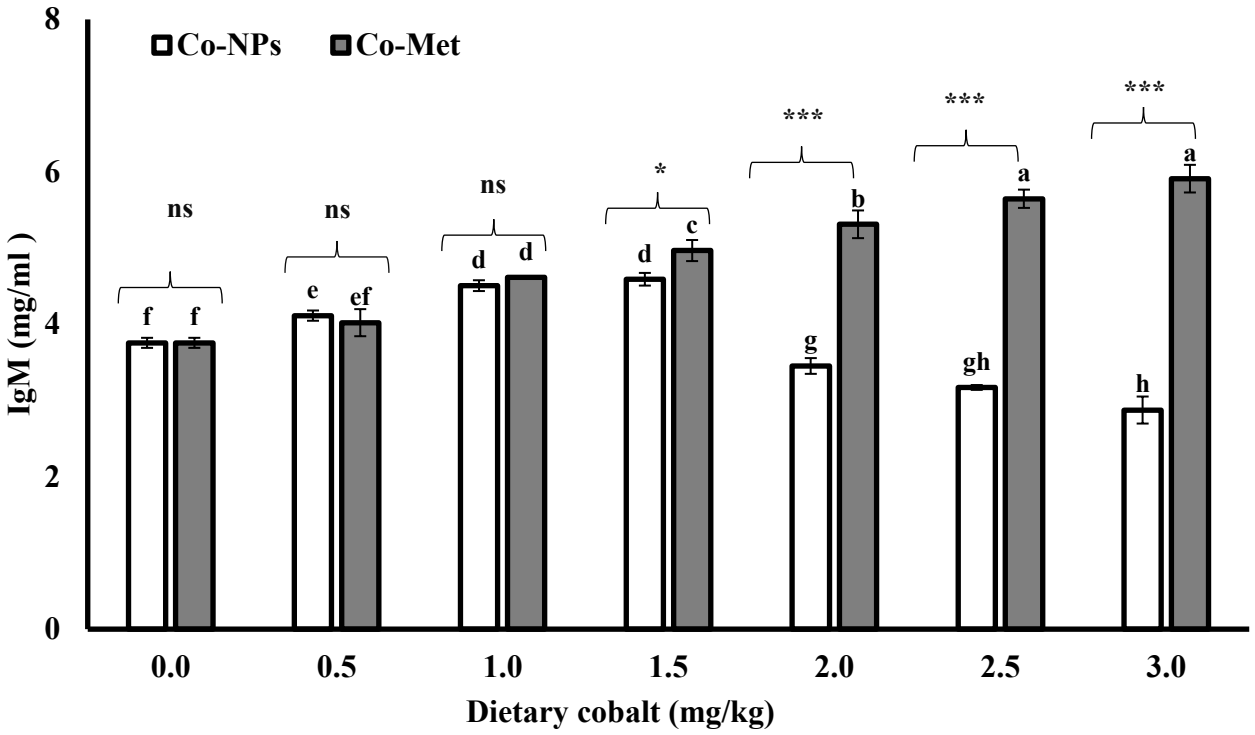

Figure 66. Plasma immunoglobulin (IgM) level of *T.putitora* fingerlings after feeding graded levels of Co-NPs, and Co-Met complex supplemented diets for 90 days

Bars show the values as average± SE (n=9). Different letters on bars show significant difference. Data was analyzed by adopting ANOVA followed by LSD test to compare the effect of different Chemical forms, and dosage of dietary cobalt.

Moreover, difference between the two diet groups i.e., Co-NPs, and Co-Met complex at specific dosage level was determined by using paired T-test at 95% confidence intervals.

ns= non-significant difference; *= P≤0.01 ; **= P≤0.001; ***= P≤0.001

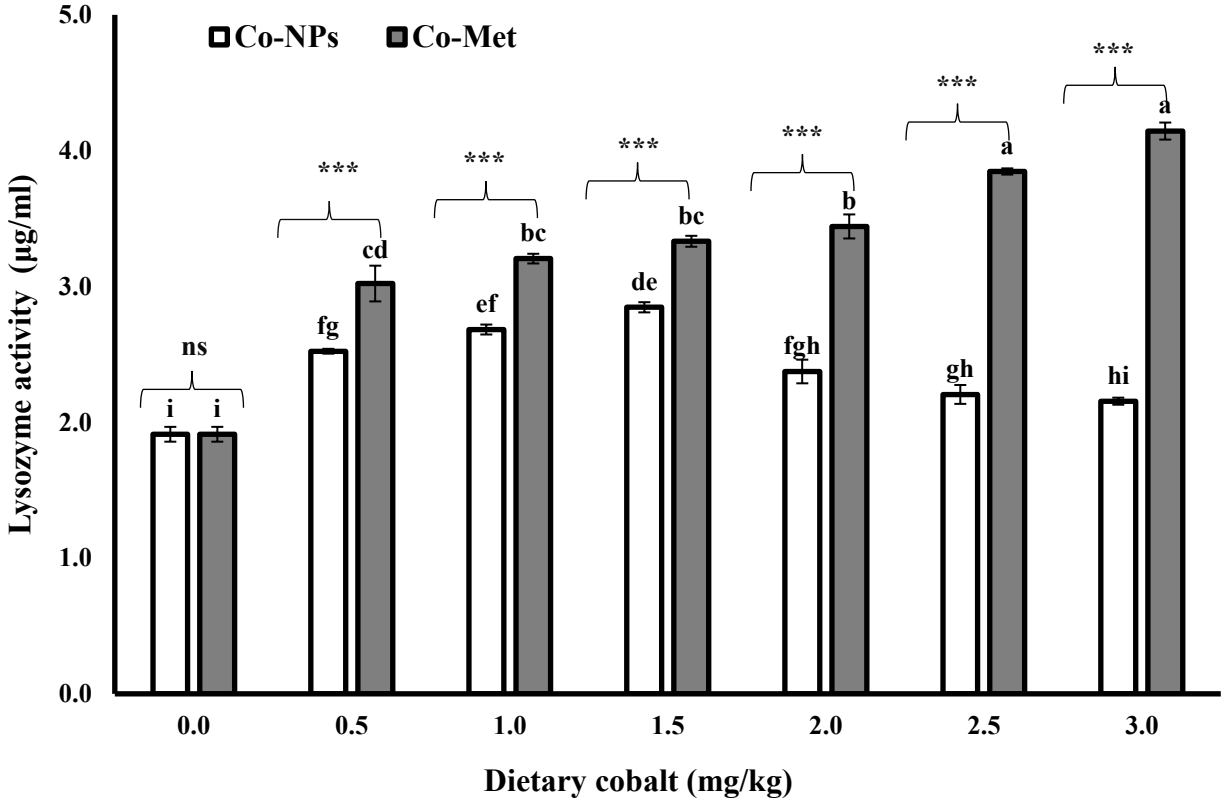

Figure 67. Serum lysozyme activity of *T.putitora* fingerlings after feeding graded levels of Co-NPs, and Co-Met complex supplemented diets for 90 days

Bars show the values as average± SE (n=9). Different letters on bars show significant difference. Data was analyzed by adopting ANOVA followed by LSD test to compare the effect of different Chemical forms, and dosage of dietary cobalt.

Moreover, difference between the two diet groups i.e., Co-NPs, and Co-Met complex at specific dosage level was determined by using paired T-test at 95% confidence intervals.

ns= non-significant difference; *= P≤0.01 ; **= P≤0.001; ***= P≤0.001

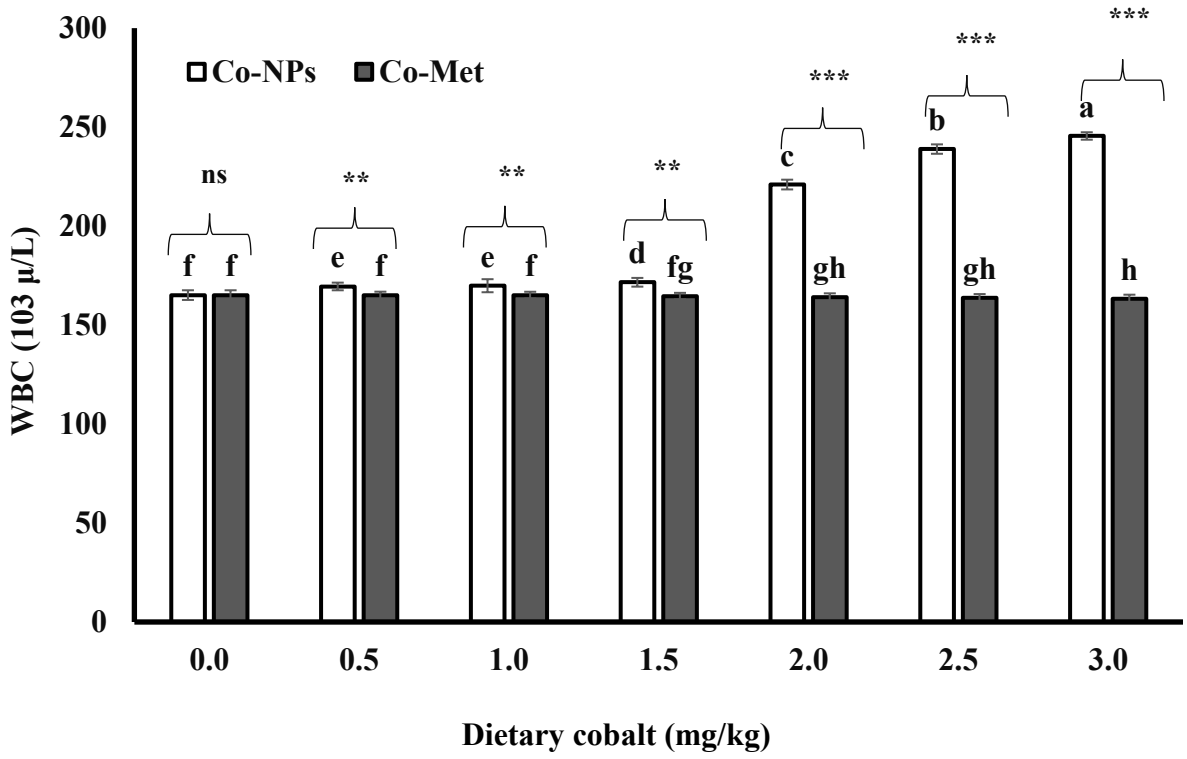

Figure 68. White blood cell count of *T.putitora* fingerlings after feeding graded levels of Co-NPs, and Co-Met complex supplemented diets for 90 days

Bars show the values as average± SE (n=9). Different letters on bars show significant difference. Data was analyzed by adopting ANOVA followed by LSD test to compare the effect of different Chemical forms, and dosage of dietary cobalt.

Moreover, difference between the two diet groups i.e., Co-NPs, and Co-Met complex at specific dosage level was determined by using paired T-test at 95% confidence intervals.

ns= non-significant difference; *= P≤0.01 ; **= P≤0.001; ***= P≤0.001

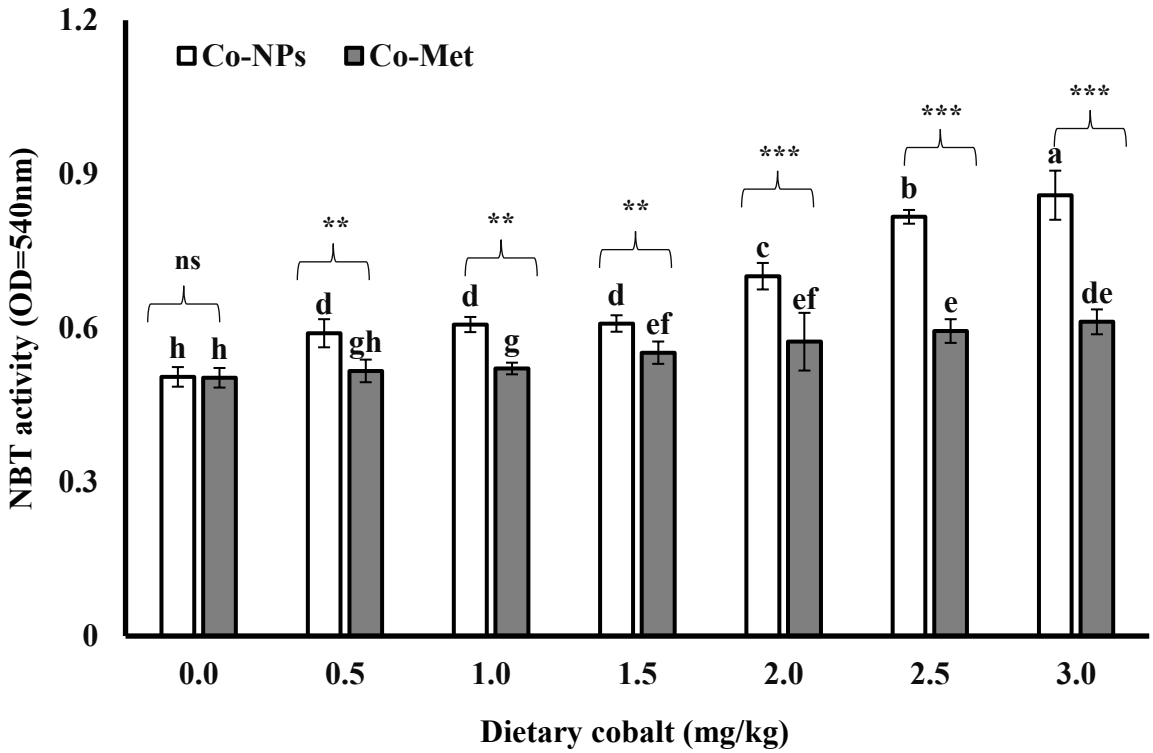

Figure 69. NBT (Nitro-blue tetrazolium) activity of *T.putitora* fingerlings after feeding graded levels of Co-NPs, and Co-Met complex supplemented diets for 90 days

Bars show the values as average± SE (n=9). Different letters on bars show significant difference. Data was analyzed by adopting ANOVA followed by LSD test to compare the effect of different Chemical forms, and dosage of dietary cobalt.

Moreover, difference between the two diet groups i.e., Co-NPs, and Co-Met complex at specific dosage level was determined by using paired T-test at 95% confidence intervals.

ns= non-significant difference; *= $P \leq 0.01$; **= $P \leq 0.001$; ***= $P \leq 0.001$

Table 40. Summary of Two way ANOVA showing the comparative effect of graded levels of different chemical forms of dietary cobalt on the intestinal enzymes activities of *T. putitora* fingerlings

	df	SS	MS	F- value	P-value
Amylase (U/mg)					
Chemical forms	1	0.207	0.207	223.21	<0.001
Dosage	6	0.024	0.004	4.396	0.001
Chemical forms × Dosage	6	0.070	0.012	12.53	<0.001
Protease (U/mg)					
Chemical forms	1	0.032	0.032	479.83	<0.001
Dosage	6	0.022	0.004	55.38	<0.001
Chemical forms × Dosage	6	0.064	0.011	157.69	<0.001
Cellulase (U/mg)					
Chemical forms	1	0.284	0.284	880.51	<0.001
Dosage	6	0.072	0.012	37.07	<0.001
Chemical forms × Dosage	6	0.132	0.022	68.35	<0.001

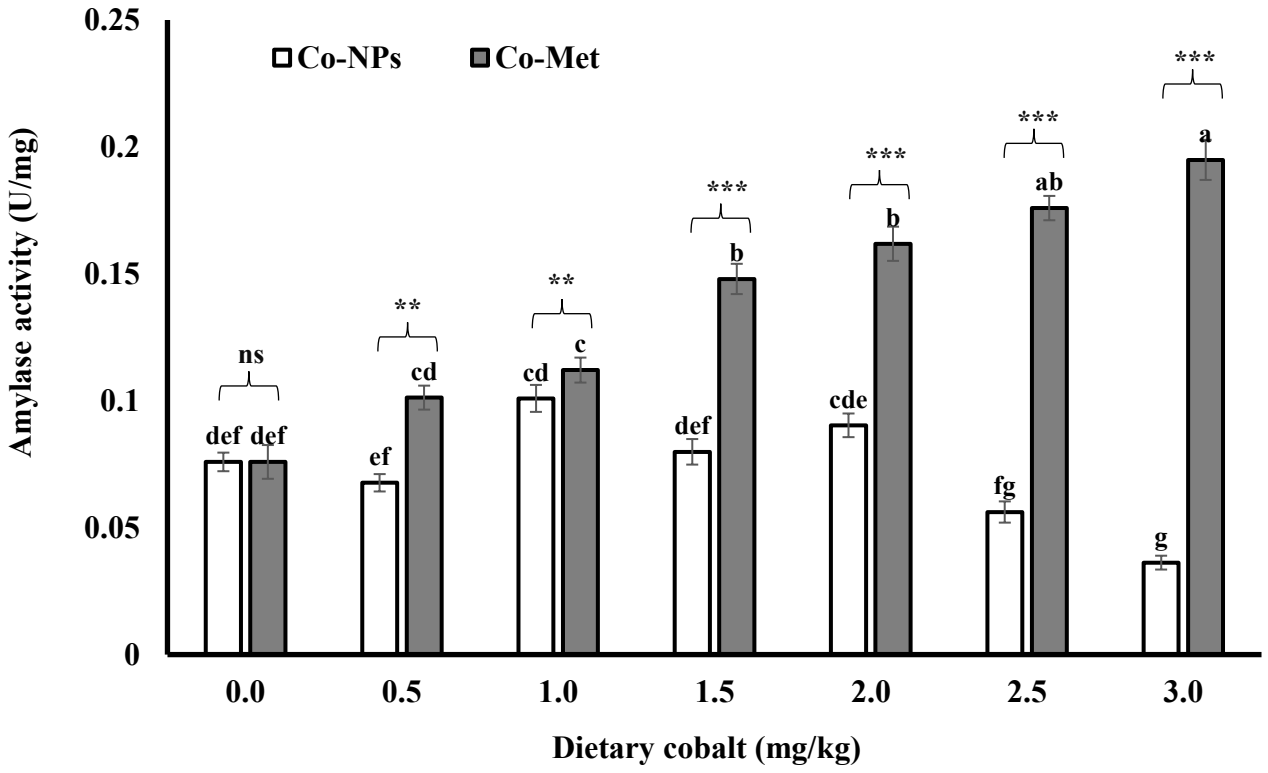

Figure 70. Amylase activity of *T.putitora* fingerlings after feeding graded levels of Co-NPs, and Co-Met complex supplemented diets for 90 days

Bars show the values as average± SE (n=9). Different letters on bars show significant difference. Data was analyzed by adopting ANOVA followed by LSD test to compare the effect of different Chemical forms, and dosage of dietary cobalt.

Moreover, difference between the two diet groups i.e., Co-NPs, and Co-Met complex at specific dosage level was determined by using paired T-test at 95% confidence intervals.

ns= non-significant difference; *= $P \leq 0.01$; **= $P \leq 0.001$; ***= $P \leq 0.001$

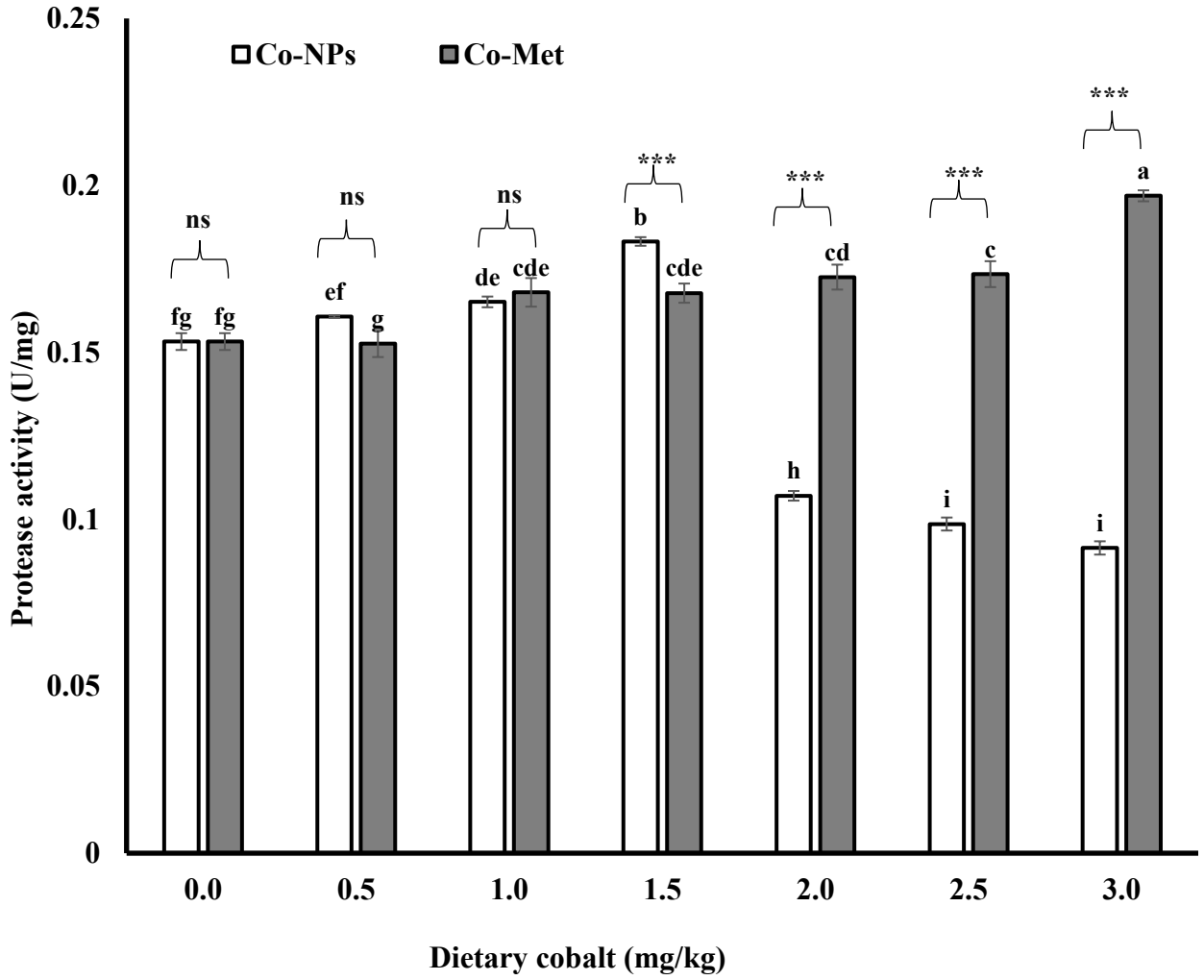

Figure 71. Protease activity of *T.putitora* fingerlings after feeding graded levels of Co-NPs, and Co-Met complex supplemented diets for 90 days

Bars show the values as average± SE (n=9). Different letters on bars show significant difference. Data was analyzed by adopting ANOVA followed by LSD test to compare the effect of different Chemical forms, and dosage of dietary cobalt.

Moreover, difference between the two diet groups i.e., Co-NPs, and Co-Met complex at specific dosage level was determined by using paired T-test at 95% confidence intervals.

ns= non-significant difference; *= P≤0.01 ; **= P≤0.001; ***= P≤0.001

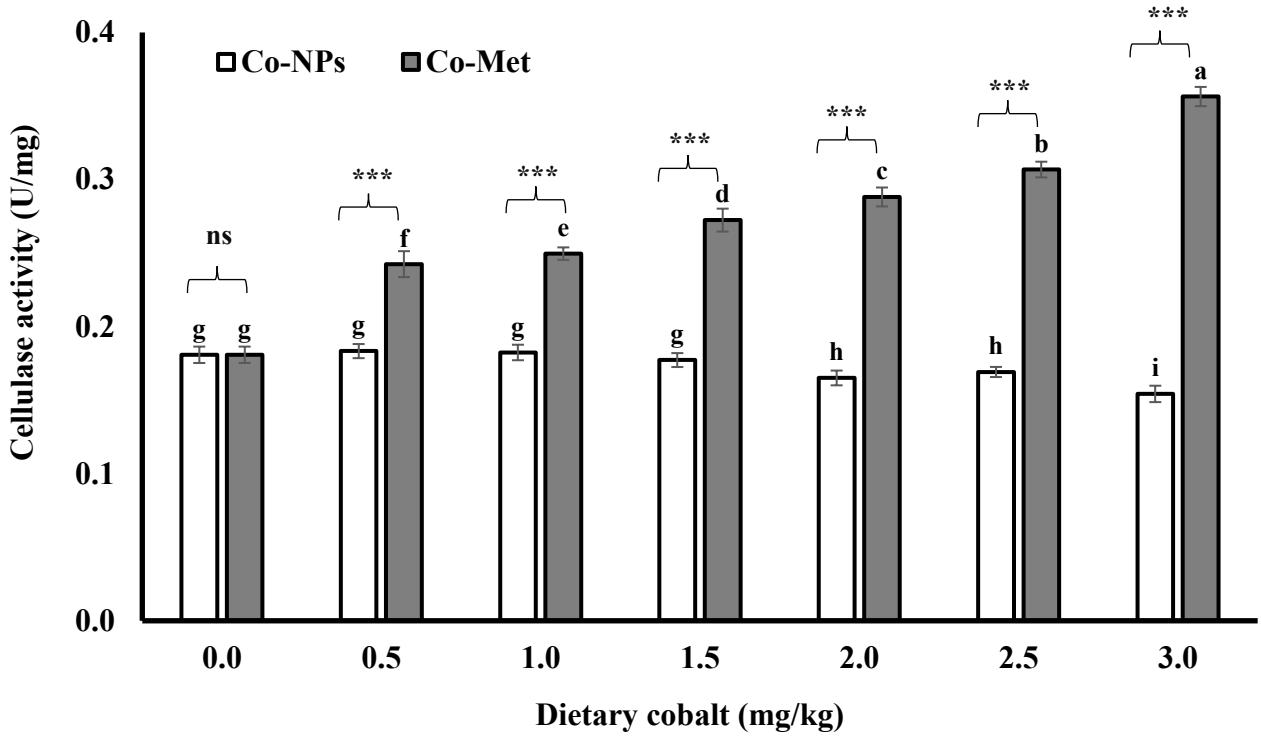

Figure 72. Cellulase activity of *T.putitora* fingerlings after feeding graded levels of Co-NPs and, Co-Met complex supplemented diets for 90 days

Bars show the values as average± SE (n=9). Different letters on bars show significant difference. Data was analyzed by adopting ANOVA followed by LSD test to compare the effect of different Chemical forms, and dosage of dietary cobalt.

Moreover, difference between the two diet groups i.e., Co-NPs, and Co-Met complex at specific dosage level was determined by using paired T-test at 95% confidence intervals.

ns= non-significant difference; *= P≤0.01 ; **= P≤0.001; ***= P≤0.001

Table 41. Summary of Two way ANOVA showing the comparative effect of graded levels of different chemical forms of dietary cobalt on the muscle proximate composition of *T. putitora* fingerlings

	df	SS	MS	F- value	P-value
Crude Protein (%)					
Chemical forms	1	902.38	902.38	1705.88	<0.001
Dosage	6	222.5	37.08	70.1	<0.001
Chemical forms × Dosage	6	846.88	141.14	266.82	<0.001
Crude Fat (%)					
Chemical forms	1	454.45	454.45	1213.88	<0.001
Dosage	6	135.16	22.52	60.17	<0.001
Chemical forms × Dosage	6	114.6	19.1	51.02	<0.001
Crude Ash (%)					
Chemical forms	1	11.901	11.901	61.82	<0.001
Dosage	6	122.638	20.44	106.17	<0.001
Chemical forms × Dosage	6	21.67	2.613	18.76	<0.001

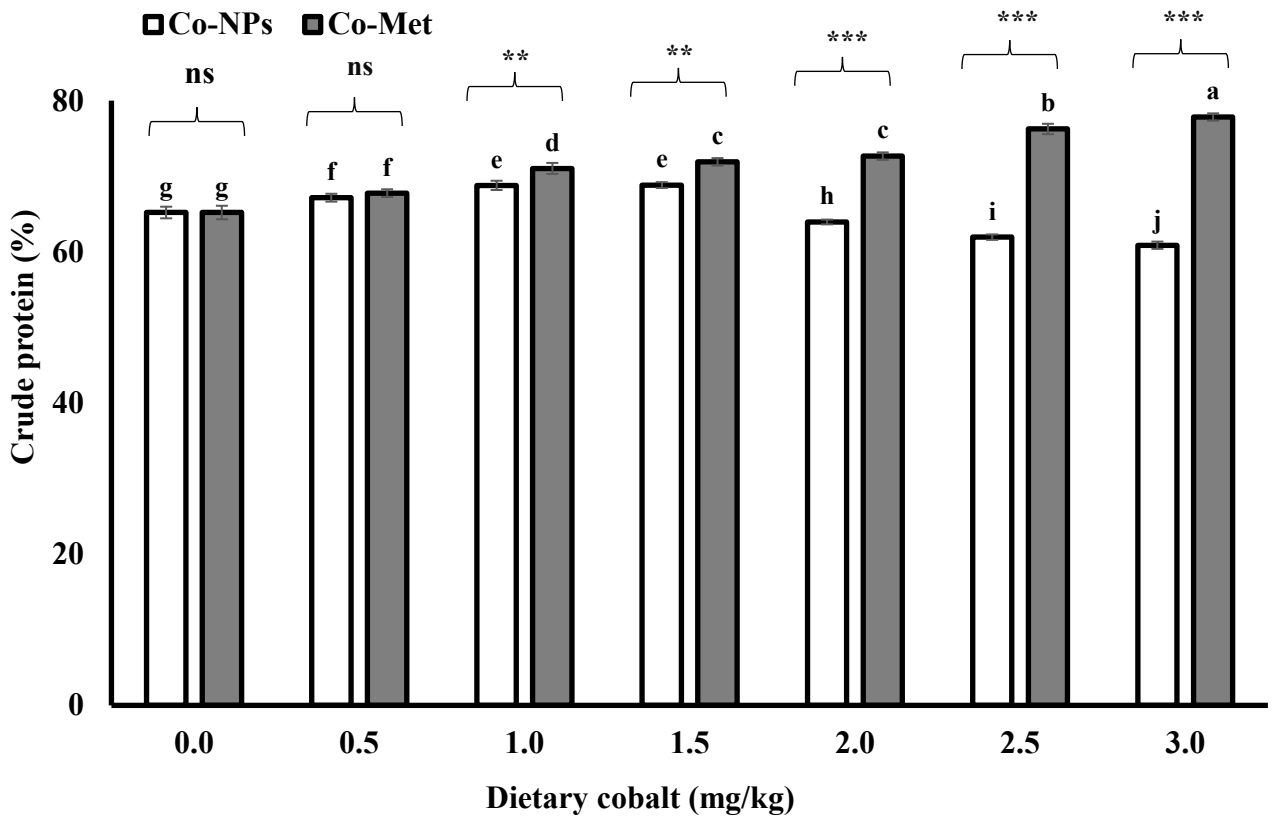

Figure 73. Muscle crude protein (%) content of *T.putitora* fingerlings after feeding graded levels of Co-NPs, and Co-Met complex supplemented diets for 90 days

Bars show the values as average± SE (n=9). Different letters on bars show significant difference. Data was analyzed by adopting ANOVA followed by LSD test to compare the effect of different Chemical forms, and dosage of dietary cobalt.

Moreover, difference between the two diet groups i.e., Co-NPs, and Co-Met complex at specific dosage level was determined by using paired T-test at 95% confidence intervals.

ns= non-significant difference; *= P≤0.01 ; **= P≤0.001; ***= P≤0.001

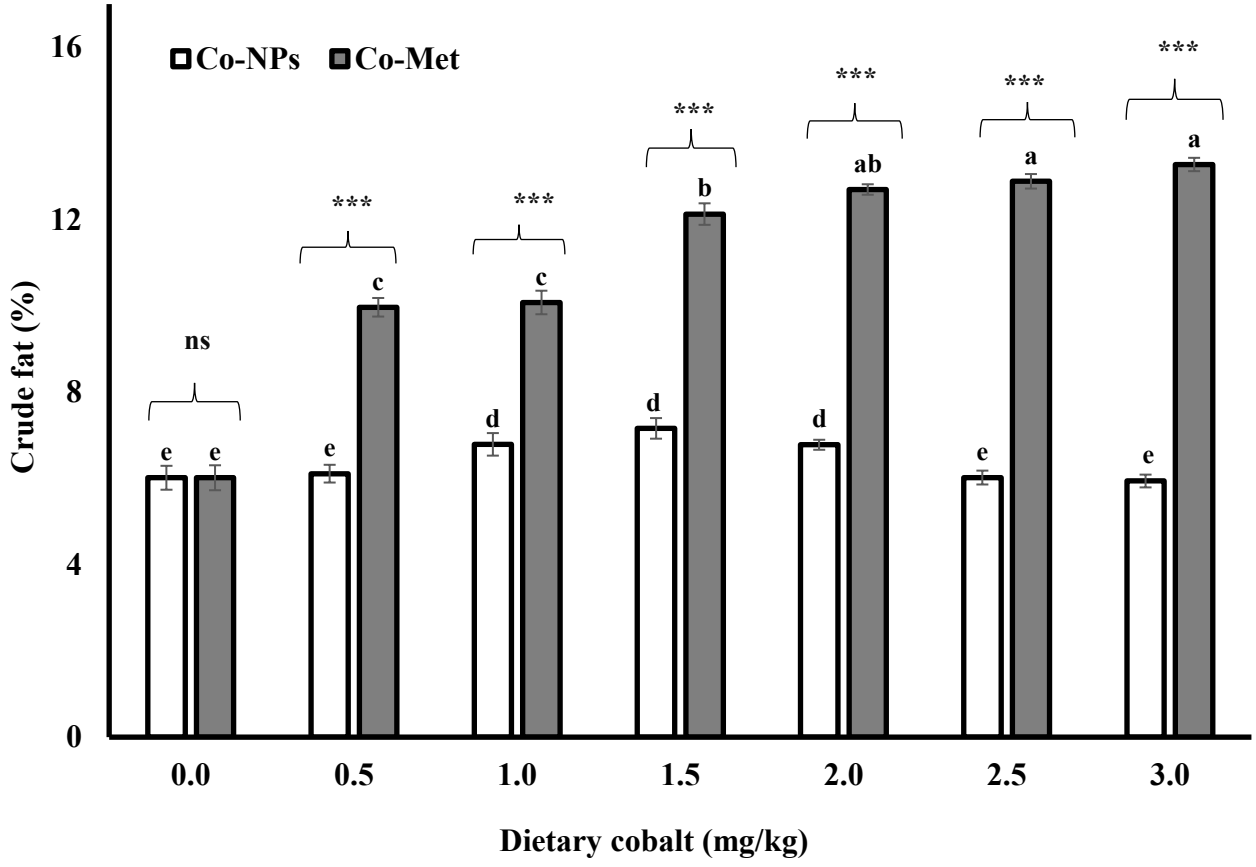

Figure 74. Muscle crude fat (%) content of *T.putitora* fingerlings after feeding graded levels of Co-NPs and Co-Met complex supplemented diets for 90 days

Bars show the values as average± SE (n=9). Different letters on bars show significant difference. Data was analyzed by adopting ANOVA followed by LSD test to compare the effect of different Chemical forms and dosage of dietary cobalt.

Moreover, difference between the two diet groups i.e., Co-NPs and Co-Met complex at specific dosage level was determined by using paired T-test at 95% confidence intervals.

ns= non-significant difference; *= $P \leq 0.01$; **= $P \leq 0.001$; ***= $P \leq 0.001$

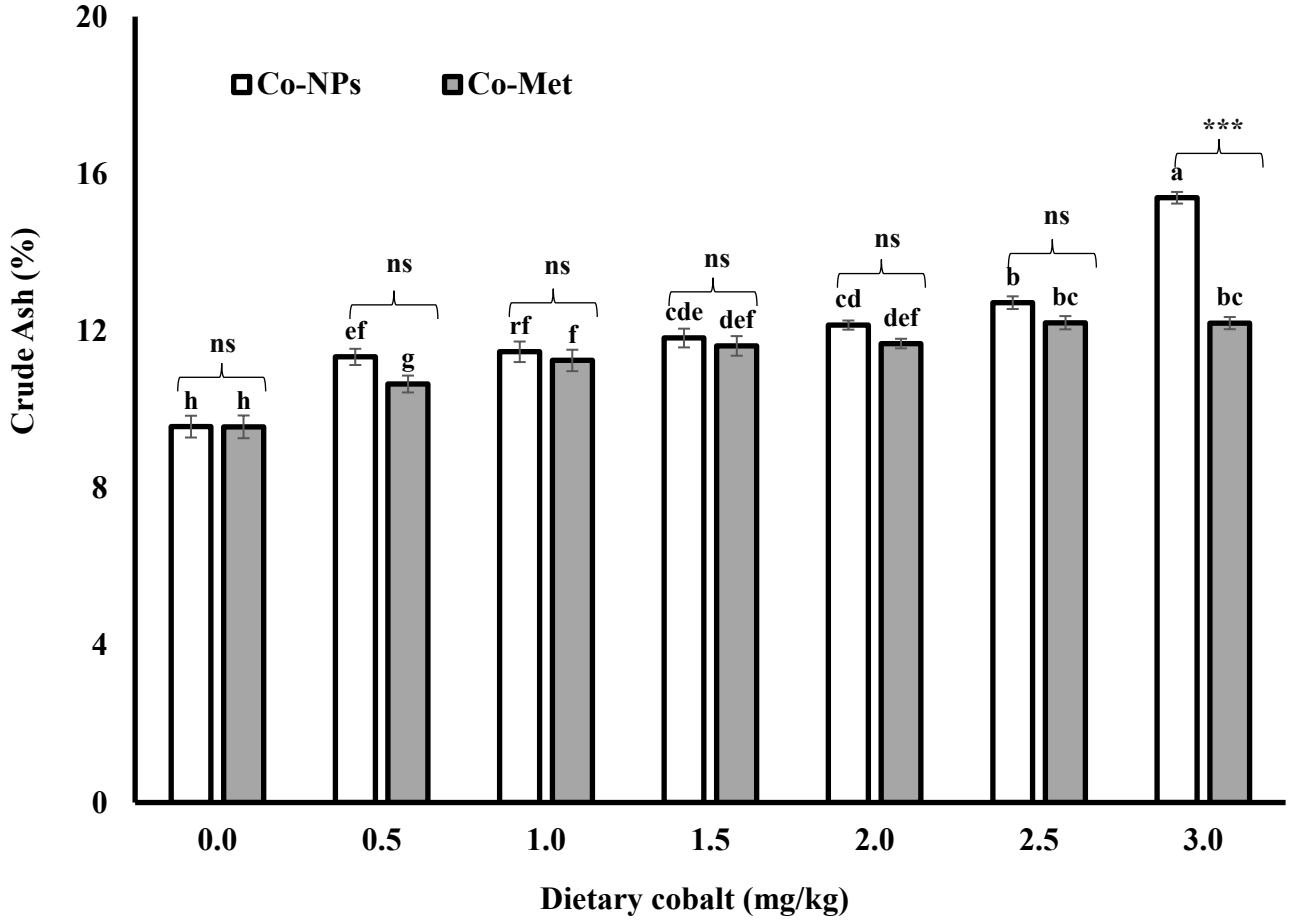

Figure 75. Muscle crude ash (%) content of *T.putitora* fingerlings after feeding graded levels of Co-NPs and Co-Met complex supplemented diets for 90 days

Bars show the values as average± SE (n=9). Different letters on bars show significant difference. Data was analyzed by adopting ANOVA followed by LSD test to compare the effect of different Chemical forms and dosage of dietary cobalt.

Moreover, difference between the two diet groups i.e., Co-NPs and Co-Met complex at specific dosage level was determined by using paired T-test at 95% confidence intervals.

ns= non-significant difference; *= P≤0.01 ; **= P≤0.001; ***= P≤0.001

Table 42.Summary of two way ANOVA showing the comparative effect of graded levels of different chemical forms of dietary cobalt on the metal bioaccumulation levels in muscle of *T.putitora* fingerlings

	df	SS	MS	F- value	P-value
Iron					
Chemical forms	1	0.001000	0.001000	990.42	<0.001
Dosage	6	0.001000	0.000085	94.5	<0.001
Chemical forms × Dosage	6	0.001000	0.000091	100.74	<0.001
Manganese					
Chemical forms	1	0.000009	0.000009	1086.86	<0.001
Dosage	6	0.000006	0.000001	126.43	<0.001
Chemical forms × Dosage	6	0.000005	0.000001	111.755	<0.001
Cobalt					
Chemical forms	1	0.000086	0.000086	10255.4	<0.001
Dosage	6	0.000054	0.000009	1058.92	<0.001
Chemical forms × Dosage	6	0.000043	0.000007	842.765	<0.001

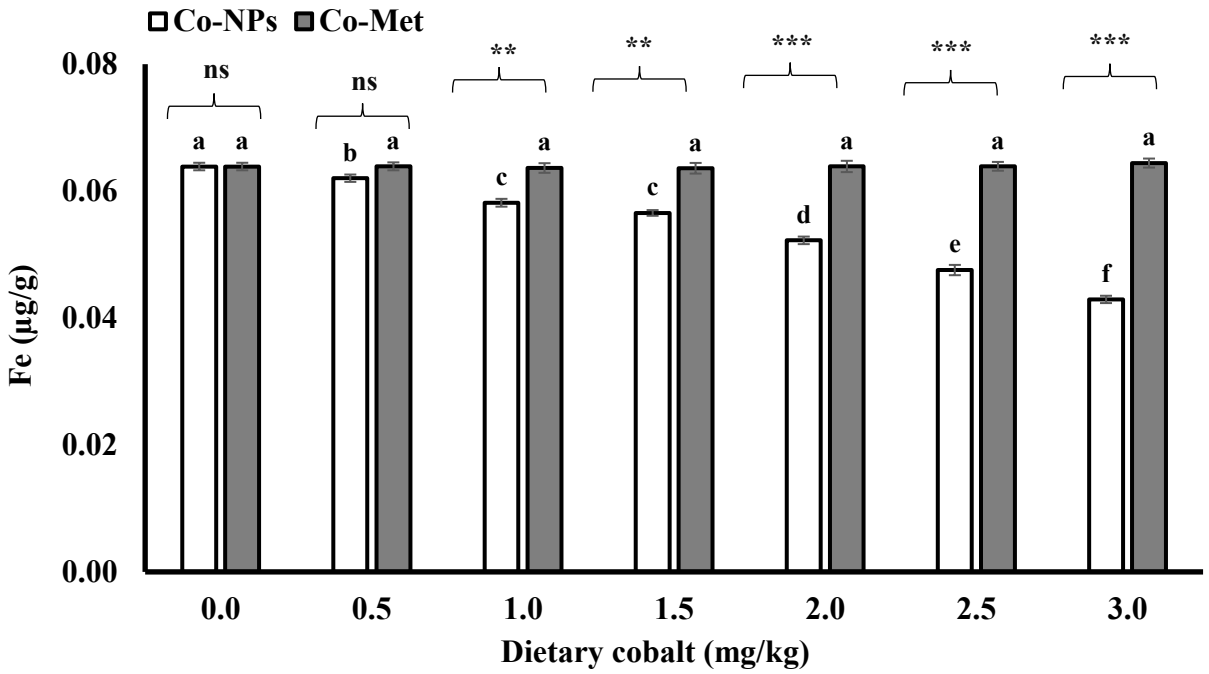

Figure 76. Muscle iron bioaccumulation level of *T.putitora* fingerlings after feeding graded levels of Co-NPs and Co-Met complex supplemented diets for 90 days

Bars show the values as average± SE (n=9). Different letters on bars show significant difference. Data was analyzed by adopting ANOVA followed by LSD test to compare the effect of different Chemical forms and dosage of dietary cobalt.

Moreover, difference between the two diet groups i.e., Co-NPs and Co-Met complex at specific dosage level was determined by using paired T-test at 95% confidence intervals.

ns= non-significant difference; *= $P \leq 0.01$; **= $P \leq 0.001$; ***= $P \leq 0.001$

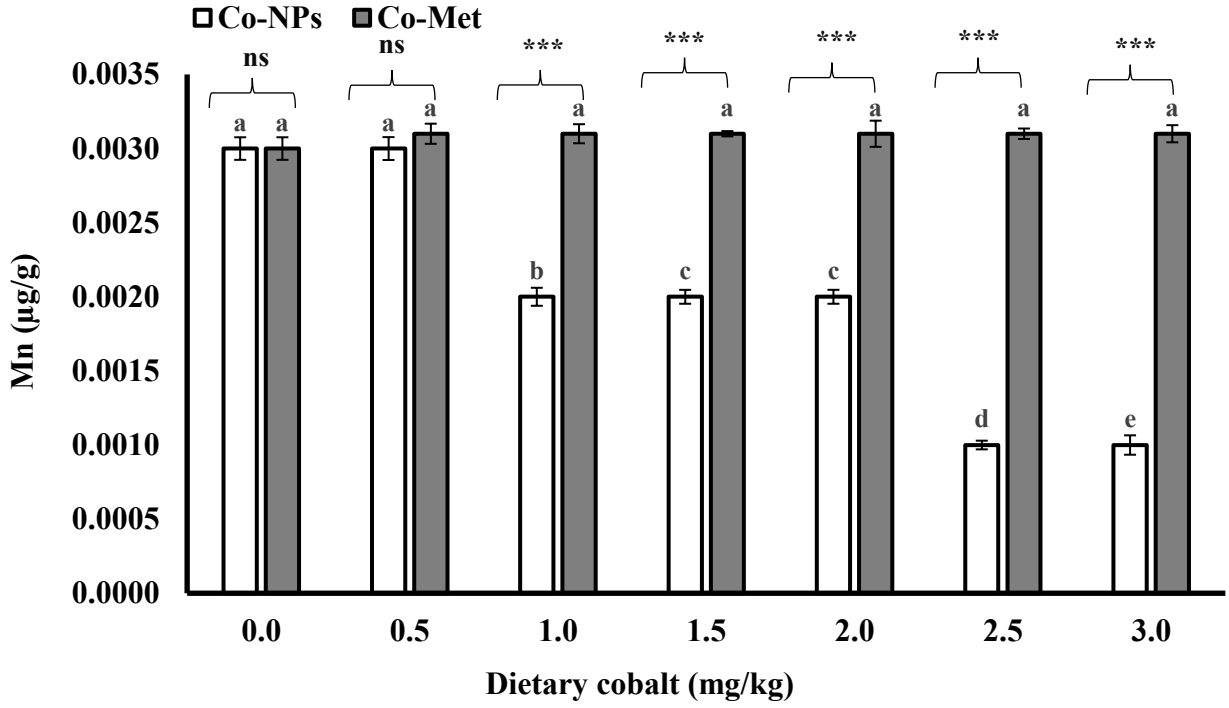

Figure 77. Muscle manganese bioaccumulation level of *T.putitora* fingerlings after feeding graded levels of Co-NPs and Co-Met complex supplemented diets for 90 days

Bars show the values as average± SE (n=9). Different letters on bars show significant difference. Data was analyzed by adopting ANOVA followed by LSD test to compare the effect of different Chemical forms and dosage of dietary cobalt.

Moreover, difference between the two diet groups i.e., Co-NPs and Co-Met complex at specific dosage level was determined by using paired T-test at 95% confidence intervals.

ns= non-significant difference; *= P≤0.01 ; **= P≤0.001; ***= P≤0.001

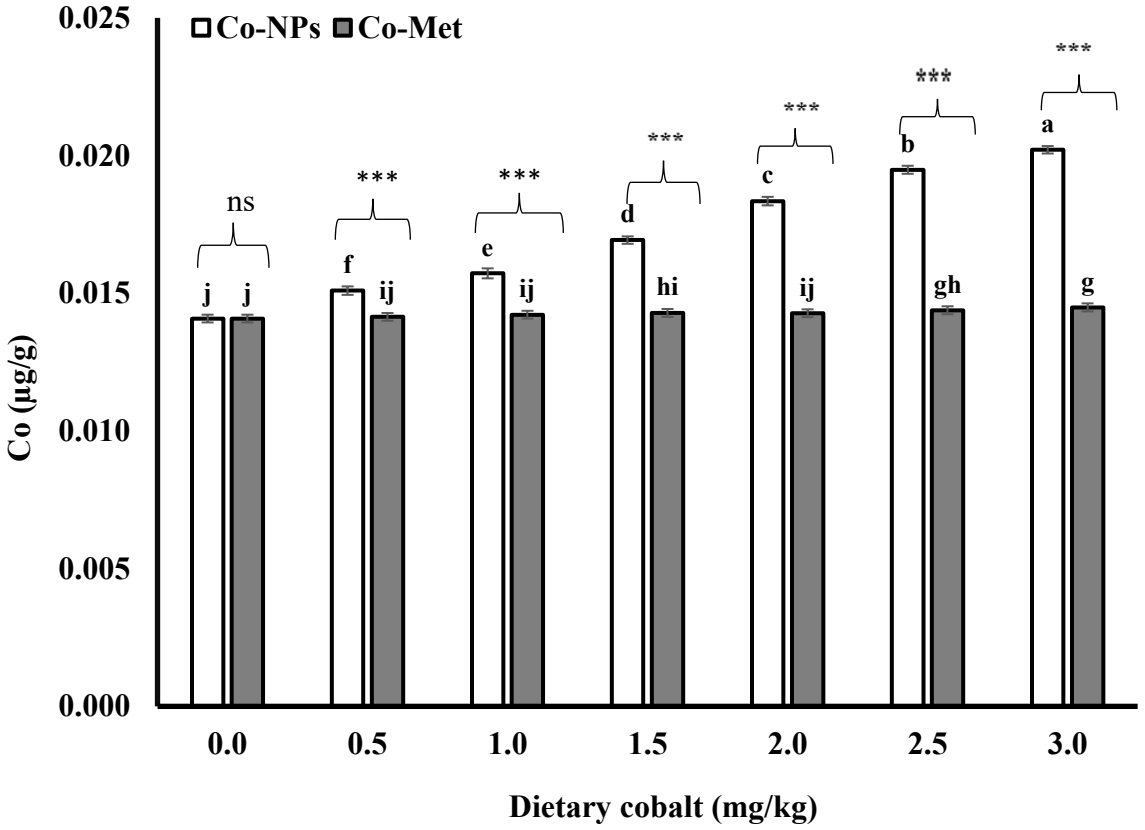

Figure 78. Muscle cobalt bioaccumulation level of *T.putitora* fingerlings after feeding graded levels of Co-NPs and Co-Met complex supplemented diets for 90 days

Bars show the values as average± SE (n=9). Different letters on bars show significant difference. Data was analyzed by adopting ANOVA followed by LSD test to compare the effect of different Chemical forms and dosage of dietary cobalt.

Moreover, difference between the two diet groups i.e., Co-NPs and Co-Met complex at specific dosage level was determined by using paired T-test at 95% confidence intervals.

ns= non-significant difference; *= $P \leq 0.01$; **= $P \leq 0.001$; ***= $P \leq 0.001$

CHAPTER 4

Evaluation of the practical application of organic and inorganic forms of cobalt supplemented diets on *T.putitora*

Growth performance.

The effect of Co-NPs and Co-Met complex supplemented diets on the growth performance of *T. putitora* after 90 days of rearing in earthen ponds is shown in table 43. Statistical analysis done by one-way ANOVA showed a significant difference in the final weight (n=3, $F_{4,10}$=303.45; PSE=0.329; P<0.001), %WG (n=3, $F_{4,10}$=458.36; PSE=1.699; P<0.001), %SGR (n=3,$F_{4,10}$=445.26; PSE=0.0078; P<0.001), AWG (n=3, $F_{4,10}$=412.92; PSE=0.283; P<0.001) and ADG (n=3, $F_{4,10}$=353.35; PSE=0.003; P<0.001) among all diet groups of fish. The post hoc pairwise comparison indicated the positive effect of dietary Co supplementation on the growth performance of TNI (Co-NPs 1.5mg/kg), TM3 (Co-Met 1.5mg/kg), and TM4 (Co-Met 3mg/kg) groups while a negative effect on a TN2 group (Co-NPs 3mg/kg), of fish. Results also showed improved growth performance parameters with an increase in Co-Met-complex supplementation while dietary Co-NPs showed a negative effect at a higher levels (3mg/kg diet). The all pairwise comparison indicated the highest growth performance parameters of TM4 followed by a TM3 and then a TNI group of fish.

Immunological indices

The effect of Co-NPs and Co-Met complex supplemented diets on the immunological indices of *T. putitora* after 90 days rearing in earthen ponds is shown in table 44. Statistical analysis done by one-way ANOVA showed a significant difference in the plasma protein (n=30, $F_{4,145}$=898.16; PSE=0.057; P<0.001), IgM (n=30, $F_{4,145}$=1644.32; PSE=0.038; P<0.001), lysozyme activity (n=30, $F_{4,145}$=888.29; PSE=0.048; P<0.001), WBCs count (n=30, $F_{4,145}$=523.45; PSE=2.403; P<0.001),respiratory burst activity (n=30, $F_{4,145}$=216.32; PSE=0.015; P<0.001) and

phagocytic activity (n=30,$F_{4,145}$=131.74; PSE=1.85; P<0.001) among all diet groups of fish. The post hoc pairwise comparison indicated the positive effect of dietary Co supplementation on the immunological indices of TNI (Co-NPs 1.5mg/kg), TM3 (Co-Met 1.5mg/kg), and TM4 (Co-Met 3mg/kg) groups whereas it showed negative effect on TN2 group (Co-NPs 3mg/kg) of fish. Furthermore, TN2 group of fish showed highest respiratory burst activity (NBT activity) and WBC count as compared to other diet groups.

Hematological indices

The effect of Co-NPs and Co-Met complex supplemented diets on the hematological indices of *T. putitora* after 90 days rearing in earthen ponds is shown in table 45. Statistical analysis done by one-way ANOVA showed a significant difference in the RBC count (n=30, $F_{4,145}$=328.82; PSE=0.014; P<0.001), Hb(n=30, $F_{4,145}$=593.51; PSE=0.031; P<0.001), HCT (n=30, $F_{4,145}$=110.41; PSE=0.521;P<0.001),MCV(n=30,$F_{4,145}$=15534.34;PSE=0.307;P<0.001),MCH(n=30,$F_{4,145}$=4951.69;PSE=0.187; P<0.001) and MCHC (n=30, $F_{4,145}$=7711.30; PSE=0.121; P<0.001) among all diet group of fish. The post hoc pairwise comparison indicated the positive effect of dietary Co supplementation on the hematological indices of TNI (Co-NPs 1.5mg/kg), TM3 (Co-Met 1.5mg/kg), and TM4 (Co-Met 3mg/kg) groups while a negative effect on TN2 group (Co-NPs 3mg/kg) of fish. Results also indicated improved blood parameters with an increase in Co-Met-complex supplementation whereas Co-NPs showed a negative effect at a higher level (3mg/kg diet). Pairwise comparison indicated significantly highest blood parameters in TM4 followed by a TM3 group of fish.

Biochemical indices

The effect of Co-NPs and Co-Met complex supplemented diets on the serum biochemical indices of *T. putitora* after 90 days rearing in earthen ponds is shown in table 46. Statistical analysis done by one-way ANOVA showed a significant difference in the serum AST (n=30, $F_{4, 145}$=161.33; PSE=0.043; P<0.001) and ALT (n=30, $F_{4,145}$=57.35; PSE=0.060; P<0.001) activities among all diet groups of fish. The post hoc pairwise comparison indicated highest AST and ALT activity in TN2 (Co-NPs 3mg/kg) followed by TN1 (Co-NPs 1.5mg/kg) and CNM group of fish whereas lowest activity was observed in TM4 (Co-Met 3mg/kg) group of fish.

Muscle proximate composition

The effect of Co-NPs and Co-Met complex supplemented diets on the on muscle proximate composition of *T. putitora* after 90 days rearing in earthen ponds is shown in table 47. Statistical analysis done by one-way ANOVA showed a significant effect on the muscle %crude protein (n=30, $F_{4,145}$=217.39; PSE=0.0478; P<0.001), %fat (n=30,$F_{4,145}$=448.25; PSE= 0.178; P<0.001) and ash % (n=30, $F_{4,145}$=114.83; PSE=0.277; P<0.001) content among all diet groups of fish. The post hoc pairwise comparison indicated highest muscle crude protein and fat content in TM4 group (Co-Met 3mg/kg) of fish followed by TM3 (Co-Met 1.5 mg/kg) and TN1 (Co-NPs 1.5mg/kg) group of fish whereas highest muscle crude ash content was observed in TN2 (Co-NPs 3mg/kg) group of fish.

Muscle amino acid profile

The effect of Co-NPs and Co-Met complex supplemented diets on the muscle amino acid profile of *T. putitora* after 90 days rearing in earthen ponds is shown in table 48. Statistical analysis

done by one-way ANOVA showed a significant effect on the muscle amino acids profile among all diet groups of fish i.e., threonine($n=6, F_{4,25}=53.4$;PSE=0.030;$P<0.001$),methionine($n=6, F_{4,25}=68.5$;PSE=0.156;$P<0.001$),leucine($n=6, F_{4,25}=190$;PSE=0.043;$P<0.001$),valine($n=6, F_{4,25}=10$.4;PSE=1.25;$P<0.001$),Isoleucine($n=6, F_{4,25}=58.6$;PSE=0.128;$P<0.001$),phenylalanine($n=6, F_{4,25}=$349;PSE=0.254;$P<0.001$),Histidine($n=6, F_{4,25}=79.2$; PSE=0.168; $P<0.001$),lysine($n=6$, $F_{4,25}=27.6$; PSE=0.278; $P<0.001$),arginine($n=6$, $F_{4,25}=209$; PSE=0.112; $P<0.001$), Aspartic acid ($n=6$, $F_{4,25}=4.08$; PSE=0.162; $P<0.001$), Serine ($n=6$, $F_{4,25}=61$; PSE=0.093; $P<0.001$), and Alanine ($n=6$, $F_{4,25}=220$; PSE=0.164; $P<0.001$). The post hoc pairwise comparison indicated highest muscle essential amino acids in TM4 group of fish followed by TM3 and TN1 group of fish.

Expression of growth and stress related genes

The effect of Co-NPs and Co-Met complex supplemented diets on the mRNA expression of MyoD, MyoG in muscle and heat shock protein 70KDa in the liver of *T. putitora* after 90 days rearing in earthen ponds is shown figure 79, 80 and 81 respectively. Statistical analysis done by one-way ANOVA showed a significant effect on the mRNA level of MyoD ($n=6, F_{4,25}=243.93$;PSE=0.061;$P<0.001$), MyoG ($n=6, F_{4,25}=36.26$;PSE=0.104;$P<0.001$) in the muscle and HSP-70KDa ($n=6, F_{4,25}=48.81$;PSE=0.176;$P<0.001$) in the liver of all diet groups of fish. The post-hoc pairwise comparison indicated a significant higher mRNA levels of MyoD and MyoG in the muscle of fish reared on diet supplemented with 3mg Co-met complex/kg diet (TM4 group) as compared to other diet groups of fish. However both of these growth genes were downregulated in muscle of TN2 group of fish. (Fig. 79 and 80 respectively). Moreover, highest expression of heat shock protein was observed in the liver of TN2 group (Co-NPs 3mg/kg) of fish which were fed 3mg Co-NPs kg diet followed by TN1 group of fish (Fig.81).

Table 43. Growth performance of *T. putitora* after 90 days rearing in earthen ponds on Co-NPs (inorganic) and Co-Met complex (organic) supplemented diets

	Diet groups					Statistical Analysis		
	CNM	**TN1**	**TN2**	**TM3**	**TM4**	**PSE [a]**	**P-value**	**F- value**
W_i (g)	15.97^{a}	15.98^{a}	15.97^{a}	15.93^{a}	15.92^{a}	0.940	0.953	0.162
W_f (g)	36.90^{d}	39.92^{c}	33.70^{e}	40.90^{b}	44.37^{a}	0.329	0.001	303.45
WG (%)	131.04^{d}	149.82^{c}	111.23^{e}	156.83^{b}	178.67^{a}	1.699	0.001	458.36
SGR(%body weight/day)	0.93^{d}	1.02^{c}	0.83^{e}	1.05^{b}	1.14^{a}	0.0078	0.001	445.26
AWG (g)	20.93^{d}	23.94^{c}	17.73^{e}	24.97^{b}	28.45^{a}	0.283	0.001	412.92
ADG (g)	0.23^{d}	0.27^{c}	0.20^{e}	0.28^{b}	0.32^{a}	0.003	0.001	353.35

Data is expressed as average value (n=3).Values with different letters within the same rows are significantly different ($P<0.05$). Data was analyzed by adopting ANOVA followed by LSD.

Wi=average initial weight of fish W_f=average final weight of fish; %WG= percentage weight gain; AWG= Average weight gain during 90 days/fish; ADG= Average daily weight gain/fish; %SGR=Specific growth rate (%body weight/day);

CNM= Control group; TN1= 1.5 mg Co-NPs/kg diet; TN2= 3.0 mg Co-NPs/kg diet; TM3= 1.5 mg Co-Met complex /kg diet; TM4= 3.0 mg Co-Met complex /kg diet.

Table 44. Immunological indices of *T.putitora* after 90 days rearing in earthen ponds on Co-NPs (inorganic) and Co-Met complex (organic) supplemented diets

	Diet groups					Statistical Analysis		
	CNM	**TN1**	**TN2**	**TM3**	**TM4**	**PSE [a]**	**P-value**	**F- value**
Plasma protein (mg/ml)	5.63^{d}	6.13^{c}	4.82^{e}	6.83^{b}	8.01^{a}	0.057	0.001	898.16
IgM (mg/ml)	3.26^{d}	3.81^{c}	2.28^{e}	4.51^{b}	5.23^{a}	0.038	0.001	1644.32
lysozyme activity (µg/ml)	6.25^{d}	6.97^{c}	6.01^{e}	7.45^{b}	8.54^{a}	0.048	0.001	888.29
WBC (10^3 µ/L)	158.79^{b}	159.52^{b}	247.87^{a}	163.40^{b}	162.49^{b}	2.403	0.001	523.45
NBT (OD=540nm)	0.642^{c}	0.675^{b}	0.804^{a}	0.462^{d}	0.394^{e}	0.015	0.001	216.32
Phagocytic activity (%)	51.94^{c}	55.63^{b}	29.14^{d}	57.08^{b}	70.67^{a}	1.85	0.001	131.74

Data is expressed as average value (n=30).Values with different letters within the same rows are significantly different (P<0.05). Data was analyzed by adopting ANOVA followed by LSD.

a=Pooled standard error mean

CNM= Control group; TN1= 1.5 mg Co-NPs/kg diet; TN2= 3.0 mg Co-NPs /kg diet; TM3= 1.5 mg Co-Met complex/kg diet; TM4= 3.0 mg Co-Met complex /kg diet

Table 45. Hematological indices of *T. putitora* after 90 days rearing in earthen ponds on Co-NPs (inorganic) and Co-Met complex (organic) supplemented diets

	Diet groups					Statistical Analysis		
	CNM	**TN1**	**TN2**	**TM3**	**TM4**	**PSE [a]**	**P-value**	**F- value**
RBC (10^6µ/L)	1.110^{c}	1.178^{b}	1.074^{d}	1.201^{b}	1.55^{a}	0.014	0.001	328.82
HB (g d/l)	6.802^{d}	7.22^{c}	6.49^{e}	7.367^{b}	7.88^{a}	0.031	0.001	593.95
HCT (%)	33.11^{c}	36.79^{b}	32.15^{c}	36.09^{b}	42.02^{a}	0.521	0.001	110.41
MCV (cm^3 erythrocyte)	171.9^{b}	172.31^{b}	114.29^{c}	175.19^{ab}	178.09^{a}	0.307	0.001	15534.34
MCH (pg)	32.68^{d}	33.14^{c}	27.08^{e}	43.93^{b}	50.03^{a}	0.187	0.001	4951.69
MCHC (Hb/100 ml blood)	42.63^{d}	45.97^{c}	39.27^{e}	55.53^{b}	55.82^{a}	0.121	0.001	7711.30

Data is expressed as average value (n=30).Values with different letters within the same rows are significantly different ($P<0.05$). Data was analyzed by adopting ANOVA followed by LSD

a=Pooled standard error mean; RBC= red blood cell count; HB= Hemoglobin level; HCT= hematocrit; MCV=mean corpuscular volume; MCH= mean corpuscular hemoglobin; MCHC= corpuscular hemoglobin concentration

CNM= Control group; TN1= 1.5 mg Co-NPs/kg diet; TN2= 3.0 mg Co-NPs/kg diet; TM3= 1.5 mg Co-Met complex /kg diet; TM4= 3.0 mg Co-Met complex /kg diet

Table 46: Biochemical indices of *T. putitora* after 90 days rearing in earthen ponds on Co-NPs (inorganic) and Co-Met complex (organic) supplemented diets

	Diet groups					Statistical Analysis		
	CNM	**TN1**	**TN2**	**TM3**	**TM4**	**PSE [a]**	**P-value**	**F- value**
AST activity (U/L)	3.21^{b}	3.17^{b}	3.53^{a}	2.96^{c}	2.48^{d}	0.043	0.001	161.33
ALT activity (U/L)	2.81^{b}	2.82^{b}	3.44^{a}	2.80^{b}	2.58^{c}	0.060	0.001	57.35

Data is expressed as average value (n=30).Values with different letters within the same rows are significantly different (P<0.05).

a=Pooled standard error mean

CNM= Control group; TN1= 1.5 mg Co-NPs/kg diet; TN2= 3.0 mg Co-NPs/kg diet; TM3= 1.5 mg Co-Met complex/kg diet; TM4= 3.0 mg Co-Met complex /kg diet

Table 47: Muscle proximate composition of *T.putitora* after 90 days rearing in earthen ponds on Co-NPs (inorganic) and Co-Met complex (organic) supplemented diets

	Diet groups					Statistical Analysis		
	CNM-2	**TN1**	**TN2**	**TM3**	**TM4**	**PSE [a]**	**P-value**	**F- value**
Protein (%)	60.31^{d}	65.88^{c}	57.94^{e}	66.87^{b}	70.16^{a}	0.478	0.0001	217.39
Fat (%)	9.125^{c}	12.81^{b}	8.90^{c}	14.31^{a}	14.22^{a}	0.178	0.0001	448.25
Ash (%)	11.83^{c}	13.05^{b}	17.26^{a}	12.76^{b}	13.30^{b}	0.277	0.0001	114.83

Data is expressed as average value (n=30).Values with different letters within the same rows are significantly different ($P<0.05$). Data was analyzed by adopting ANOVA followed by LSD a=Pooled standard error mean

CNM= Control group; TN1= 1.5 mg Co-NPs/kg diet; TN2= 3.0 mg Co-NPs/kg diet; TM3= 1.5 mg Co-Met complex/kg diet; TM4= 3.0 mg Co-Met complex /kg

Table. 48. Muscle amino acid profile of *T.putitora* after 90 days rearing in earthen ponds on Co-NPs (inorganic) and Co-Met complex (organic) supplemented diets

	Diet groups					Statistical Analysis		
	CNM	**TN1**	**TN2**	**TM3**	**TM4**	**PSE [a]**	**P-value**	**F- value**
Essential amino acids (EAA)								
Threonine	1.37^{c}	1.72^{b}	0.49^{d}	1.70^{b}	2.41^{a}	0.030	0.001	534
Methionine	4.94^{b}	6.71^{a}	3.76^{c}	6.50^{a}	6.56^{a}	0.156	0.001	68.5
Leucine	1.71^{b}	1.77^{b}	0.69^{c}	1.74^{b}	2.35^{a}	0.043	0.001	190
Valine	7.96^{d}	10.94^{c}	6.83^{de}	16.47^{b}	19.21^{a}	1.250	0.001	10.4
Isoleucine	2.13^{d}	2.64^{c}	1.46^{e}	3.32^{b}	3.96^{a}	0.128	0.001	58.6
Phenylalanine	6.06^{d}	10.44^{c}	6.27^{d}	13.70^{b}	17.05^{a}	0.254	0.001	349
Histidine	1.53^{d}	2.56^{c}	0.31^{e}	3.23^{b}	4.15^{a}	0.168	0.001	79.2
Lysine	3.55^{c}	4.26^{bc}	1.91^{d}	4.71^{b}	5.85^{a}	0.278	0.001	27.6
Non-Essential amino acids (NEAA)								
Arginine	5.67^{d}	6.18^{c}	3.52^{e}	7.16^{b}	7.59^{a}	0.112	0.001	209
Aspartic acid	1.17^{a}	1.07^{ab}	1.13^{a}	0.61^{b}	1.53^{a}	0.162	0.001	4.08
Serine	3.74^{c}	4.29^{b}	2.73^{d}	3.63^{c}	4.65^{a}	0.093	0.001	61
Alanine	6.22^{c}	5.66^{d}	11.53^{a}	6.57^{c}	8.93^{b}	0.164	0.001	220

Data is expressed as average value (n=6).Values with different letters within the same rows are significantly different ($P<0.05$). Data was analyzed by adopting ANOVA followed by LSD.
a=Pooled standard error mean
CNM= Control group; TN1= 1.5 mg Co-NPs/kg diet; TN2= 3.0 mg Co-NPs/kg diet; TM3= 1.5 mg Co-Met complex/kg diet; TM4= 3.0 mg Co-Met complex/kg diet

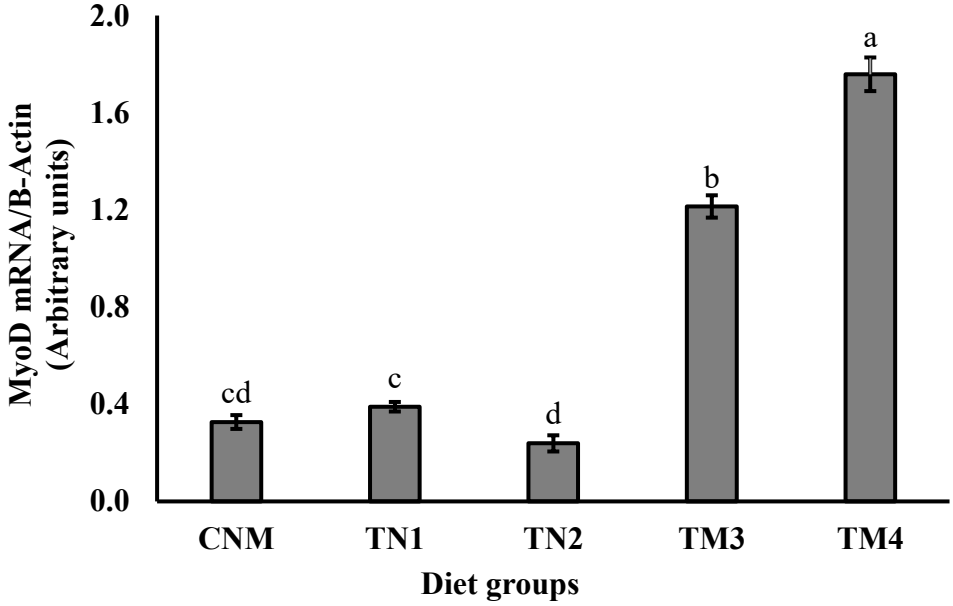

Figure 79. mRNA expression level of myoblast determination protein 1 homolog MyoD gene in muscle tissues of *T.putitora* after 90 days rearing in earthen ponds on Co-NPs (inorganic) and Co-Met complex (organic) supplemented diets

Bars shows the values as average± SE (n=6). Data was analyzed by adopting ANOVA followed by LSD. Different letters on bars shows significant difference.

CNM= Control group; TN1= 1.5 mg Co-NPs/kg diet; TN2= 3.0 mg Co-NPs /kg diet; TM3= 1.5 mg Co-Met complex/kg diet; TM4= 3.0 mg Co-Met complex/kg diet

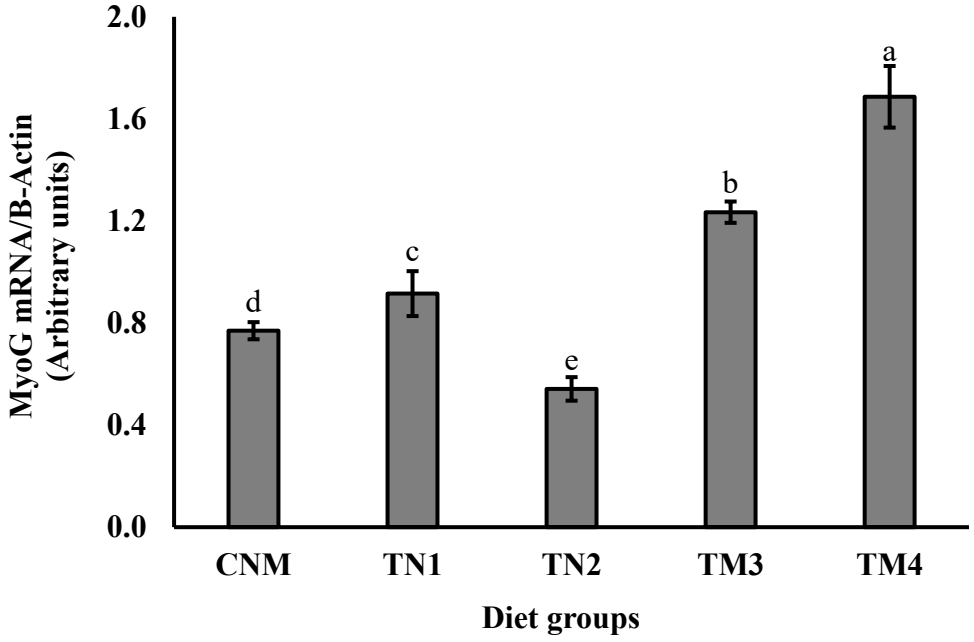

Figure 80. mRNA expression level of myogenin gene in Muscle tissues of *T. putitora* after 90 days rearing in earthen ponds on Co-NPs (inorganic) and Co-Met complex (organic) supplemented diets

Bars shows the values as average± SE (n=6). Different letters on bars shows significant difference. Data was analyzed by adopting ANOVA followed by LSD

CNM= Control group; TN1= 1.5 mg Co-NPs/kg diet; TN2= 3.0 mg Co-NP /kg diet; TM3= 1.5 mg Co-Met complex/kg diet; TM4= 3.0 mg Co-Met complex/kg diet

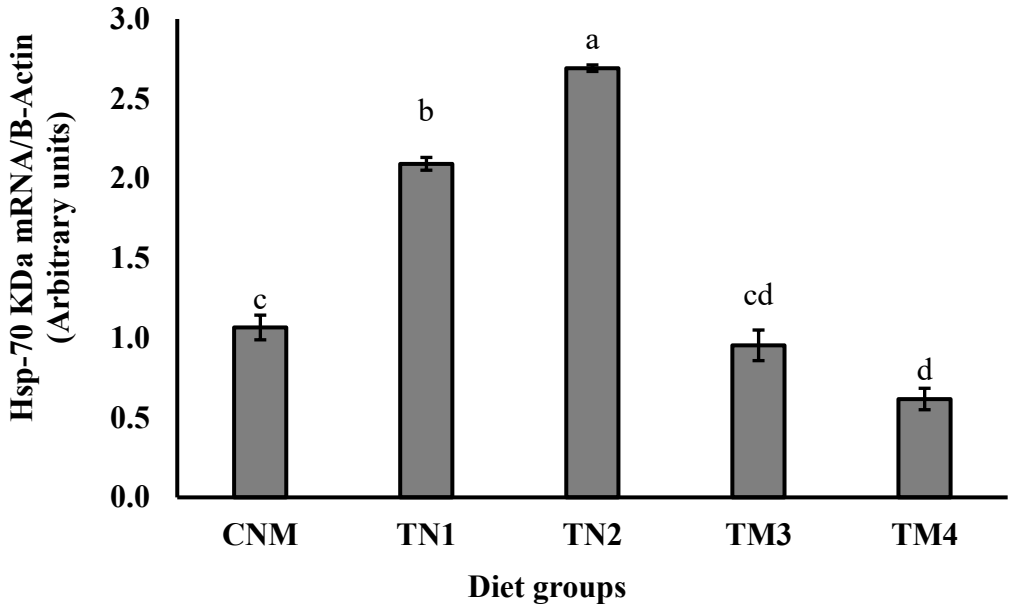

Figure 81. mRNA expression level of heat shock protein 70KDa in liver of *T. putitora* after 90 days rearing in earthen ponds on Co-NPs (inorganic) and Co-Met complex (organic) supplemented diets

Bars shows the values as average± SE (n=6). Different letters on bars shows significant difference. Data was analyzed by adopting ANOVA followed by LSD

CNM= Control group; TN1= 1.5 mg Co-NPs/kg diet; TN2= 3.0 mg Co-NPs /kg diet; TM3= 1.5 mg Co-Met complex/kg diet; TM4= 3.0 mg Co-Met complex/kg diet

DISCUSSION

The important role of many minerals in various intra and extracellular physiological functions of the body and their impact at an optimum level on the growth and annual production of fish is well documented (Lall, 2003). A dietary mineral requirement of fish depends on different factors, like chemical form, particle size of the mineral, dosage level, genetic makeup, nutritional status, and developmental stage of fish (Kratzer, 2018). Based on concentration and consumption, many investigators reported dose-dependent toxicity of various metals in different fish species (Jaishankaret al., 2014; Green and Planchart, 2018; Ale et al., 2018).

Though the effect of dietary cobalt supplementation on the growth performance, survival, protein synthesis, hematological, and immune indices of fish has been reported by some investigators (Blust, 2011; Nasri et al., 2019), no information is available regarding cobalt uptake, its utilization, bioaccumulation, and nutritional requirement in *T.putitora*. Therefore, the present study was designed to determine the optimum dietary supplementation levels of an organic and inorganic form of cobalt for *T. putitora*. The study was conducted in three phases. First optimum dietary supplementation level of the conventional inorganic form of cobalt, cobalt chloride ($CoCl_2$) was determined by feeding graded levels of $CoCl_2$ as feed supplement for 90 days to different groups of fish and evaluating its effect on growth performance, proximate body composition, and metal bioaccumulation level in the muscle of advanced fry of *T. putitora.*

Fish growth performance is considered a reliable indicator to evaluate the beneficial and toxicological effects of metal (Jaishankar et al., 2014). In the present study, the improved growth performance of the fish i.e., higher %WG, %SGR and AWG was observed with the increasing level of cobalt up to supplementation level of 2mg/kg diet (R^2= 0.889; Fig.34) and beyond that fish showed reduced growth (Table 22). Like our results, Sapkale et al. (2011) also reported improved growth performance of *Lates calcarifer* at the same dosage level, i.e., 2mg Co/kg diet. However, other fish species showed optimum growth performance at different dosage levels, i.e., greater than or lower

than the dosage level observed in the present study. For instance, Mukherjee and Kaviraj, (2009) reported a 40% increase in weight gain and improved survival rates of *C. carpio* hatchlings when reared on diet supplemented with 1mgCo/kg diet, while at the same dosage level (1mg Co/kg diet), Blust, (2011) reported a 229% increase in weight gain, and 108% increase in total body length of *Liza parsia*. However, *Ictalurus punctatus* showed optimum growth performance at lower dosage levels, i.e., 0.5mg Co/kg diet (Sapkale et al., 2011), while 0.1% and 0.003% dietary cobalt supplementation had been proved as an important candidate to improve the growth indices of *Heteropneustes fossilis* (Mukherjee and Kaviraj, 2011), and *C.carpio* fingerlings (Mukherjee and Kaviraj, 2009) respectively. Moreover, some fish showed better performance at higher dosage level e.g. *Clarias batrachus* at 5mg Co/kg diet (Sapkale and Singh, 2011), and *Epinephelus malabaricus* at 10mgCo/kg diet (Lin et al., 2010).The dietary requirement of minerals depends on species, developmental stage, size, sex, feeding, and culture conditions of fish (Hans and Jana, 2018).Therefore, differences in optimal dietary requirement of cobalt in different fish species may be due to variation in feeding habits, physiology, and metabolic demand of micro minerals. The improved growth performance observed here or reported by others in different fish species in response to dietary cobalt could be due to the involvement of Co as a cofactor in many metabolic enzymes and regulation of physiological and biochemical processes, manufacturing of muscular protein and hemoglobin synthesis in fish (Paul, 2001; Blust, 2011). Moreover, Hertz et al.(1989) reported an increase in the labeled amino acid incorporation into fish muscle proteins in response to dietary Co supplementation and indicating a protein-sparing effect of cobalt in fish.

Cobalt is an essential micro-mineral (Blust, 2011), but beyond optimum requirement, it becomes toxic (Mukherjee and Kaviraj, 2009). For instance, at higher cobalt supplementation, *Oncorhynchus mykiss* showed a reduced growth rate, hemorrhage in the gastrointestinal tract, and changes in WBCs count (Atamanalp et al., 2011). In our study, dietary cobalt above the 2mg/kg diet

inclusion level negatively affected the growth performance of fish, i.e., groups of fish fed 2.5mg Co/kg diet, and 3mg Co /kg diet showed 8% and 11% reduced %WG respectively (Table 22) as compared to control group of fish, thus indicating a dosage-dependent toxic effect of dietary cobalt in fish. Similar observations have been reported to show reduced growth performance of fish in response to dietary cobalt above the optimal level, e.g. *Heteropneustes fossilis* showed reduced weight gain when a dietary supplementation level of Co was above 0.01% (Mukherjee et al., 2011). Similarly, in rainbow trout, Co at higher dietary supplementation level, i.e., above 5g/kg diet caused toxicity (Chanda et al., 2015), while *L. rohita* showed decrease growth rate when exposed to sub-lethal concentrations of cobalt (Alkesh and Bharat, 2014). The reduced growth rate may be due to reduced feed intake (Javed, 2013) or inhibition of certain enzymes. It is well-documented that cobalt has a high affinity with SH (sulfhydryl) groups and at higher concentration it displaced the cations (Fe, Zn, etc.) from metal activated enzymes and inhibits important mitochondrial enzymes involved in respiration (Additives et al., 2009), thus resulting in oxidative stress and damage DNA, protein, and fat (Marr et al., 1998; Blust, 2011; Simonsen et al., 2012). Many investigators reported cytotoxic, carcinogenic, and genotoxic effects associated with the inflammatory response and necrosis in response to concentrations of Co above optimal levels in different organisms (De Boeck et al., 2003; Simonsen et al., 2012).

Dietary cobalt up to an inclusion level of 2mg/kg also showed a significant dose-dependent positive effect on the proximate composition of *T. putitora*. The impact of the feed formulation on the overall proximate composition of fish is well documented (Shearer, 1994; Rasmussen, 2001; Karapanagiotidis et al., 2019). The proximate composition, i.e., crude protein, fat, and ash contents provides information about the efficiency of nutrient transfer from feed to the fish (Pangle and Sutton, 2005) and the overall physiological condition of fish (Yeannes and Almandos, 2003). It is considered an important quality indicator that depends on multiple exogenous (water temperature, salinity, DO,

etc.), endogenous (age, strain, size of fish), and dietary factors (composition, feeding frequency, ration level, etc.) (Alemu et al., 2013). In the present experiment, all groups of fish were reared on the same 40% crude protein diet under similar culture conditions.

Fish are nutritious source of proteins, vitamins, omega-3 fatty acids, and minerals. Protein content of fish has biological importance due to the presence of all essential amino acids and higher digestibility value (Kaushik and Seiliez, 2010; Sujatha et al., 2013). In our experiment, crude protein level of advanced fry of *T. putitora* increased in response to cobalt up to an inclusion level of 2mg/kg as compared to a non-supplemented group of fish (control) (Table 23). According to Finley et al. (2012), cobalt is involved in the synthesis of proteins, RBC, and fatty acids and repairs of the myelin sheath of central nervous system (CNS). Consistent with our results, different scientists had reported an increase in crude protein content of fish in response to dietary cobalt. Mukherjee and Kaviraj (2009) have reported an increase in the muscle crude content of *C. carpio* when reared on diet supplemented with 1% cobalt, whereas 0.10% cobalt supplemented diet significantly improved the crude protein level of *Heteropneustes fossilis* (Mukherjee and Kaviraj, 2011). Furthermore, similar results were reported by Tonye and Sikoki (2014) in *Oreochromis niloticus* and Al-Ghanem (2011) in *C. carpio.* The increase in protein content in response to cobalt observed here and reported by others may be due to the involvement of Co in protein-sparing effect and the incorporation of amino acids in fish muscle (Hertz et al., 1989; Kawakami et al., 2012). According to Tonye and Sikoki (2014) cobalt is a biologically active element which plays several metabolic function inside the body; enhanced synthesis of muscular protein, and nitrogen assimilation. In our experiment, increase in dietary cobalt supplementation above 2mg/kg reduced the whole body crude protein content of fish, which may be due to the dose-dependent toxic effect of cobalt inside the body (Finley et al., 2012). In contrast, body crude protein and fat contents showed an increasing trend with an increased level of Co in the diet (Fig.37). Similar to our results, an increase in crude fat in response to dietary cobalt

was observed in common carp (Mukherjee and Kaviraj, 2009; Al-Ghanem, 2011), *Heteropneustes fossilis* (Bloch) (Mukherjee and Kaviraj, 2011), and *Oreochromis niloticus* (Tonye and Sikoki, 2014). Other than fish, dietary cobalt up to 0.15-0.25 mg/kg feed is commonly used as a fattening agent in cattle farming (Schwarz et al., 2000; Spears, 2002; Kincaid et al., 2003; Nocek et al., 2006).

Fish are a healthy source of essential micro minerals for human consumption (Steffens, 2006). The fish's proximate composition analysis also includes analysis of ash content: mineral content in fish. Unfortunately, the inclusion of dietary minerals above their optimum supplementation levels results in toxic effects, whereas deficiency of any particular micro mineral leads to the bioaccumulation of other metals inside the fish body such as gut, liver, kidney, and muscle. Thus, their inclusion in the diet should be carefully monitored so that they do not bio-accumulate (Tchounwou et al., 2003; Soetan et al., 2010). We did not find any previous report regarding the RDA (recommended daily allowance) standardization of cobalt supplementation in *T. putitora.* In our study, crude ash content was found to be directly correlated with the dietary cobalt dosage level, with a maximum level in a group of fish fed 3mg Co/kg diet. It may indicate an increase in metal bioaccumulation in different tissues with an increase in the dosage level of Co (Lall, 2003). Cobalt does not strongly accumulate in most fish species like other metals, but its bioaccumulation and distribution mechanism in fish is still not precisely known.

The International Atomic Energy Agency (IAEA) published a report in 2010 on cobalt bioaccumulation levels in different fish species. According to them, cobalt bioaccumulation in fish depends on various factors, including routes of exposure, water temperature, feeding rate, size, species and metabolic state of the fish. Thus, literature revealed variation in the rate of bioaccumulation of Co in different fish species (Özparlak et al., 2012; Visetpotjanakit and Khrautongkieo, 2018; Niemiec, et al., 2019). Cobalt is mostly taken up in the body via a dietary source (Blust, 2011), water-soluble cobalt compounds are easily absorbed across the small intestine

(Simonsen et al., 2012). Tissue partitioning of cobalt is time and dosage-dependent and in general most of the ingested cobalt is accumulated in kidneys, liver, gut and to some extent in the skeletal muscles. International Commission on Radiological Protection (ICRP) had developed the biokinetics of inorganic forms of ingested cobalt inside the body. According to them, 50% of the ingested cobalt is removed via excretion, while 5% store in the liver and 45% of the remaining cobalt is stored in other body organs (Unice et al., 2012; Czarnek et al., 2015).

In the present study, we found a direct correlation between dietary cobalt and the muscle cobalt bioaccumulation level ($R^2 = 0.952$) (Fig.41). Similar results were reported by Yildiz (2008) in different fish species. Most metals are competitive inhibitors of each other for their uptake across the intestinal membranes (Bertolo et al., 2001). Here, an increased inclusion level of cobalt in diet negatively affected bioaccumulation of divalent metals, i.e., Fe and Mn (Table 24). It seems that Co by competitive-concentration-dependent interaction restricts mucosal uptake of these metals across intestinal lumen. Cobalt is mostly absorbed across the tissues via DCTI (di-valent metal transporter) voltage-gated calcium channels (VGCC) (Okamoto and Eltis, 2011), and the same DCTI is also involved in the transport and absorption of Fe and other divalent ions (Hediger et al., 2002)

For the validation of results, we also determined the effect of Co supplementation on the expression of growth-related genes (myoblast determination protein 1MyoD and Myogenin MyoG) in the muscle, and the stress response gene (heat shock protein, HSP-70KDa) in the liver. The physiological function and health status of fish depend on genetic makeup, environmental factors (including nutrients), and interactions among them (Bimal et al., 2020). Therefore, basic molecular level knowledge is required to understand growth mechanisms and enhance annual aquaculture production (Hakim et al., 2018). Nowadays, nutrigenomics is gaining much attention (Martin and Król, 2017) and research is in progress to evaluate the effect of feed supplements on gene expression and regulation of gmechanisms (Martin and Król, 2017; Williams and Watts, 2019). Unfortunately,

genomic study related to dietary cobalt effect on fish is inadequate and is limited to particular fish species and specific genes (Blust, 2011).

Yet, the whole genome of *T.putitora* is not sequenced, therefore, we first cloned the genes of interest and then sequenced them to obtain their nucleotide sequence. Since mahseer belongs to the Cyprinidae family, the multiple species alignments of peptide sequences of MyoD, MyoG, and HSP-70kDa with other closely related fish species such as *Danio rerio*, *Cyprinus carpio* and *Ctenopharyngodon idella* showed the highest sequence similarities, i.e., with *C. idella* (100%), *D. rerio* (99.3%) and *C. Carpio* (97.9%) whilst it was 87.1 to 92.4% similar to other fish species. However, it was less similar to mammalian orthologues i.e., 82.1% with *Homo sapiens* and 82.9% with *Mus musculus* (Fig. 14 and 15). Additionally, the MyoG gene was 90.2% and 94.1% similar with *D. rerio* and *C. carpio* (Fig. 19 and 20). Furthermore, HSP-70kDa was 98.3% to *D. rerio* (Fig. 24 and 25). After gene cloning, MyoD, MyoG, and HSP-70kDa were submitted to NCBI to obtain their accession numbers. Here the expression of both growth-related genes MyoD and MyoG in muscle is in accord with growth performance data, i.e., up-regulated in response to dietary cobalt at 2mg/kg inclusion level. It may be due to activation of hypoxia-inducible factor HIFα in the presence of cobalt (Wagatsuma et al., 2011), and the stimulation of several myogenic regulating factors (MRF), i.e., MYF- 4 and 5, and MyoD (Rescan, 2001). HIFα is localized in the nuclear and cytoplasmic regions of growing myoblasts and myotubes. Co increases the expression of HIFα which then stimulates several myogenic regulating factors (MRF), i.e., MYF- 4 and 5, and MyoD (Rescan, 2001). These myogenic factors encode the basic helix looped helix (bHLH) proteins in fish, whose increased expression plays an important role in the regulation of myogenesis and specification of newly synthesized muscles (Lin et al., 2006). Since MyoD regulates the proliferation, and activation of satellite cells towards the myogenic lineage while MyoG is involved in the myoblast fusion, and cell differentiation to synthesize new muscle fibers during fish muscle growth; a key regulator of

skeletal muscle synthesis (Braun and Gautel, 2011), thus the expression of both of these genes resulted in the increased number of muscle fibers size of fish skeletal muscle and overall growth performance of the fish. Besides myogenesis, cobalt also induces the expression of glucose transporters and various glycolytic enzymes, which in turn facilitates the anaerobic energy metabolism of newly formed myofibers (Wagatsuma et al., 2011).

At the cellular level, fish possess highly developed stress response mechanisms to cope with the changing internal and external environmental conditions (Kayhan and Duman, 2010). According to Oksala et al. (2014), the cell can respond to chemical stress by inducing the activation of a series of stress proteins including heat shock proteins. Several heat shock proteins have been identified in fish which are activated in response to both biotic and abiotic stress (Iwama et al., 1998). Among them, HSP-70KDa is commonly used as a stress biomarker due to its significant role in cytoprotection and rapid expression under cellular stress (Basu et al., 2002). Here, we found a significant increase in expression of HSP-70KDa in the liver of groups of fish reared on a diet supplemented with 3mg Co/kg than other groups of fish, indicating cobalt toxicity. It seems that at higher dosage levels of Co, the cytoprotective mechanisms of *T. putitora* became activated. Over expression of HSP-70KDa gene, i.e., activation of cytoprotective mechanisms against metal toxicity is also reported by Piano et al. (2004). It is documented that under stress conditions, fish reduce the feed intake (Trenzado et al., 2006), thus the poor growth performance at higher dosage levels of cobalt may also be due to poor feed intake and insufficient availability of essential nutrients required for optimal growth of fish.

Several intrinsic and extrinsic factors affect the mineral bioavailability inside the body (Forbes and Erdman Jr, 1983; Harvey, 2001). Among them, the size of the supplemented mineral is an important extrinsic factor that affects mineral absorption and its utilization in animals (Miles and Henry, 2000; Kratzer, 2018). New techniques including nanotechnology have recently become attractive in fish nutrition for improving the stability, absorption, and bioavailability of the

micronutrients in the body (Handy, 2012). Nanotechnology involves the synthesis and application of materials/elements at their molecular levels i.e., at their nanometer scales (Lin, 2007). Metal nanoparticles (MNPs) are very small in size (<100 nm) and possess unique physical and chemical properties. Generally, the reduction in size, i.e., from bulk to nano-scale, also changes the physiological and chemical properties of materials e.g. convert the non-soluble, non-diffusible, and inert materials into soluble, diffusible and reactive ones (Wang and Li, 2010). The high absorption and assimilation of nano forms of several minerals, including zinc (Zn), selenium (Se), and iron (Fe) and their positive impact on growth and immune responses of different fish species is well documented e.g. *Pangasius hypophthalmus* (Kumar et al., 2017), *Macrobrachium rosenbergii* (Muralisankar et al., 2014), *Argyrosomus regius* (Mansour et al., 2017), *Clarias batrachus* (Akter et al., 2018), and *Sparus aurata* (Rigos et al., 2010). By realizing the efficiency of NPs, we also prepared cobalt nanoparticles (Co-NPs) by adopting a top-down approach and evaluated their efficacy and optimum dosage for *T. putitora.*

MNPs can be prepared by physical, chemical, and biological methods (Rajput, 2015). Two approaches, i.e., bottom-up and top-down are commonly under consideration during the preparation of NPs. The bottom-up approach involves the assembly of atoms, ions, and molecules as building blocks, while top-down involves the decomposition of bulk solid into nano-scale by mechanical force, which also controls the shape and size of particles. Top-down approach is the cheapest, fastest, and most suitable method for the synthesis of nanoparticles at industrial level (Ghiuță et al., 2017). We also adopted this approach and used a ball mill device for the synthesis of Co-NPs. Our prepared particles (Co-NPs) were <20nm in size and had a spongy surface (Fig. 45). Like us, Mirzaie et al. (2013) also used a similar method for the synthesis of cobalt chloride nanoparticles. Due to the high chance of contamination, some scientists have been concernecd (Rajput, 2015). However, the electron dispersal spectrum (EDS) of our Co-NPs did not show any impurities (other metals close to these

synthesized NPs) and indicated the presence of 21% oxygen, 40.33% chloride, and 37% cobalt (Fig. 50A).

The chemical form is also the main extrinsic factor that affects mineral utilization in living organisms (Kratzer, 2018). Metal-amino acid complexes are commonly present in biological systems and perform an important function in bio-inorganic chemistry such as absorption, storage, and transport of metal ions and metalloproteins to the target sites, where they play different roles e.g., act as enzyme catalysts, transport of oxygen, part of important vitamins, etc. (Ashmead, 2012). In our experiment, we converted the inorganic form of cobalt i.e., $CoCl_2$ into its organic form by its chemical conversion into cobalt methionine complex (Co-Met). We used methionine for complexing cobalt, which is an important amino acid for the living body and is considered a limiting amino acid in the fish diet (Luo et al., 2005). Synthesized Co-Met complex was characterized for size and surface morphology by using x-rays diffraction spectroscopy (XRD) (Fig.46), and scanning electron microscope (SEM) (Fig. 48). XRD is mostly used for solid crystalline substances, as they can diffract x-rays at their inter planer surfaces that allow us to study their accurate crystalline phases and structure (Sharma et al., 2012). Unfortunately, XRD analysis of Co-Met did not show any peak, which might be due to the amorphous nature of the complex. We further characterized the Co-Met complex by using a scanning electron microscopy. SEM image revealed the average size of complex 82.69 nm with a honeycomb like octahedral structure, which might be due to the bonding of central cobalt with methionine (Ashry and Melegy, 2015). Literature revealed that cobalt methionine complex has been used as a feed supplement (Qadir et al., 2014; Ayub et al., 2017), as an antimicrobial agent (McAuliffe et al., 1966; Rusu et al., 2009).

In the present study, we used graded levels of different chemical forms of dietary cobalt (Co-NPs and Co-Met complex), and evaluated their beneficial and toxicological effect on *T.putitora* fingerlings. No previous work so far has been done to evaluate the effect of dietary Co-NPs and Co-

Met complemented diets in *T.putitora.* The results of our feeding trials indicated the significant effect of graded levels of both forms of dietary cobalt on the growth performance of fish. Improved growth performance (%WG, %SGR and ADG) was observed in groups of fish reared on diets supplemented with Co-NPs up to an inclusion level of 1-1.5mg Co/kg (Table 25). Though some scientists had reported growth the stimulating effect of cobalt in different fish species (Mukherjee and Kaviraj, 2009; Al-Ghanem, 2011; Blust, 2011; Mukherjee and Kaviraj, 2011), however, we did not find any literature regarding the effect of nanoform of cobalt chloride supplemented diet on growth performance of *T.putitora.* Like our results, many scientists reported the improved growth performance of different fish species in response to the dietary inclusion of other metals NPs supplemented diets e.g. Zhou et al. (2009) reported the improved growth performance, increased muscle protein, and reduced feed conversion ratio of *Carassius auratus gibelio* in response to Se-NPs, and Faiz et al. (2015) reported the improved the growth performance of *Ctenopharyngodon idella* in response to ZnO-NPs supplemented diet.

In the present study, improved growth performance in response to Co-NPs supplemented diet as compared to the control group of fish could be due to increased mineral absorption across the gastrointestinal membranes (Bhattacharyya et al., 2015). NPs an have different effect on different fish species at different developmental stages, depending on their chemical nature, form, and size (Zhu et al., 2012; Baker et al., 2014; Koelmans et al., 2015). According to Sajid et al. (2015), dietary metals NPs significantly affect the growth performance of aquatic organisms due to their increased absorption from the gut lining into the body rather than partly or unused excreted from the gastrointestinal tract. Due to their small size and high surface area to volume ratio, NPs are highly bio-available thus required in less quantity which makes them cost-effective (Griffitt et al., 2008). Our results also confirm this, as we observed improved growth performance of *T. putitora* at lower

dosage levels of Co-NPs (1-1.5 mg/Kg diet) as compared to conventional $CoCl_2$ (macro-scale) which is required at higher dosage levels (2 mg/kg diet).

In this part of the study, dietary Co-NPs, above 1.5mg Co/kg inclusion level significantly reduced the growth performance of the fish in a dosage-dependant manner with the lowest growth performance in a N6 group of fish reared on 3mg Co-NPs/kg diet. Reduced growth performance may indicate the toxicological effect of NPs (Sarkar et al., 2015). Several scientists had reported NPs toxicity in aquatic animals (Kovacic and Somanathan, 2013; Zaichenko and Stoika, 2014; Ahmad et al., 2015; Mansouri et al., 2015; Sajjadi and Sarasiab, 2018). It is well documented that higher dosage levels of MNPs negatively affect the development and survival of fish and result in concentration-dependent increased mortality (Shaw and Handy, 2011). Kwon et al. (2009) reported the cytotoxic effect of Co-NPs at high concentration levels that negatively affected the growth performance of *L. rohita*. Several factors affect the MNPs toxicity in fish including size, shape, surface morphology, structural properties, and chemical composition. These factors affect the physiological interaction between metal nanoparticles (MNPs), and the target tissue (Handy et al., 2008).

Overdosing of trace minerals is a major concern to wild populations of aquatic organisms, due to the discharge of aquaculture effluent in natural water bodies (Gräslund and Bengtsson, 2001). An excess of these minerals not only poses a health risk to fish but also unhealthy for human consumption. Commercial fish feed usually contained a considerably high level of zinc, selenium, manganese, iron, and copper than the optimum requirement of fish (Nguyen et al., 2019). Mostly inorganic forms of minerals like acetate, nitrate, sulfate, chloride, and carbonate are less costly, so they are generally overdosed in commercial fish feeds to make sure their generous safety factor (Nguyen et al., 2019).

In aquaculture feed industry, amino acid chelated mineral (an organic form of minerals) for trace mineral supplementation is also getting importance due to their enhanced absorption (Rider et

al., 2010). In our study, Co-Met complex enriched diet also showed dose-dependent improved growth performance of *T.putitora* fingerlings as compared to a non-supplemented group of fish ($P<0.05$) (Table 31). As compared to Co-NPs, Co-Met did not show any toxicological effect and showed optimum growth performance at 3 mg Co-Met/kg diet dosage level. No literature is available for the comparison of our results. However, some scientists reported significantly improved bioavailability of other trace elements chelated with amino acid and their positive impact on the growth performance of different fish species. For instance, Apines-Amar et al. (2004) reported improved growth performance of *Oncorhynchus mykiss* when reared on a diet supplemented with Zn-Met complex (organic) as compared to the inorganic form of Zn. Similarly, Wang and Lovell (1997) observed improved growth performance in channel catfish *Ictalurus punctatus* in response to selenium methionine complex supplemented diet in contrast to the inorganic form of selenium. The high bioavailability and most significant positive effect of amino acid chelated minerals could be due to their stable configuration. According to Bharadwaj et al. (2014), the presence of heterocyclic ring structure in amino-chelated trace elements protect them from any chemical reaction in the gastrointestinal lumen and made them resistant for their binding to different antagonists/ inhibitors, thus making them less susceptible to precipitating out into intestinal lumen, easily attach to the intestinal receptor sites and absorbed. We further observed that groups of fish fed Co-Met complex performed better as compared to fish fed the same dosage levels of conventional $CoCl_2$ or Co-NPs. It seems the Co-Met complex is required in less amount as compared to inorganic form of Co. Many investigators indicated the less dietary requirement of amino acid chelated trace minerals (organic form) as compared to an inorganic form of metals that suggested that it may be due to the higher bioavailability, better utilization, minimum loss of metals to the environment (Spears, 1996; Nollet et al., 2007). According to Taylor-Pickard and Tucker (2005), in natural fish feed ingredients minerals are bonded to amino acids and protein, so using amino acid chelated elements (organic form) is a

natural way to supplement trace elements and protect both culture and wild fish stocks from metal toxicity.

For the determination of the optimum supplementation level of dietary inorganic and organic Co, we used several biological and molecular markers. In aquaculture, hematological indices are an important tool to monitor the physiological status, immune potential, and pathological changes in fish.It depends on many extrinsic and intrinsic factors including species, sex, size, stocking density, culture system, diet compositions, water quality, etc. (Fazio, 2019). Many investigators reported changes in hematological indices of different fish species in response to various toxicants, including metals (Atamanalp et al., 2010; Ololade and Oginni, 2010; Abarghoei et al., 2015; Burgos-Aceves et al., 2019). In this part of the study, we observed significant effects of graded levels of both organic and inorganic forms of Co on the hematological indices such as RBC, Hb, MCH, MCHC, and MCV of *T. putitora*. All groups of fish fed Co-Met complex supplemented diets showed dose-dependent, increasing trend in hematological parameters (RBCs, Hb, HCT%, MCV, MCH, and MCHC) of fish, with a maximum at a dosage level of 3mg Co-Met /kg diet (Table 32). However, in response to dietary Co-NPs, an increasing trend in hematological parameters was observed up to 1.5mg Co-NPs/kg diet, then all indices showed a decreasing trend with an increasing dosage level of Co-NPs (Table 26).

Cobalt plays an important role in hemopoiesis via activation of hypoxia-mediated signaling mechanisms HIF-1α, and stimulation of erythropoietin (EPO) gene expression (Maxwell and Salnikow, 2004; Ke and Costa, 2006). Besides these, cobalt ions also induce several hematopoietic and non-hematopoietic effects of erythropoietin such as modification of glucose and lipid metabolism (Hojman et al., 2009), and the expression of other HIF response genes like multiple angiogenic growth factors e.g., VEGF (vascular endothelial growth factor) and angiopoietin, glucose transporters, cell proliferation, etc. (Simonsen et al., 2012). EPO is an important glycoprotein that stimulates the production of red blood cells and modification of glucose, and lipid metabolism

(Hojman et al., 2009). Increased EPO gene expression in response to cobalt has been observed in rats after a single intraperitoneal injection of cobalt chloride (60 mg/kg body weight) (Lison, 2015). Here, the improved hematological indices of *T.putitora* especially RBCs and HB, in response to Co may indicate the stimulation of erythropoietin production and improvement in oxygen-carrying capacity and overall health of fish (Lippi et al., 2006; Finley et al., 2012). Unfortunately, no comparable study is available for comparison of our results of dietary organic (Co-Met complex) and inorganic (Co-NPs) cobalt effect on the hematological indices of fish. However, Atamanalp et al. (2010) reported increased RBCs count and %HCT in *Salmo trutta fario* and RBCs count and MCHC level in *Oncorhynchus mykiss* after 180 µg/L $CoCl_2$ exposure. Moreover, Kubrak, et al. (2012) reported increased blood hemoglobin level in *Carassius auratus* in response to cobalt (50–150 mg/L).

The presence of toxic levels of metal in the aquatic environment and food exerts a toxic effect at both cellular and molecular levels that ultimately results in physiological and biochemical changes in fish (Jiwan and Ajah, 2011; Liu et al., 2016). Though we did not notice any toxic signs on the hematological indices of fish fed Co-Met complex supplemented diet yet, a dose-dependent decreasing trend was observed in groups of fish fed Co-NPs above 1.5mg/kg diet. Like our results, many scientists reported a dose-dependent toxic effect of cobalt exposure on hematological indices of fish (Finley et al., 2012). For instance, *Oreochromis mossambicus* showed reduced RBC and Hb level, when exposed to Co at higher concentration (30-150 ppm) (Suganthi et al., (2015). Similarly, Saravi et al. (2009) also reported a decrease in RBC count in *Cyprinus carpio* after exposure to 200 mg/L $CoCl_2$. In our study, dose-dependent reduced HCT (%) and RBC count at higher dosage levels of Co-NPs could be due to an insufficient supply of healthy RBC to blood. Moreover, reduced MCHC and Hb level might be due to reduced oxygen-carrying capacity of blood during cobalt toxicity (Atamanalp et al., 2011). According to EM (2011), cobalt interferes with the binding ability of hemoglobin to oxygen during respiration. Due to the insufficient supply of oxygen, respiration could

not be maintained efficiently. Furthermore, reduced MCV at higher dosage levels of Co-NPs may be due to competitive inhibition of Fe (Massey 1992 et al., 2015). Literature revealed the dose-dependent toxic effect of many other MNPs on hematological indices of fish. For instance, CuO-NPs at 4000 mg/l resulted in reduced RBC, Hb, and HCT in *Rutilus rutilus* (Jahanbakhshi et al., 2015), and at 100ppm in *Oncorhynchus mykiss* (Khabbazi et al., 2015). Moreover, Bukhari and Suganthi (2017), also reported significantly reduced hematological indices i.e., RBC, Hb, HCT, MCH, MCHC, and MCV in *Oreochromis mossambicus* when exposed to CuO. According to Burgos-Aceves et al. (2019), the toxic effect of concentration-dependent CuO-NPs could be due to transient changes in blood chemistry and depletion of plasma sodium ions.

Nutrition plays a vital role in enhancing fish growth, improves health status, and maintain the fish's ability to overcome stress and protect themselves from disease-causing agents in aquaculture (Pohlenz and Gatlin III, 2014). According to Trichet (2010), several dietary feed ingredients including both essential and non-essential nutrients have a profound effect on immune systems when supplied at specific pharmacological dosage levels. Therefore, before considering the beneficial aspects of specific dietary nutrients, it is essential to evaluate its optimal dosage level. Though essential trace minerals are required in trace quantity but are vital for physiological functioning and maintaining fish health. Minerals act as co-factors of important enzymes involved in several metabolic, biochemical, and physiological processes, thus help to improve immune competence and performance of fish (Paul, 2001; Kiron, 2012; Alpert, 2017). The deficiency of a particular mineral results in reduced disease resistance and makes the animal more susceptible to disease (Teles, 2012). According to Eisa and Elgebaly (2010), micro minerals such as Cu, Fe, Zn, etc., at optimum level improve the immune functions in fish by enhancing the functioning of several immune cells e.g. lymphocytes, leukocytes, and neutrophils.

In our experiment, graded levels of both organic and inorganic forms of cobalt supplemented diets showed a significant effect on immune responses of fish such as the plasma protein, IgM level, serum lysozyme activity, and resistance against pathogens. Co-Met complex showed a dosage dependant increase in plasma protein level, IgM level, serum lysozyme activity but did not show a profound effect on WBCs count and NBT activity up to 2.5mg/kg diet while further increase of dosage level (3mg/kg diet) did not show further improvement, and effect was somewhat statistically comparable to 2.5mg/kg dosage level (Table 33). However, fish fed Co-NPs supplemented diet showed an increase in plasma protein, IgM level, and lysozyme activity up to 1.5mg/kg diet while further increase showed a negative effect, i.e., decrease in the level of these immunological indices and increase in WBCs count and NTB activity (Table 27). These results indicated the higher efficiency of the organic form of Co (Co-Met complex) as compared to inorganic form (Co-NPs) could be due to higher bioavailability, stable structural configuration, and bactericidal activities (Stănilă et al., 2011).

According to Kadim et al. (2003) dietary cobalt improve the protein availability, digestion, and absorption of other nutrients. In our study, increased plasma protein levels in response to cobalt supplemented diet might be due to the protein-sparing effect (Hertz et al., 1989) or the increase in the synthesis of protein (Hsu et al., 1953). Unfortunately, no literature is available to compare our results, however, Al-Habsi et al. (2007) reported low levels of plasma protein in cobalt deficient Omani goats as compared to the Co-treated group. Furthermore, in our experiment both chemical forms of dietary cobalt showed a significant increase level of plasma IgM, thus indicating a strong innate immune response (Asadi et al., 2012).

Cobalt is a precursor of vitamin B_{12} which is involved in cellular and humoral immunity (Erkurt et al., 2008). Vitamin B_{12} plays an important role in the regulation of the immune system, and its deficiency suppresses the protective immune response mechanisms against pathogenic bacteria in

animals (Maggini et al., 2007). Unfortunately, literature regarding the immuno-stimulatory effect of dietary cobalt in fish is poorly documented. However, some scientists observed increased plasma IgM level in goats in response to parenteral injection of hydroxyl-cobalamin (Johnson et al., 2016), and humans in response to cyanocobalamin treatment (Erkurt et al., 2008). Moreover, Tamura et al. (1999), observed increase plasma IgM level, when vitamin B_{12}-deficient patients were treated with methyl-vitamin B_{12}. According to them, vitamin B_{12} is essential for the proliferation of B cells into plasma cells, which are involved in the secretion of antibodies and required adaptive immune responses. The negative effect, i.e., impairment in antibody production and functioning of neutrophil and phagocytic cells in response to the low dosage of dietary cobalt is well documented (Spears, 2000; Johnson et al., 2010; Johnson et al., 2016).

Nowadays, research is in progress to explore the antimicrobial activity of MNPs (Ravishankar Rai, 2011). The small size and high surface area to volume ratio enhance the interaction of positively charged NPs with microbes and carry out the wide array of antibacterial activities (Wang et al., 2017). In the present study, the improved disease resistance and immunological indices of *T. putitora* in response to the optimum supplementation level of both forms of Co could be direct i.e., destruction of the cell wall of bacteria due to the strong affinity of positively charge Co to the negatively charged bacterial cell or may be indirect because of hypoxia-mimic property of Co that results in the increased expression of series of hypoxia-responsive genes. Recently, HIF-1 has been reported as a macrophage regulator that plays an important role in innate immune and neutrophil inflammatory function. It is a transcriptional regulator that controls some of the key aspects of several immune responses like acts as a promising agent for immune-boosting cures (Zinkernagel et al., 2007). HIF-1 boosting therapeutic drugs are being used against several infectious diseases since it is considered as the main regulator of several cellular stress responses and is involved in controlling and activation of immune cells against pathogenic microbes (Hellwig-Bürgel et al., 2005; Palazon et al., 2014) i.e., production

of phagocytic cells, including macrophages and neutrophils (Oda et al., 2006). Being considered as a master regulator of innate immune responses, the expression of HIF in epithelial cells controls the release of chemo-attractant that stimulates the migration of macrophage to the site of infection (Bhandari and Nizet, 2014; Palazon et al., 2014). Inside these macrophages, HIF increases the bactericidal activities via increased production of a wide range of different types of antimicrobial agents such as peptides, proinflammatory cytokines, and NO (nitric oxide) (Mancino et al., 2008; Elks et al., 2011). Besides these HIF also extends the lifespan of functional neutrophils by inhibiting NF-JB-dependent apoptotic pathway (Elks et al., 2011).

In the past few years, amino acid complexes with transition metals had received much attention due to their chemotherapeutic properties which allow them to behave as antifungal and antibacterial agents against various pathogenic bacteria e.g. *S. aureus, E. coli*, etc. Cobalt and copper ions have been used in the past to inhibit the growth of pathogenic bacteria (Chang et al., 2010). Cobalt and its complexes show a diverse array of antibacterial and antiviral properties that can be used for antibacterial and antiviral drugs (Rasu et al., 2009). These complexes possess diverse antibacterial activities against *S. cerevisiae* and *C. albicans.* Adewuyi et al. (2011) reported broad-spectrum antibacterial actions of cobalt complexes with chitosan due to the positively charged metal ion which facilitates the polycationic interaction of the complex with the negatively charged bacterial cell wall, resulting in bacterial death. According to Chohan et al. (2006), Schiff bases-transition metals complexes shows a wide range of anti-fungal/bacterial activities, thus introducing a new, and novel era of metal-based therapeutic drugs against bacteria and fungi.

In aquaculture, Infectious diseases present a major challenge, causing high mortality, and impaired growth due to infection (Houston, 2017). It is documented that cobalt possesses a diverse array of antibacterial and antiviral properties that can be used in many drugs (Heffern et al., 2013). Nowadays, cobalt and its complexes are widely used as antibacterial and antiviral agents in many

therapeutic drugs (Rusu et al., 2009). In our experiment, after 90 days feeding trial, both forms of Co, showed improved resistance of fish against *A. hydrophila* infection. Here like growth, immunity indices, group of fish reared on Co-NPs supplemented diets above 1.5mg Co/kg inclusion level showed less resistance against bacteria with the highest % cumulative mortality in the N6 group fed diet supplemented with 3mg Co-NPs/kg diet (Fig. 53), may be due oxidative stress and suppression of immune response (Mansouri et al., 2015). The oxidative stress generated reactive oxygen species (ROS), which are responsible for the majority of the diseases in fish (Sevcikova et al., 2011). The oxidative stress and toxicity due to the higher Co-NPs inclusion level are evident in our study while observing the WBCs count, NBT, and serum AST activity of fish. The higher NBT (Fig. 52) and AST activity (Fig. 51) may be due to the production of higher amounts of intracellular ROS by phagocytic cells (Sakai, 1999). Hosseini et al. (2014) also reported increased ROS production and AST activity during cobalt toxicity. Moreover, Ale et al. (2018) also reported increased AST activity in *Prochilodus lineatus* when exposed to Ag-NPs (25 µg/l). It is well documented that ROS weaken the immune response and damage the cellular bio-molecules (proteins, carbohydrates, lipids, enzymes and, nucleic acids) (Weidinger and Kozlov, 2015; Kanwal et al., 2016). Kanwal et al. (2019) reported increased WBCs count in *Labeo rohita* when exposed to the toxic level of cobalt. Similar results were reported by Atamanalp et al. (2011) and Suganthi et al. (2015) in *O. mykiss* and *O. mossambicus* respectively when exposed to cobalt chloride at a toxic level (180µg/l and 150ppm respectively). Hosseini et al. (2014) also reported increased ROS production and AST activity during copper toxicity.

Nutrient digestibility is an important factor since it reduces the excretion of undigested nutrients in the surrounding water. Determination of intestinal enzyme (amylase, protease, and cellulase) activities helps to quantify the digestive capacity and optimization of micronutrient concentration in the feed. The activity of these enzymes is directly related to the composition of feed

(RØnnestad et al., 2013). These enzymes take part in degradation mechanisms, which enable the fish to efficiently metabolize nutrients (Furne et al., 2005; Chaudhuri et al., 2016). The capabilities of gastrointestinal enzymes, their activities, and metabolism plays an important role while formulating fish feed, since nutrient utilization in fish is dependent on the availability of these enzymes in the GI-tract.

The current study showed a significant effect of graded levels of both chemical forms of dietary cobalt on intestinal enzyme activities of *T. putitora*. Amylase and protease activities increased in both diet groups but a decreasing trend was observed in the Co-NPs group of fish when fed a diet supplemented with Co-NPs at a toxic level, i.e., above 1.5.mg Co-NP/kg diet (Table 28). Even though no literature is available on the effect of Co-NPs and Co-Met complex supplemented diets on intestinal enzyme activities in fish, but few scientists reported positive effects of conventional cobalt on intestinal enzyme activities of fish e.g. Al-Ghanem (2011) and Mukherjee and Kaviraj (2011) reported the improved intestinal enzyme activities in *Cyprinus carpio* and *Heteropneustes fossilis* when they were fed diet supplemented with 1.5% and 0.1% cobalt respectively.

The GI tract of fish comprises a complex microfloral ecosystem which is essential for pathological, physiological, and nutritional points of view. According to Nayak (2010), a diverse range of different types of microbes from surrounding water/sediment and food colonize the gastrointestinal tract (GI tract) of fish. Among these, obligate and facultative anaerobic bacteria are the main colonizers in the GI tract. In aquaculture, gut microbiota plays a significant role in nutrient metabolization thus has a direct impact on the digestive physiology of the host (Rowland et al., 2018). Intestinal microflora has a symbiotic relationship with the fish that is important for immune responses, maintaining physiological functioning, and mainly helps in digestion (Neish 2009). According to Skrodenyte-Arbaciauskiene et al. (2006), GI microflora can synthesize several types of growth factors, vitamins, amino acids, metabolites, and release of enzymes including amylase, esterase,

carbohydrase, phosphatase, cellulase, protease, lipase, and peptidase, thus playing an important role as a contributory source of GI enzymes in fish. For instance, Ray et al. (2012) reported the microbial amylase and cellulase activity in *C. idella, Dicentrarchus labrax, Plecoglossus altivelis*, *Oreochromis niloticus*, and *Ictalurus punctatus,* and indicated that the only source of cellulase activity in omnivorous and carnivorous fish is bacteria which harbor the GI tract of the fish from feed or surrounding.

In our experiment, increased intestinal enzyme activities might also be due to the indirect involvement of cobalt via microbial vitamin B_{12} production (Lin et al., 2010). Cobalt is the integral component of vitamin B_{12} (Zhang et al., 2009), thus considered an essential trace mineral in animal diets (González-Montaña et al., 2020). Intestinal bacteria synthesize vitamin B_{12} by utilizing cobalt from dietary sources (Lin et al., 2010). Li et al. (2016) reported the increased intestinal enzyme activities of *Megalobrama amblycephala* fingerlings in response to vitamin B_{12} supplemented diet at the inclusion level of 0.12mg/kg, and suggested that an increase in enzyme activity is related to the increased digestive and assimilative mechanisms which are activated by vitamin B_{12}. In the present study, no significant difference in cellulase activity was observed in *T. putitora* in response to Co-NPs supplemented diet up to 1.5mg Co-NP/kg. However, a further increase in Co-NPs supplementation level negatively affected the cellulase and amylase activity which could be due to the toxicity produced by cobalt chloride nanoparticles at higher concentrations. It seems that at the higher dosage level Co accumulated in the intestinal tract and hinder the colonization of bacteria, thus drastically affects the concentration of these enzymes.

Fish muscle is composed of different components such as protein, fat, fiber, mineral, vitamins, and moisture, which altogether contribute to the overall proximate composition of fish (Alemu et al., 2013; Puke and Galoburda, 2020). In our study, a graded level of both chemical forms of dietary cobalt showed a significant effect on muscle contents of crude protein, fat, and ash. Unfortunately,

no relevant literature is available to compare our results. However, few scientists reported increased muscle content of crude protein and fat in different fish species, *H. fossilis* (Mukherjee and Kaviraj, 2011), *O. niloticus* (Tonye and Sikoki, 2014), and *C. carpio* (Al-Ghanem, 2011) in response to Co supplemented diets.

In our feeding experiment, all groups of fish fed Co-Met complex supplemented diets showed a dose-dependent increasing trend in muscle crude protein and fat contents with a maximum level at an inclusion level of 3mg Co-Met /kg diet (Table 35). However, in response to dietary Co-NPs, an increasing trend was observed up to 1.5mg Co-NPs/kg diet, then both muscle crude % protein and fat showed a decreasing trend with an increase in the concentration of Co-NPs in the diet. The decrease in muscle protein content in groups of fish reared on toxic levels of dietary Co-NPs could be due to the use of protein as an energy source for detoxification and maintenance of homeostasis (Zheng et al., 2013; Wang et al., 2015). According to Kanwal et al. (2019), the toxic level of MNPs induces toxicity is destructive to the energy stores, thus causing overall reduced weight gain of fish.

In our study, we also observed higher muscle ash content in a N6 group of fish reared on a 3mg Co-NPs/kg supplemented diet, indicating higher mineral accumulation in fish muscle. No literature is available that indicates the effect of dietary Co-NPs on the proximate composition of fish. However, Wang et al. (2015) reported a decrease in muscle crude protein and fat content and an increase in crude ash of Juvenile *Epinephelus coioides* in response to Cu-NPs (100 μg/l) exposure as compared to the control group of fish. Moreover, crude ash content was considerably lower in the muscle of fish reared on Co-Met supplemented diets than those reared on Co-NPs supplemented diet indicated their low bioaccumulation level in fish muscle. Hunt et al. (2011) also reported an increase in muscle crude protein and fat contents of rainbow trout when fed 3mg/kg organic selenium as Sel-Plus®. Moreover, they did not observe any effect on crude ash content of fish in response to organic selenium supplemented diet.

The nutritional value of minerals depends on their supplementation level in the diet and bioavailability in living organisms (Richards et al., 2010). In our study, dietary Co-NPs above 1.5mg/kg supplementation level negatively affected the iron and manganese absorption across the internal membrane and subsequently their accumulation in the muscle of fish. Different factors such as mineral interaction with other dietary components, their chemical form, solubility in water and lipids, size and charge of metal, diet-related factors, presence of antagonists and host-related factors, i.e., expression of cellular transporters affect the mineral absorption inside the GI tract (Harvey 2001). Usually, minerals are absorbed across the GI tract via simple diffusion, facilitated diffusion, active transport, different types of carries protein, and metals specific receptor sites that facilitate their absorption (Venugopal, 2013). Since Fe, Mn, and Co are metabolically similar, thus they compete for their absorption at receptor sites in the GI tract (Norwood et al., 2003; Younus et al., 2020). It seems that at a higher concentration of Co-NPs, a saturation of carrier proteins resulted in a decrease in absorption and accumulation of Fe and Mn.

In this part of the study, we also observed an increase in cobalt bioaccumulation with the increasing Co-NPs supplementation level in the diet (Table 30). The bioaccumulation of metals in different tissues of fish depends on the chemical form and size of metals, dosage level, duration, and nature of tissues (Güner, 2010; Mansouri et al., 2011; Mansouri et al., 2012). The dose-dependent increase in Co bioaccumulation in the muscle of *T.putitora* in response to Co-NPs supplemented diet may be due to their small size which facilitates the faster penetration of NPs through the intestinal luminal mucosal epithelial membranes (Venugopal, 2013; Bunglavan et al., 2014). Moreover, the muscle ash content and their relationship with Co-NPs supplementation level in the diet also confirm the dose-dependent accumulation of Co in the muscle of fish.

Contrary to Co-NPs, we did not observe any dose-dependent effect of Co-Met complex supplemented diet on the bio-accumulation of Fe and Mn in the muscle of *T. putitora* (Table 36) may

be due to the inert nature of amino acid-metal chelates which protect them from anti-nutritional factors present in the diet and intestinal lumen (Apines-Amar et al., 2004). It seems that organic forms of cobalt do not interfere with the absorption of other divalent cations. According to Paripatananont and Lovell (1997), chelated minerals are highly bio-available and their accumulation level in tissue is low. This is in accord with our results, we also did not observe their accumulation in the muscle tissue up to 2.5 mg/kg diet however, further increase in dosage level (3mg/kg diet) showed slight accumulation of Co. It seems that beyond optimum requirement, organic form of Co could be accumulated in the muscle of fish. Further study is required to determine the toxic level of the dietary Co-Met complex.

Statistical analysis of all results of this part of the study indicated the significant effect on the dosage level and chemical form of dietary Co on the growth performance, health status, immunity, and muscle proximate composition of fish (Table 37→42). Moreover, significant ($P<0.05$) interaction between dosage levels and chemical forms of Co indicated the importance of chemical form in the determination of the optimum dietary supplementation level. Here both forms of Co (organic and inorganic) showed variable optimum supplementation level requirement, i.e., 1.5 mg/kg diet for Co-NPs and 2.5mg/kg diet for Co-Met complex, we observed the highest growth performance in a M6 group of fish reared on 3mg/kg Co-Met supplemented diet, whereas N6 group of fish reared on 3mg/kg Co-NPs supplemented diet showed the poor growth performance. All other biological markers (hematological and immunological parameters) followed the same trends. Moreover, pairwise comparison at each dosage level also indicated the higher efficiency of the organic form of Co (Co-Met complex) in contrast to inorganic form (Co-NPs). Our results are in accord with literature which indicated that mineral bioavailability depends on supplementation levels and its chemical form (Rider et al., 2010).

Many scientists studied the comparative efficacy of organic and inorganic form of minerals in fish and reported the higher efficiency of the organic form of minerals e.g. Domínguez et al. (2017) reported improved growth performance of *Sparus aurata* (Linnaeus, 1758) when reared on diet supplemented with organic Zn, Mn, and Se as compared to their inorganic forms. Furthermore, Wang et al. (1997) also reported the improved post-challenge immune response of channel catfish against *Edwardsiella ictaluri* when they were previously reared on diets supplemented with organic selenium as compared to inorganic form. Moreover, Rider et al. (2010) reported high efficiency of organic selenium supplemented diets in *Oncorhynchus mykiss*. Katya et al. (2017) reported the beneficial effect of the chelated mineral complex on the growth performance of *Sebastes schlegeli*as as compared to inorganic mineral premix supplemented diets. Furthermore, Lin et al. (2013) reported the improved growth performance, survival rate and immune response of *Litopenaeus vannamei* when reared on a diet supplemented with Zn-Methionine as compared to the control group of shrimps. Apines-Amar et al. (2004) and Tan and Mai (2001) also reported improved growth and immune response in fish when fed chelated minerals supplemented diets. Paripatananont and Lovell (1997) studied the comparative absorption of organic and inorganic minerals supplemented diets in *Ictalurus punctatus* and reported the higher bio-availability and less requirement of chelated organic minerals compared to inorganic form. Other than fish, some reports are also available which indicates the improved efficiency of organic zinc as compared to $ZnSO_4$ when used as feed supplemented in poultry feed (Chang, 2001; Caoet al., 2002).

Keeping in view the above results we can conclude that Co-NPs supplemented diet resulted in improved growth performance, hemato-immunological indices, intestinal enzyme activities, and muscle proximate composition of fish at a lower inclusion level in diet i.e. 1.5mg Co/kg. However, their further supplementation is detrimental to fish. On the other hand, no toxic signs were observed in Co-Met supplemented diet group of fish even at higher dosage levels, and at each dosage level,

Co-Met complex supplemented showed the most positive effect on all studied parameters as compared to Co-NPs supplemented diets.

Worldwide, the fisheries and aquaculture sector is increasing annually and is considered as a reliable and fastest sector to overcome protein shortage in human diets (Little et al., 2016). With time, fish farmers had adopted various advanced technologies and intensification approaches to increase their annual production (Watanabe et al., 2002). Advancement in fish nutrition is considered a key point that should be properly addressed for sustainable and successful aquaculture (Subasinghe et al., 2009). Low cost, non-contaminated, nutritionally complete, and high-quality artificial feed play an important role in effective aquaculture production (Hixson, 2014). According to Prabu et al. (2017), higher fish production can be achieved by feeding them nutritionally balanced diet via using animal and plant-based feed ingredients along with essential supplements such as vitamins, minerals, etc.

The literature revealed that the optimum level of essential vitamins and minerals determined in a controlled environment does not always consistent with semi-intensive earthen pond culture systems. It is documented that a controlled environment, prevents the interaction of environmental effects, like temperature, water quality, and live food organisms with the studied nutrient (Lovell, 1989; Cuzon et al., 2004). Thus, before recommending the optimum requirement of any nutrient for practical feed formulations, it is suggested to conduct experiments under conditions as similar as possible to the conditions where the results will be applied (FAO, 2020). *T.putitora* in captivity is mostly cultured in semi-intensive culture condition, there we further expanded our study and conducted experiment under semi-intensive culture conditions. According to Edwards al. (2000) optimum level of essential vitamins and minerals in the diet is a prerequisite while rearing fish in fertilized ponds. Unfortunately, mineral supplementation in aquatic feed is facing major concern as most of the farmers are not well aware of the actual quantity of particular mineral supplementation required in semi-intensive culture systems (Robb et al., 2013).

In this phase of our study, based on results of the previous experiment, we selected two dosage levels (1.5 and 3mg Co/kg diet) of both Co-NPs and Co-Met complex and conducted a completely randomized feeding trial for the evaluation of the practical application of both forms of cobalt as a feed supplement. An experiment was conducted in earthen ponds in a semi-intensive culture conditions. All the ponds were adjacent to each other in the same vicinity and were under the similar environmental conditions. Moreover, to minimize the other factors and obtaining the effects of only Co supplements, efforts were made to maintain water quality by the addition of fresh water at dawn and dusk, regular monitoring of pond fertility. Additionally, fish in each pond was stocked at the same stocking density. Results indicated no noticeable difference in water quality parameters of different ponds. Thus any difference in growth and hemato-immunological responses among different groups of fish are likely due to the effect of different dosage levels of organic and inorganic form of Co.

In this part of the study, growth performance indices (WG%, SGR%, AWG and, ADG) (Table 43), immunological indices (Table 44), hematological indices (Table 45) showed a similar trend in response to Co-NPs and Co-Met complex supplemented diets as observed in the previous experiment i.e., organic form of Co (Co-Met complex) showed a most significant positive effect in a dose-dependent manner on all studied parameters as compared to the inorganic form of Co (Co-NPs) which showed toxicological effect (poor growth performance and lowest hemato-immunological response) at higher level i.e., 3mg/kg diet. Although, group of fish fed 1.5mg Co-NPs supplemented feed (TN1) also showed improved growth performance, health status, and immunity of fish as compared to the control group of fish but the impact of Co-NPs was less significant in contrast to the effect of Co-Met complex at the same dosage level i.e., 1.5mg/kg diet. Furthermore, the highest NBT activity in a group of fish (TN2) fed a higher dosage of Co-NPs also indicated toxicological effect caused by oxidative stress i.e., increased production of reactive oxygen species (Tewari et al., 2002).

In this part of the study, we also studied the serum enzymes, i.e., aspartate transaminase (AST) and alanine transaminase (ALT) levels (Table 46). Serum biochemical analysis in fish is considered a powerful tool for quantitative measurement of metal toxicity and a source of valuable information regarding the ecological significance of metallic ions and their effect on the body (Ghoreishi et al., 2013). AST and ALT are commonly considered stress indicators, and are frequently used to determine pesticide and heavy metal toxicity in fish (Authman et al., 2015). Both of these enzymes are sensitive to metal exposure and are involved in amino acid and protein metabolism (Öner et al., 2008). The higher serum levels of both AST and ALT in a group of fish reared on 3mg Co-NPs/kg diet is the indication of toxicological effects of Co-NPs and maybe due to liver damage; hepatocellular necrosis due to loss of functional integrity of plasma membrane of liver cells and cellular leakage. Many scientists reported an increase in serum AST and ALT activity in response to heavy metal toxicity in animals. For instance, Ghoreishi et al. (2013) reported increased activity of both of these enzymes in lambs in response to the higher dosage level of cobalt nano-particles. Similarly. Öner et al. (2008) also reported increased ALT and AST activity in Nile tilapia when they were exposed to heavy metals. Moreover, Atli et al. (2015) reported increased activities of both of these enzymes during increased Cd and Pb exposure in *O. niloticus* resulting in impaired metabolism and tissue damage. According to Zikić et al. (2001), increased activities of these enzymes indicate the tissue damage that occurs due to the stress-induced by metal toxicants.

Fish is an excellent source of high quality protein, omega-3 fatty acids and vitamins and minerals (Chalamaiah et al., 2012). The nutritive of fish depends on many factors, including the amount and quality of food that the fish eats (Beveridge et al., 2013). The literature revealed the impact of feed supplement on the proximate composition of fish e.g. significant effect of dietary Zn on the proximate composition of *Labeo rohita* (Akram et al., 2019), *Ctenopharyngodon idella* (Liang et al., 2012), *Ctenopharyngodon idella* (Val.) (Wu et al., 2015), and *Acipenser baerii* (Moazenzadeh

et al., 2018). Moreover, others reported a significant effect of Cu supplemented diets on the proximate composition of *Pelteobagrus fulvidraco* and *Acipenser baerii* (Tan et al. 2011; Moazenzadeh et al. 2020). Furthermore, Ling et al. (2010) reported significant effect of Fe supplemented diets on proximate composition of *Cyprinus carpio* while Zhou et al. (2009), and Zhu et al. (2012) reported a significant effect of dietary Se on the proximate composition of *Carassius auratus gibelio* and *Micropterus salmoide* respectively. We also observed a significant effect of dietary Co supplementation on the proximate composition of *T.putitora*. Our results showed highest muscle crude protein and fat contents in a TM4 group of a fish fed diet supplemented with 3mg Co-Met complex/kg diet followed by a TM3 and TN1 groups of fish fed diets supplemented with 1.5mg/kg diet Co-Met complex and Co-NPs respectively indicated the higher efficiency of Co-Met Complex as compared to Co-NPs. The highest ash content and significantly less contents of protein and fat in a TN2 group of fish fed diet supplemented with 3 mg Co-NPs /kg diet in contrast to Co-Met complex indicated that Co-NPs at higher dosage level could cause toxicity and negatively affect the proximate composition of fish (Table 47). No comparable study is available to compare our results; however, many investigators reported the negative impact of a higher dosage of other metals on the proximate composition of different fish species. For instance, Abdel-Tawwab et al. (2016) reported reduced crude protein and fat content of *Oreochromis niloticus* when exposed to the toxic level of Zn. Moreover, Berntssen et al. (1999) reported reduced whole body crude protein of *Salmo salar* when reared on toxic levels of dietary copper.

The muscle amino acid profile of the different groups of fish further validated our results and indicated a higher level of essential amino acids in the TM4 group of fish followed by the TM3 and TN1 group of fish (Table 48). Scientists had reported improved amino acid profile of fish in response to mineral supplemented diets e.g. Jiang et al. (2016) reported significantly improved muscle amino acid profile of grass carp in response to dietary Mn. However, a decrease in muscle amino acid

concentration was observed when fish were reared on cobalt-NPs supplemented diet at higher dosage level i.e., 3mg/kg diet (TN2 group) indicating the negative effect of Co-NPs supplemented diet on the nutritive quality of fish. Like our result, Bharat and Alkesh (2014) reported a decrease in quality and quantity of amino acids in the muscle of *Labeo rohita* exposed to cobalt sulfate at a higher concentration level for long duration.

Here the expression trend of growth and stress-related genes were also in accord with our other observations and indicated the higher efficiency of dietary Co-Met complex i.e., dose-dependent significantly higher expression of MyoD and MyoG genes in muscle and lower expression of heat shock protein HSP-70KDa in the liver as compared to Co-NPs. However, the impact of dietary Co-NPs at a dosage level of 1.5mg/kg diet on gene expression was significantly higher than control and statistically lower than dietary Co-Met complex. Moreover, the toxicological effect of Co-NPs at higher dosage level i.e., 3mg Co-NPs/kg was evident i.e., significantly suppressed expression of MyoD and MyoG genes in muscle and higher expression of heat shock protein HSP-70KDa in the liver as compared to genes expression observed in a control group of fish. The overall result indicated the most significant dose-dependent positive effect of the Co-Met complex supplemented diet on the myogenesis. Since HSP-70KDa is considered as a stress indicator in fish, its increased expression indicated disruption of normal cellular processes in response to toxicity. Different scientists reported increased production of heat shock protein in response to metal toxicity e.g. in *Tanichthys albonubes* (Jing et al., 2013), *Danio rerio* (Guo et al., 2018), *O. niloticus* (Girilal et al., 2015), and *Chanos chanos* (Rajeshkumar et al., 2013).

Conclusion

Overall results of this study indicate that the beneficial and toxicological effect of dietary supplementation depends on the particle size and chemical form of cobalt. Conventional $CoCl_2$ up to dosage level of 2mg/kg diet showed dose-dependent positive effect on the growth and proximate body

composition of fish but at greater doses showed a negative effect on study parameters. However, Co-NPs supplementation showed somewhat more pronounced beneficial effects at lower dosage level i.e., 1-1.5mg/kg diet and above these levels toxicological effects appeared. Moreover, as compared to inorganic form of cobalt (Co-NPs), the Co-Met complex (organic) showed the most pronounced dose-dependent positive effect on growth performance, hemato-immunological response, proximate body composition, intestinal enzyme activities, and myogenesis in *T.putitora*. It is clear that supplementation that Co-Met complex supplemented diet minimizes negative effects associated with higher doses or other forms and is an alternative possible candidate for cobalt supplementation during practical feed formulations.

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Dietary cobalt supplementation improves growth and body composition and induces the expression of growth and stress response genes in *Tor putitora*

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Abstract A 90-day randomized feeding experiment was performed to assess the effects of dietary cobalt (Co) supplementation on the growth performance, muscle composition, status of iron and manganese in the muscle as well as the expression of growth-related genes in the muscle (myoblast determination protein 1 homolog (MyoD) and myogenin) and the stress-related gene heat shock protein 70 KDa (Hsp-70) in the liver of mahseer (*Tor putitora*). Feeding trial was conducted in triplicate under controlled semi-static conditions, and graded levels of dietary cobalt (0.5–3 mg/kg) were fed to six groups of advanced fry of *T. putitora*. The results obtained indicated a curvilinear relationship of dietary Co levels with body crude protein content and weight gain (%). A positive correlation was observed with up to 2 mg Co/kg diet. However, a decreasing trend was found with values over 2 mg Co/kg diet. The expression of muscle growth biomarkers MyoD and myogenin showed a similar response, upregulation up to 2 mg Co/kg diet and decreased expression at 3 mg Co/kg diet. Indeed, the highest dietary Co supplementation increased the expression of Hsp-70, a key gene expressed in response to stress. Moreover, the muscle content of iron and manganese showed an inverse relationship with the dietary Co supplementation. Our findings suggest that 2 mg/kg Co dietary supplementation stimulates myogenesis and optimize muscle growth and body composition, while higher levels enhanced the expression of stress response genes and impaired growth of *T. putitora*.

Keywords Cobalt chloride · Myoblast determination protein 1 homolog · Myogenin · Heat shock protein 70 KDa · Mahseer

Introduction

Properly balanced feed formulation according to developmental stage and species is a prerequisite for optimal health status and growth performance in fish. Dietary protein and other essential nutrients such as lipids, vitamins, carbohydrates and minerals are required in optimum quantity for maintaining growth and improving health (Whitney and Rolfes 1993). Fortified feed with essential nutrient supplementation can prevent susceptibility to disease and nutrient-related deficiencies that may impair and disturb body functions.

Fish can absorb minerals and other compounds from surrounding water. Nevertheless, due to low concentration in water, freshwater fish generally require minerals

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as dietary supplements to fulfill body requirements (Beveridge et al. 2013). Supplementation of any particular mineral in feedstuffs should be carefully screened to avoid metal accumulation at toxic levels (Malomo and Ihegwuagu 2017). The current knowledge about supplementation of metallic minerals in fish is mostly limited to iron, manganese, zinc and selenium, which are the main elements of body fluids and the basic components of non-enzymatic macromolecules and can also act as cofactors in enzymatic reactions (Robbins 1993). However, various spiteful symptoms associated with deficiencies of other minerals like chromium, copper, fluorine, iodine and molybdenum, are well known (Lall 2002; Terech-Majewska et al. 2016).

Cobalt is considered as an important essential micromineral in fish and other vertebrates (Perrault et al. 2014; Rahal and Shivay 2016). It regulates blood glucose levels, the activity of many enzymes and intestinal microbial synthesis of vitamin B_{12}, which is a cofactor of two important enzymes, i.e. methylmalonyl-CoA mutase (MCM) and methionine synthase (MS) (Speich et al. 2001; Siddiqui et al. 2014). MS is involved in the synthesis of methionine and nucleic acids (McDowell 1989), while MCM plays an important role in the Krebs cycle by contributing to the synthesis of succinyl-CoA (Kräutler 2005; Takahashi-Iñiguez et al. 2012; Moll and Davis 2017).

Worldwide Co abundance in the Earth's crust and in water is relatively low (Karadede-Akin and Ünlü 2007; Swanner et al. 2014). Therefore, it must be supplied with the feed (Robbins 1993; Mukherjee and Kaviraj 2009). Dietary Co requirement in fish varies with age, size, species and culture conditions (Wilson 1991). Although plant- and animal-based proteins are a source of Co for aquafeeds, some freshwater fish require additional Co in their diet for optimum growth (Wilson 1991). Though Co is ranked as an essential dietary mineral, knowledge about the optimum dietary requirement of cobalt in many fish species is limited.

Mahseer (*Tor putitora*) is a cyprinid fish, widely distributed throughout the Indian subcontinent. It is an important game fish and regarded as a valued fish due to its large size and high commercial value (Bhatt and Pandit 2016). It is classified as an endangered species due to the depletion of natural stocks. In Pakistan, it is cultured in captivity under controlled environment conditions. Contrary to natural bodies of water, culturing fish in captivity requires fortified nutrition and adequate feeding (Oliva-Teles 2012). Bearing in mind the importance of Co as an essential micromineral in feedstuffs for fish farming and the fact that limited literature has addressed the dietary requirement of Co, the present study was designed to determine optimal dietary supplementation levels of Co for *T. putitora*. To this end, we analyzed the effect of graded levels of dietary Co supplementation on growth rate, body composition, metal bioaccumulation and the expression of genes involved in growth and stress responses.

Material and methods

Experimental design

Mahseer (*T. putitora*) advanced fry were used for this study. Uniform-sized mahseer (average wet weight ± SEM, 1.35 ± 0.02 g) were selected and randomly distributed (stocking density = 1.5 g/L, i.e. 30 fish/tank) in 21 rectangular fish rearing fiberglass troughs (capacity = 45 L) equipped with heaters and an aeration system for maintaining water temperature (22.5°C) and dissolved oxygen (DO) level (6.0 mg/L). A basal diet containing 39.4% of crude protein was used as a control without Co supplementation (Table 1). Experimental diets A–F were designed on the basis of the basal diet but including increasing amounts of cobalt chloride hexahydrate (Sigma, USA), which was added to finely ground feed ingredients (A, 0.5 mg/kg diet; B, 1.0 mg/kg diet; C, 1.5 mg/kg diet; D, 2.0 mg/kg diet; E, 2.5 mg/kg diet; and F, 3.0 mg/kg diet). Feed pellets were prepared, dried and saved in Ziploc bags by following a standard method described previously (Amir et al. 2018). Fresh feed was prepared after every 15 days. Before providing feed to the respective groups, feed pellets were crushed by using a pestle motor and sieved through a fine mesh (150–250 μm), while feed particle size was adjusted after 1 month on the basis of the size of the fry. The experiment was conducted using three tanks per treatment. Feeding trial started after 1 week of acclimatization to corresponding tanks (control fish fed with the basal diet and fish fed with experimental diets A–F). At the beginning of the experiment, fish were fed three times a day (9:00, 13:00 and 16:00) at a daily rate of 8% body weight. After 1 month, daily rate was reduced to 4% body weight and supplied twice a day (9:00 and 18:00) until the end of the trial. Remaining feed was removed after 2 h of feeding while fish excreta were removed daily. Both were filtered and collected

separately. Every day, the total volume of water was maintained by addition of fresh water. Throughout the experiment, water quality parameters were checked by means of Multi-parameter Hanna HI 9829-01102. The feeding trial lasted for 90 days, during which temperature and DO levels fluctuated slightly, i.e. temperature ± 0.2°C and DO level ± 0.35 mg/L, while total ammonia remained < 0.5 mg/L. All experimental procedures were approved by the Quaid-i-Azam University's animal welfare committee in compliance with local legislation (BEC-FBS-67-QAU-2017).

Sampling and growth measurements

At the end of the trial (90 days), fish were starved for 24 h before sampling. Fish from each tank were captured separately, weighed and counted for evaluation of growth performance. Standard formulas reported previously (Zhou et al. 2013; Munir et al. 2016) were adopted for determining growth performance:

$$\text{Average weight gain} = \text{average } w_f - \text{average } w_i$$

$$\text{Weight gain } (\%) = w_f - w_i \div w_i \times 100$$

$$\text{Feed conversion ratio (FCR)} = \text{Feed intake (g)} \div \text{Weight gain (g)}$$

$$\text{Specific growth rate } (\%) = (\ln w_f - \ln w_i) \div \text{experimental duration} \times 100$$

where w_i = initial body weight of fish, w_f = final body weight of fish.

About 6 fish per tank (18 fish/treatment) were anesthetized with buffered MS222 (0.1 mg/L) and dissected at low temperature (on an ice pad), and the muscle and liver were immediately collected and preserved in RNA later (Thermo Scientific CAT# AM7020) for molecular-based studies. To obtain sample in enough quantity for proximate analysis, 21 fry from each tank were caught and divided into three pools consisting of 7 fry each, thus representing three pools/tank (nine pools/treatment).

Body proximate composition

Standard protocols were adopted to determine mahseer body composition (AOAC 2000). All analyses were conducted at the ISO 17025 accredited laboratory facility of the Poultry Research Institute (PRI, Islamabad, Pakistan). The crude protein and fat contents were determined by using the Kjeldahl and Soxthlet extraction procedure, respectively, while ash content was determined by heating sample in a muffle furnace at 550°C.

Table 1 Formulation and composition of basal diet for *T. putitora*

Ingredients	Quantity (g/100 g)
White fish meal	45
Soybean meal	15
Sunflower meal	15
Gluten 60%	15
Rice polish	4
Wheat bran	4
CMC[a]	1
Vitamin premix[b]	1
Cobalt chloride[c]	0
Proximate composition (%)	
Crude protein	39.42
Crude fat	12.57
Ash	8.73

[a] Carboxy-methyl-cellulose

[b] Vitamin premix contains vitamins, amino acids and minerals premix/100g

[c] Cobalt chloride hexahydrate was added to experimental diets A (0.5 mg/kg diet), B (1.0 mg/kg diet), C (1.5 mg/kg diet), D (2.0 mg/kg diet), E (2.5 mg/kg diet) and F (3.0 mg/kg diet). The basal diet was not supplied with cobalt chloride

Metal bioaccumulation

Atomic absorption spectrometry was used to determine the muscle Co, iron and manganese contents. For this purpose, 1 g fish muscle was added to conical flasks containing 5 ml of HNO_3 and 1 ml $HClO_4$. The reaction mixture was digested on a hot plate at 200°C to 250°C until a clear and transparent solution was obtained. Samples were cooled at room temperature, filtered through a Whatman No. 42 filter paper, diluted by adding 50 ml of distilled water and analyzed for Co (λ = 240 nm), Fe (λ = 248 nm) and Mn (λ = 279.5 nm) by using Varian AA240FS (Palo Alto, CA, USA) for fast sequential atomic absorption spectroscopy. For each metal studied, standard calibration curves were performed to determine metal concentrations in samples.

Molecular cloning of *T. putitora* myoblast determination protein 1 homolog, myogenin and heat shock protein 70 KDa cDNA fragments

Total RNA from muscle and liver samples was isolated by using High Pure RNA tissue kit (Roche, Basel, Switzerland) and Illustra RNAspin Mini Isolation Kit (GE Healthcare, Chicago, IL, USA), respectively. Following quantification with NanoDrop ND-1000 (Thermo Scientific, Waltham, MA, USA), 1 μg of total RNA was used to synthesize cDNA by incubating with M-MLV RT (Promega, Madison, WI, USA) for 1 h at 37°C in the presence of random hexamers. Reverse transcription polymerase chain reaction (RT-PCR) was performed with primers designed from conserved regions of myoblast determination protein 1 homolog (MyoD), myogenin and heat shock protein 70 KDa (Hsp-70) coding-domain sequences in closely related fish species (*Danio rerio*, *Ctenopharyngodon idella*, *Cyprinus carpio* and *Labeo rohita*) (Table 2). PCR products, MyoD and myogenin from the muscle, and Hsp-70 from the liver were purified and then ligated into pGEM-T Easy (Promega, Madison, WI, USA). The resulting constructs were used to transform competent *E. coli* cells. Positive colonies were grown overnight at 37°C in liquid LB media supplemented with ampicillin to isolate plasmid DNA by using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich, San Luis, MI, USA). Plasmids with inserted fragments were totally sequenced on both strands. The cloning of amplified products and sequencing of three independent clones for each gene allowed us to obtain cDNA fragments of 421 bp for MyoD, 615 bp for myogenin and 353 bp for Hsp-70. The nucleotide sequences isolated for *T. putitora* MyoD, myogenin and Hsp-70 were submitted to the DDJB/EMBL/GenBank databases under accession numbers MH545701, MH545702 and MH545703, respectively. The inferred amino acid sequences of *T. putitora* MyoD, myogenin and Hsp-70 were aligned with GDH orthologues in other fish species and vertebrates to explore evolutionary relationships and generated phylogenetic trees by pair-wise alignments (Sievers et al. 2011).

Quantitative RT-PCR

Total RNA (1 μg) isolated from tissue samples was reverse transcribed for 1 h at 37°C with M-MLV RT (Promega, Madison, WI, USA). MyoD, myogenin and Hsp-70 mRNA levels were determined with StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). To this end, 0.4 μM of the corresponding primer pair (Table 3), 10 μl of SYBR Green (Applied Biosystems, Foster City, CA, USA) and 1.6 μl of diluted cDNA were mixed in a 20-μl reaction. The temperature cycle protocol for amplification was 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 62°C for 1 min. A dissociation curve was run after each experiment to ensure amplification of single products. The efficiency of the PCR reaction was checked for each gene by generating standard curves with serial dilutions of a control cDNA sample. MyoD, myogenin and Hsp-70 mRNA levels were normalized to the expression of *T. putitora* β-actin (primers shown in Table 3). Variations in gene expression were calculated by the standard $\Delta\Delta C_T$ method (Pfaffl 2001).

Statistics

Statistical package program SPSS (version 20) was used to analyze data. Univariate generalized linear model (GLM) followed by LSD test was applied to determine significant differences ($P < 0.05$) among treatments for growth performance, proximate composition and metal accumulation in the muscle of *T. putitora*. One-way ANOVA was used to analyze gene expression data.

Results

Growth performance

The relative growth performance of *T. putitora* in response to different levels of dietary Co supplementation is shown in Table 4. At the end of the experiment, a positive relationship was observed between % weight gain and Co dietary dosage level up to 2 mg Co/kg diet ($R^2 = 0.889$). However, values over 2 mg Co/kg diet negatively affected weight gain ($R^2 = 0.98$). Among different treatments, the highest percentage of weight gain was observed in fish fed 2 mg/kg dietary Co ($P < 0.05$). However, in fish fed diets supplemented with 2.5 mg/kg (group E) and 3 mg/kg (group F), dietary Co reduced fish weight compared to the control by 8% and 11.3%, respectively.

Table 2 Putative primers used in the present study for isolation of MyoD, myogenin and Hsp-70 cDNA fragments

Gene	Primer type	Sequence 5′ to 3′	Amplicon size
MyoD	Forward	TTTCTACGACGACCCTTGCTTC	464 bp
	Reverse	TGCCATCAGAGCAGTTGGATC	
Myogenin	Forward	CCAGCGTTTTTACGAAGGCG	665 bp
	Reverse	ACGTCAGAGACCTCAGGTTGG	
Hsp-70	Forward	ATGGTCCTGGTGAAGATGAAG	396 bp
	Reverse	GATGTCCTTCTTGTGCTTCCTC	

Whole body composition

Whole body proximate composition in response to different levels of dietary Co supplied to mahseer is shown in Table 5. Crude protein content showed a positive correlation ($R^2 = 0.99$) with dietary Co supplementation up to 2 mg/kg diet (group D), having values significantly higher than those of the control fish ($P < 0.05$). However, the highest level of dietary Co (3 mg/kg, group F) showed a negative effect (deceased content) on muscle protein. In contrast to protein content, muscle fat and ash exhibited a positive correlation ($R^2 = 0.8587$ and $R^2 = 0.756$, respectively) with dietary Co even at the highest supplementation level.

Metal bioaccumulation

The effect of graded levels of dietary Co concentration on the accumulation of Fe, Mn and Co in muscle is shown in Table 6. The concentration of Mn and Fe in the muscle of *T. putitora* showed a negative correlation (Mn, $R^2 = 0.907$; Fe, $R^2 = 0.993$) while Co showed a positive correlation ($R^2 = 0.953$) with dietary Co supplementation (Fig. 1a–c).

Cloning of *T. putitora* MyoD, myogenin and Hsp-70 cDNA fragments

Among fish, *T. putitora* MyoD exhibited higher similarity with sequences reported for *C. idella*, *D. rerio* and *C. carpio* (100%, 99.3% and 97.9% of identity, respectively), while the identity with other fish species ranged from 87.1% to 92.4%. A lower similarity was observed when compared with mammalian orthologues: identity with *Homo sapiens* and *Mus musculus* was 82.1% and 82.9%, respectively (Supplementary Fig. S1). As seen in MyoD, *T. putitora* myogenin also exhibited higher similarity with *D. rerio* and *C. carpio* (90.2% and 94.1% of identity, respectively). However, the percent identity with other fish species ranged from 68.9% to 74.0%, while 59.3% and 58.7% similarity were found with *H. sapiens* and *M. musculus*, respectively (Supplementary Fig. S2). For *T. putitora* Hsp-70, we found 98.3% similarity with amino acid sequences reported for *D. rerio*, while for other fish species, similarity ranged from 88.9% to 93.2%. The similarity of *T. putitora* Hsp-70 with that of *H. sapiens* and *M. musculus* was 85.5% and 90.6%, respectively (Supplementary Fig. S3).

Table 3 Putative primers used in the present study for RT-qPCR

Gene	Accession no.(GenBank)	Primer type	Sequence 5′ to 3′	Amplicon size
β-actin	KU714644.1	Forward	GCTGTGCTGTCCCTGTATGC	99 bp
		Reverse	GGCGTAACCCTCGTAGATGG	
MyoD	MH545701	Forward	CGCTTTCGAGACCCTCAAGAG	107 bp
		Reverse	GCGCCTGCAGAGACTCAATG	
Myogenin	MH545702	Forward	GGACAAACCGTCTCCATCTTC	165 bp
		Reverse	CCTCCTCTTCTCCCTCAAAGTG	
Hsp-70	MH545703	Forward	GATTGCTGAAGCCTATTCTGG	83 bp
		Reverse	TTGCCTCTGGGAGTCATTG	

Table 4 Effect of graded levels of dietary cobalt supplement on the growth performance of *T. putitora*

Diet groups	Mean initial weight (g)	Mean final weight (g)	Average weight gain (g)	Weight gain (%)	Specific growth rate (%)	FCR
Control	1.36 ± 0.006^{a}	2.39 ± 0.051^{d}	1.03 ± 0.201^{e}	75.73 ± 0.413^{e}	0.63 ± 0.003^{bc}	5.61 ± 0.001^{c}
A	1.35 ± 0.005^{a}	2.41 ± 0.020^{cd}	1.06 ± 0.043^{d}	78.44 ± 0.132^{d}	0.64 ± 0.001^{bc}	5.52 ± 0.002^{d}
B	1.34 ± 0.001^{a}	2.46 ± 0.012^{c}	1.12 ± 0.054^{c}	83.61 ± 0.213^{c}	0.68 ± 0.005^{b}	5.13 ± 0.001^{e}
C	1.34 ± 0.003^{a}	2.73 ± 0.032^{b}	1.39 ± 0.023^{b}	103.98 ± 0.432^{b}	0.79 ± 0.009^{a}	4.06 ± 0.005^{f}
D	1.33 ± 0.005^{a}	2.98 ± 0.017^{a}	1.65 ± 0.053^{a}	123.47 ± 0.332^{a}	0.81 ± 0.006^{a}	3.32 ± 0.004^{g}
E	1.33 ± 0.003^{a}	2.24 ± 0.053^{e}	0.91 ± 0.033^{f}	67.77 ± 0.543^{f}	0.58 ± 0.002^{c}	6.36 ± 0.006^{b}
F	1.34 ± 0.002^{a}	2.20 ± 0.042^{e}	0.86 ± 0.013^{g}	64.46 ± 0.324^{g}	0.55 ± 0.031^{c}	6.19 ± 0.005^{a}

Data are expressed as mean ± SEM ($n = 3$). Different letters within the columns indicate significant differences between groups ($P < 0.05$)

A = 0.5 mg Co/kg diet, B = 1 mg Co/kg diet, C = 1.5 mg Co/kg Co, D = 2 mg/kg Co, E = 2.5 mg/kg Co, F = 3 mg/kg Co

Expression levels of MyoD, myogenin and Hsp-70

Availability of cDNA sequences for *T. putitora* MyoD, myogenin and Hsp-70 allowed us to design primers to determine the mRNA levels of the three genes by quantitative RT-PCR (RT-qPCR) in fish fed basal/control diet (devoid of Co supplementation) and diets supplemented with 2 and 3 mg /kg Co. Feeding dietary Co resulted in significant changes of mRNA levels for muscle MyoD and myogenin as well as hepatic Hsp-70 in *T. putitora*. In the muscle, the highest mRNA level for both MyoD and myogenin was found in fish fed 2 mg/kg dietary Co, while feeding 3 mg/kg dietary Co resulted in the lowest expression levels for both proteins, even when compared with control fish (Fig. 2a,b). Albeit MyoD and myogenin behaved similarly, the expression of myogenin showed better correspondence with weight gain and growth rate. In contrast to MyoD and myogenin expression in the muscle, the highest Hsp-70 mRNA levels in the liver were observed in fish fed 3 mg Co/kg diet (Fig. 2c).

Table 5 Effect of different levels of dietary Co chloride on muscle proximate composition of *T. putitora*

Diet groups	Protein (%)	Fats (%)	Ash (%)
Control	16.31 ± 0.042^{e}	1.53 ± 0.23^{f}	1.56 ± 0.035^{f}
A	16.90 ± 0.023^{d}	1.61 ± 0.45^{e}	1.63 ± 0.052^{e}
B	18.14 ± 0.032^{c}	1.63 ± 0.021^{e}	1.65 ± 0.075^{e}
C	18.98 ± 0.043^{b}	1.82 ± 0.065^{d}	1.71 ± 0.034^{d}
D	20.02 ± 0.023^{a}	2.13 ± 0.032^{c}	2.06 ± 0.065^{c}
E	17.01 ± 0.067^{d}	2.48 ± 0.012^{b}	2.26 ± 0.043^{b}
F	15.09 ± 0.054^{f}	3.14 ± 0.054^{a}	2.75 ± 0.032^{a}

Data are expressed as mean ± SEM ($n = 9$). Different letters within the columns indicate significant differences ($P < 0.05$)

A = 0.5 mg/kg Co, B = 1 mg/kg Co, C = 1.5 mg/kg Co, D = 2 mg/kg Co, E = 2.5 mg/kg Co, F = 3 mg/kg Co

Discussion

The results of the present study demonstrate beneficial effects of dietary Co supplementation on growth performance, body composition, muscle content of manganese and iron as well as expression of growth-regulating genes in *T. putitora*. Similarly to our results, many investigators observed beneficial effects of dietary Co supplement on growth, survival, protein synthesis, glucose metabolism, insulin effectiveness in utilizing glucose, and efficiency of protein in fish species such as rainbow trout (Blust 2011), *Labeo rohita* (Adhikari and Ayyappan 2002) and common carp (Satoh 1991).

In the present study, we observed a positive correlation between weight gain (%) and dietary Co supplementation ranging from 0 to 2 mg/kg. The highest percentage of weight gain was observed in fish fed 2 mg/kg dietary Co, while higher levels of Co supplementation had negative impact on weight gain. Diets supplemented with 2.5 mg/kg (group E) and 3 mg/kg (group F) dietary Co reduced fish weight compared to the control by 8% and 11.3%, respectively. According to our results, 2 mg/kg is the optimum level of dietary Co supplementation for early rearing of *T. putitora*. This level is somewhat higher than the 1 mg/kg diet, which was reported for gaining 40% increase in survival rates of *C. carpio* hatchlings (Mukherjee and Kaviraj 2009), and 0.05 mg/kg dietary Co as reported for channel catfish (Wilson 1991), while lower than the 2.5 and 5 mg/kg diet which are known to improve growth

Table 6 Effect of different levels of dietary Co chloride on metal accumulation in *T. putitora* muscle

Diet groups	Metal concentrations (μg/g of sample tissue)		
	Iron	Manganese	Cobalt
Control	0.0303 ± 0.0015^{a}	0.00422 ± 0.0054^{a}	0.01075 ± 0.0021^{d}
A	0.0281 ± 0.0023^{b}	0.00442 ± 0.0044^{a}	0.01082 ± 0.0043^{d}
B	0.0250 ± 0.0045^{c}	0.00415 ± 0.0032^{a}	0.01115 ± 0.0012^{c}
C	0.0211 ± 0.0023^{d}	0.0038 ± 0.0056^{b}	0.01135 ± 0.0065^{c}
D	0.0163 ± 0.0034^{e}	0.0034 ± 0.00432^{c}	0.01214 ± 0.0054^{b}
E	0.0133 ± 0.0014^{f}	0.003451 ± 0.002^{c}	0.01221 ± 0.0062^{b}
F	0.0096 ± 0.0052^{g}	0.00310 ± 0.002^{d}	0.01260 ± 0.0042^{a}

Data are expressed as mean ± SEM ($n = 3$). Different letters within the columns indicate significant differences ($P < 0.05$)

A = 0.5 mg/kg Co, B = 1 mg/kg Co, C = 1.5 mg/kg Co, D = 2 mg/kg Co, E = 2.5 mg/kg Co, F = 3 mg/kg Co

performance of sea bass (*Lates calcarifer*) and catfish (*Clarias batrachus*), respectively (Sapkale and Singh 2011). Reports indicating different optimum levels of Co supplement may reflect variations in metabolic and functional demand of micronutrients, which depend on species, age, size, sex, feed, feeding practices and farming conditions (Biesalski Hans and Jana 2018). The improved growth performance observed in the present study in response to dietary Co supplementation may result from the well-known involvement of Co in nitrogen assimilation, hemoglobin synthesis, manufacturing of muscular protein as well as in fish metabolism and biochemical processes (Silvers and Scott 2002).

Dietary Co supplements above optimum level (2 mg/kg diet) showed negative effects on the growth performance of *T. putitora*. Like our results, Mukherjee and Kaviraj (2011) also reported a decrease in the weight gain of catfish, *Heteropneustes fossilis* (Bloch), with dietary Co supplementation above 0.1%. According to Chanda et al. (2015), high dietary cobalt levels over 5 g/kg are toxic to rainbow trout. This might be due to toxicity resulting from increased dietary Co levels, leading to inhibition of key enzymes and biochemical pathways by displacing cations of metal-activated enzymes in their ion centers, and oxidative damage to DNA, lipids and protein structure due to the generation of reactive oxygen species (ROS). In fact, Co ions are considered cytotoxic at high concentrations (Simonsen et al. 2012), which may induce necrosis and inflammation (Abudayyak et al. 2017).

Exogenous factors such as environment and feed composition affect the proximate composition of cultured fish (Alemu et al. 2013). Dietary feed ingredients play significant roles in fish body composition. Body composition assessment allows us to study the efficiency of nutrient transfer from feed to fish (Whitney and Rolfes 1993). Fish is considered as a rich source of important minerals, vitamins and essential amino acids and fatty acids. The protein content of fish has a marked biological significance due to the presence of essential amino acids. Our findings indicate that dietary Co up to 2 mg/kg increased protein content in the muscle as compared to the control fish. Since dietary Co facilitates amino acid incorporation into fish, therefore, it can promote a protein sparing effect by improving glucose tolerance and reducing gluconeogenesis (Kawakami et al. 2012; Ghica et al. 2013). Consistent with our results, Tonye and Sikoki (2014) reported increased crude protein content in juvenile tilapia (*Oreochromis niloticus*), while a similar effect was observed in *C. carpio* fed dietary supplement of Co (Mukherjee and Kaviraj 2009). In contrast to protein content, crude fat in our experimental trial positively correlated with dietary Co concentration, even at levels above 2.5 mg/kg. Likewise with *T. putitora*, dietary Co chloride also increased crude fat content in *C. carpio* (Mukherjee and Kaviraj 2009). This might be due to the involvement of Co in lipid metabolism by decreasing circulating levels of LDL cholesterol and triglycerides and increasing HDL cholesterol (Kawakami et al. 2012).

Fish is also a source of essential minerals to consumers (Steffens 2006). However, inclusion of metals in aquafeeds needs to be cautiously screened to certify that the metal is not accumulated at levels that may elicit toxicological effects. We found no reports that addressed the standardization of recommended dietary

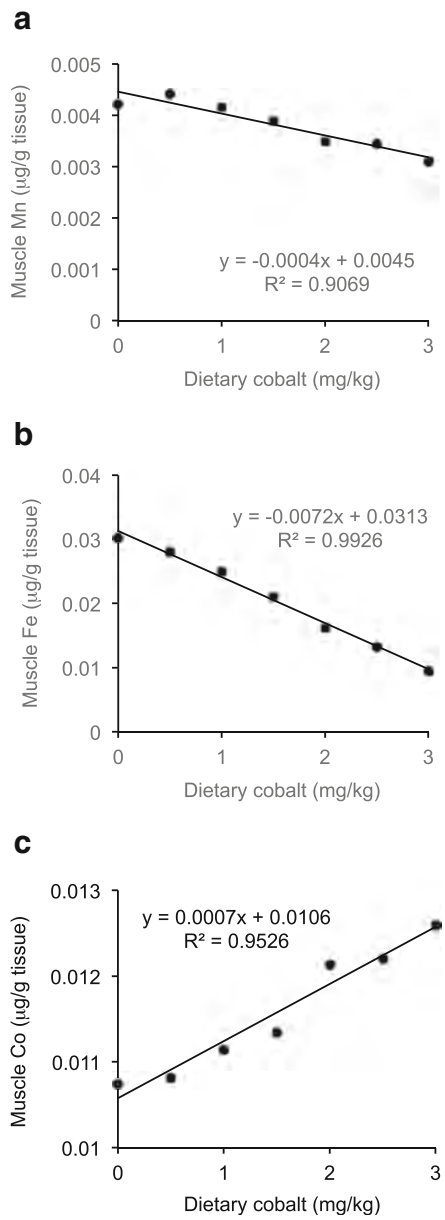

Fig. 1 Correlation between muscle accumulations of metals with graded level of dietary cobalt. Linear regression analysis for muscle accumulation of Mn (**a**), Fe (**b**) and Co (**c**) is shown. Each point represents the mean metal concentration in the muscle of three fishes

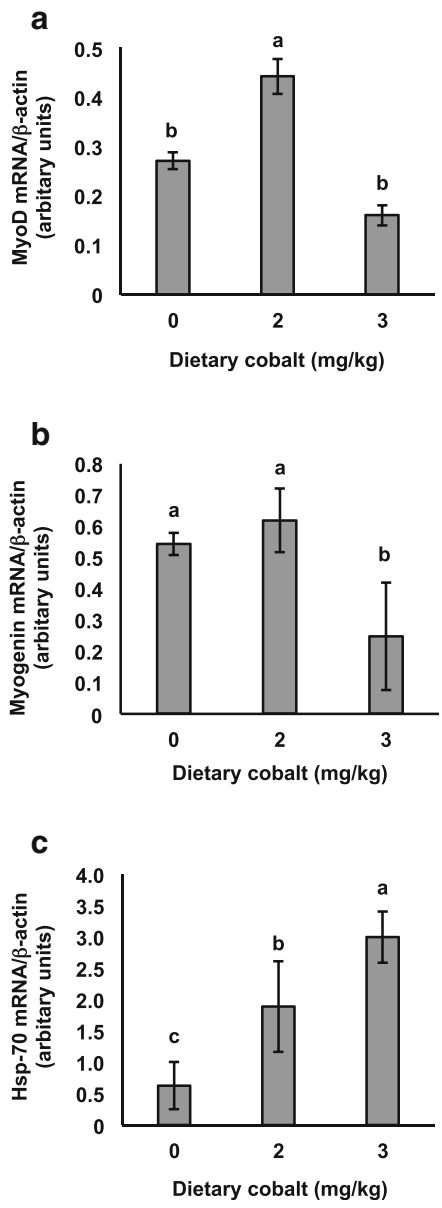

Fig. 2 MyoD, myogenin and Hsp-70 mRNA levels in the muscle and liver of *T. putitora*. Analysis of MyoD (**a**), myogenin (**b**) and Hsp-70 (**c**) mRNA levels relative to β-actin was performed by RT-qPCR in muscle and liver samples of *T. putitora* fed for 90 days with a diet supplemented with Co (2 mg/kg and 3 mg/kg) or without dietary Co (control). The values are expressed as mean ± SEM (n = 4). Statistical significance related to the control (without dietary Co supplementation) is indicated with different letters ($P < 0.05$)

allowance (RDA) of Co for fish. Indeed, micronutrient deficiency may result in increased accumulation of heavy metals from the environment (Golovanova 2008). In fish, the kidney, liver, gill, and gut are the main tissues where heavy metals are accumulated (Farkas et al. 2003; Ambreen et al. 2015). However, accumulation of dietary Co and its distribution in different tissues of fish are not precisely known. In our feeding trials, Co accumulation in the muscle positively correlated with dietary levels ($R^2 = 0.952$). Our findings are consistent with previous observations in other fish

species (Yildiz 2008). Although Co is an essential mineral that participates in biochemical processes, a high concentration may disrupt many enzymatic functions, causing toxicity (Javed 2013; Rai et al. 2015). In our experimental trial, iron accumulation shows a negative linear relationship with respect to increasing dietary Co supplementation. Most of the metals are competitively taken up in the intestinal tract of fish (Norwood et al. 2003). However, some intercellular transport systems are specific to one metal only, yet some are less selective, such as divalent metal transporter DMT1. Co is most commonly absorbed across the tissues through voltage-gated calcium channels and ligand-sensitive channels (Simonsen et al. 2012). Therefore, decreased levels of iron and manganese in the muscle resulting from increased dietary Co might be related to decreased absorption due to concentration-dependent interactive effects between metals (Kwong and Niyogi 2009).

For validating the results at the molecular level, the expression of growth and stress response genes through RT-qPCR was determined. Due to the unavailability of sequence information for *T. putitora* MyoD, myogenin and Hsp-70 messengers, genes of interest were first cloned and then sequenced. Isolation of cDNA fragments for these genes allowed us to assess changes in the expression levels of MyoD, myogenin and Hsp-70. *T. putitora* belongs to Cyprinidae, and as expected, alignment of the inferred peptide sequences of MyoD, myogenin and Hsp-70 with other cyprinids such as *D. rerio*, *C. carpio* and *C. idella* gave higher identities. Higher identity of amino acid sequences of *T. putitora* MyoD and Hsp-70 as compared to phylogenetically distant fish species and even with mammals suggest a high degree of structural conservation and conceivably functionality of MyoD and Hsp-70 during vertebrate evolution.

In contrast to MyoD and Hsp-70, *T. putitora* myogenin amino acid sequence displayed higher identity with species belonging to the same order (Cypriniformes), while the identity was markedly lower when comparing Cypriniformes to fish species belonging to Salmoniformes and Pleuronectiphormes as well as to mammals. Our findings argue for less conserved evolution of myogenin orthologues in vertebrates or specific evolution of myogenin in Cypriniformes.

MyoD and myogenin mRNA expression in the muscle of fish fed different dietary Co levels provided further insight about involvement of this mineral in the growth of *T. putitora.* The mRNA levels of MyoD and myogenin in the muscle showed a positive correlation with weight gain up to 2 mg/kg dietary level of Co. Cobalt chloride is a bioactive compound which induces the expression of a series of hypoxia response genes such as hypoxia-inducible factor (HIFα) by acting as a hypoxia-mimicking agent (Ji et al. 2012). HIFα is localized in the nucleus and cytoplasm of myotubes and myoblasts. Its increased expression in response to Co stimulates the myogenic differentiation and expression of myogenin and MyoD proteins (Wagatsuma et al. 2011). Co supplement-dependent increase in weight gain in our study might be due to the stimulation of myogenic regulating factors (MRFs) such as myogenic factor 5 (MyF5), MyoD, MRF4 and myogenin as well as increased skeletal muscle fiber formation (myogenesis) (Rescan 2001). These MRFs are highly conserved helix-loop-helix proteins in fish, and increased MyoD expression has a major role in regulating myogenesis and specification of newly formed skeletal muscles. MyoD is basically involved in the proliferation and activation of satellite cells towards the myogenic pathway while myogenin controls cell differentiation and myoblast fusions to form new myofibers. Additionally, Co is involved in the expression of several glycolytic enzymes and glucose transporters which enable anaerobic energy metabolism for regenerating myofibers (Wagatsuma et al. 2011). Since newly formed myofibers possess less capillary development to provide oxygen enough to regenerate skeletal muscle fibers, activation of glycolytic enzymes in response to Co may help newly formed myofibers.

Fish have well-developed networks of stress responses for adapting to environmental changes at the cellular level(Barton 2002). Such stress responses include the transcription of several stress proteins such as heat shock protein (Hsp) family members (Kregel 2002). Transcription of Hsp genes in response to different xenobiotics is considered a useful biomarker to assess the effect of metals in fish. We found a significant upregulation of Hsp-70 in the liver of *T. putitora* fed 3 mg/kg dietary Co compared to the control fish or fish fed with lower levels of dietary Co. This finding confirms the toxicity of Co when its level exceeded the optimum level. The liver plays a central role in metabolism, and it can be greatly affected by metal absorption in the gut (Soetan et al. 2010). Overexpression of Hsp-70 in response to higher dietary Co levels may indicate the stimulation of a cytoprotective mechanism, i.e. repair of metal-induced damaged proteins or bringing

them back to their normal conformation (Sener et al. 2003). Given that fish under stress conditions reduce feed intake (Lupatsch et al. 2010), retarded growth at higher dietary levels of Co could be the result of inadequate availability of nutrients required for proper growth performance (Yengkokpam et al. 2008).

In conclusion, our study revealed that 2 mg/kg of dietary Co supplementation is the optimum level where *T. putitora* showed improved growth performance by a mechanism involving increased protein content and enhanced expression of genes involved in muscle growth and differentiation. In contrast, higher levels of dietary Co increased the expression of the stress response gene Hsp-70 and had negative effects on growth.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Zhou Y, Yuan X, Liang X-F et al (2013) Enhancement of growth and intestinal flora in grass carp: the effect of exogenous cellulase. Aquaculture 416–417:1–7

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LOCUS       Seq1                    421 bp    mRNA    linear   VRT 27-JUN-
2018
DEFINITION Tor putitora myoblast determination protein 1 homolog (myod) mRNA,
            Partial cds.
ACCESSION   Seq1
VERSION
KEYWORDS    .
SOURCE      Tor putitora (golden mahseer)
  ORGANISM  Tor putitora
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi;
            Cypriniformes; Cyprinidae; Tor.
REFERENCE   1  (bases 1 to 421)
  AUTHORS   Younus,N., Zuberi,A. and Meton,I.
  TITLE     Biological and toxicological effects of dietary organic and
            inorganic forms of cobalt on Mahseer (Tor putitora)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 421)
  AUTHORS   Younus,N., Zuberi,A. and Meton,I.
  TITLE     Direct Submission
  JOURNAL   Submitted (27-JUN-2018) Bioquimica i Fisiologia, Universitat de
            Barcelona, Joan XXIII, 27, Barcelona 08028, Spain
COMMENT     Bankit Comment: TOTAL # OF SEQS:3.

            ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /mol_type="mRNA"
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                     /country="Pakistan"
     gene            <1..>421
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     CDS             <1..>421
                     /gene="myod"
                     /codon_start=1
                     /product="Myoblast determination protein 1 homolog"

/translation="NTNDMHFFEDLDPRLVHVSLLKPDEHHHIEDEHVRAPSGHHQAG

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                     LRNAISYIESLQALLRSQEENYYPVLEHYSGDSDASSP"
BASE COUNT      120 a    125 c    110 g     66 t
ORIGIN
        1 aacacaaacg atatgcactt ctttgaagac ctggacccca ggctcgtcca cgtgagtctg
       61 ctcaagcccg acgagcatca ccacatcgag gacgagcacg taagggcgcc cagcgggcat
      121 catcaggccg gcaggtgcct gctgtgggca tgcaaagcct gcaagagaaa aactaccaac
      181 gctgaccgcc gcaaagccgc caccatgagg gagaggagaa gactgagcaa agtcaacgac
      241 gctttcgaga ccctcaagag atgcacgtcc accaacccca accagaggct gcccaaagtg
      301 gagattctga gaaacgccat tagttacatt gagtctctgc aggcgctgct taggagtcaa
      361 gaggaaaact actaccctgt tctggaacat tacagtggag actccgatgc ctccagcccg
      421 a
//
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```
LOCUS       Seq2                    615 bp    mRNA    linear   VRT 27-JUN-
2018
DEFINITION  Tor putitora myogenin (myog) mRNA, partial cds.
ACCESSION   Seq2
VERSION
KEYWORDS    .
SOURCE      Tor putitora (golden mahseer)
  ORGANISM  Tor putitora
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi;
            Cypriniformes; Cyprinidae; Tor.
REFERENCE   1  (bases 1 to 615)
  AUTHORS   Younus,N., Zuberi,A. and Meton,I.
  TITLE     Biological and toxicological effects of dietary organic and
            inorganic forms of cobalt on Mahseer (Tor putitora)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 615)
  AUTHORS   Younus,N., Zuberi,A. and Meton,I.
  TITLE     Direct Submission
  JOURNAL   Submitted (27-JUN-2018) Bioquimica i Fisiologia, Universitat de
            Barcelona, Joan XXIII, 27, Barcelona 08028, Spain
COMMENT     Bankit Comment: TOTAL # OF SEQS:3.

            ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /mol_type="mRNA"
                     /db_xref="taxon:210638"
                     /country="Pakistan"
     gene            <1..>615
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     CDS             <1..>615
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                     /codon_start=3
                     /product="Myogenin"

/translation="DNFFQSRLTGGFDQAGYQDRSSMVGLCGDGRLLSNGVGMEDKPS

PSSSLGLSMSPHQEQQHCPGQCLPWACKVCKRKSVTMDRRKAATLREKRRLKKVNEAF

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                     SSSSDQGSGSTCCSSPEWSSTSEHCAPAYSSTHEDLLNDDSTEQ"
BASE COUNT      148 a    171 c    184 g    112 t
ORIGIN
        1 gcgataactt cttccagtca aggctgaccg gaggcttcga ccaagcagga tatcaggacc
       61 gaagctccat ggtgggcttg tgtggcgacg gaaggctgct gtcaaacgga gtggggatgg
      121 aggacaaacc gtctccatct tctagcctcg gtctgtccat gtctcctcac caggagcagc
      181 agcactgtcc gggtcagtgt ctgccctggg cctgcaaggt gtgtaagcgc aagtcggtga
      241 caatggatcg acggaaagcc gccactttga gggagaagag gaggctgaag aaagtcaacg
      301 aggcctttga ggctcttaag aggagcacgc tcatgaatcc caaccagagg ctgcctaagg
      361 tggagatcct gcgcagcgcc atccagtaca tcgagagact ccaggcgctg gtcagctctc
      421 tcaaccagca ggaacatgag cagggaaaca tgcattacag agccgcggct cctcaaggga
      481 tgtcgtcctc tagcgatcag ggctctggca gcacctgctg tagcagtcca gagtggagca
      541 gtacgtctga gcactgtgcc cccgcctaca gctccaccca tgaggatctc ctgaatgacg
      601 actccacaga gcaaa
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```
//
LOCUS       Seq3                    353 bp    mRNA    linear   VRT 27-JUN-
2018
DEFINITION  Tor putitora heat shock protein 70 KDa (hsp70) mRNA, partial cds.
ACCESSION   Seq3
VERSION
KEYWORDS    .
SOURCE      Tor putitora (golden mahseer)
  ORGANISM  Tor putitora
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi;
            Cypriniformes; Cyprinidae; Tor.
REFERENCE   1  (bases 1 to 353)
  AUTHORS   Younus,N., Zuberi,A. and Meton,I.
  TITLE     Biological and toxicological effects of dietary organic and
            inorganic forms of cobalt on Mahseer (Tor putitora)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 353)
  AUTHORS   Younus,N., Zuberi,A. and Meton,I.
  TITLE     Direct Submission
  JOURNAL   Submitted (27-JUN-2018) Bioquimica i Fisiologia, Universitat de
            Barcelona, Joan XXIII, 27, Barcelona 08028, Spain
COMMENT     Bankit Comment: TOTAL # OF SEQS:3.

            ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /mol_type="mRNA"
                     /db_xref="taxon:210638"
                     /country="Pakistan"
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     CDS             <1..>353
                     /gene="hsp70"
                     /EC_number="3.6.4.10"
                     /codon_start=1
                     /product="Heat shock protein 70 kDa"

/translation="EIAEAYLGQKVTNAVVTVPAYFNDSQRQATKDAGVIAGLNVLRI

INEPTAAAIAYGLDKGKASERNVLIFDLGGGTFDVSILTIEDGIFEVKATAGDTHLGG
                     EDFDNRMVNHFVEEF"
BASE COUNT       93 a     82 c     96 g     82 t
ORIGIN
        1 gagattgctg aagcctatct ggggcagaag gtgacaaatg cagttgtcac agttcctgcc
       61 tatttcaatg actcccagag gcaagcgact aaagatgctg gagtaatcgc tggactgaat
      121 gtcctgagaa tcatcaacga gcccacagct gcagctattg cctacggcct tgacaaaggc
      181 aaagcttcag agcgcaacgt cctgatcttt gacctgggcg gaggcacctt tgatgtgtcc
      241 atcctgacca ttgaagatgg catctttgag gtgaaggcca cagctggaga cactcatctg
      301 ggtggggagg actttgacaa ccgcatggtg aatcactttg ttgaagaatt caa
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