Dietary cobalt supplementation improves the growth, 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 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; 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 curvilinear relationship of dietary Co levels with body crude protein content and weight gain (%). A positive correlation was observed 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, up-regulation 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 fish feed formulation is a prerequisite for optimal health status and growth performance in fish. Throughout fish feed manufacturing practices, protein is considered as the main limiting nutrient. Nevertheless, additional essential nutrients such as lipids, vitamins, carbohydrates and minerals are also 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.

The importance of dietary trace minerals is evident in fish (Chanda et al. 2015). There are about thirteen essential elements that are required at optimum levels to sustain proper body functions in fish. When formulating fish feeds, it has to be considered that fish can absorb minerals and other compounds from surrounding water (Beveridge et al. 2013). Since the concentration of essential minerals in freshwater is low, freshwater fish usually require minerals as dietary supplements to fulfill body requirements (Velasco-Santamaría and Corredor-Santamaría 2011). However, 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, basic components of non-enzymatic macromolecules and also can act as cofactors in enzymatic reactions (Robbins 1993). However, various spiteful symptoms associated with deficiencies of other minerals like chromium, copper, fluorine, iodine, molybdenum, are well known (Lall 2002; Terech-Majewska et al. 2016).

Cobalt is considered as an important essential micro-mineral in fish and other vertebrates (Perrault et al. 2014; Rahal and Shivay 2016). It regulates the blood glucose levels and affects the activity of many enzymes (Speich et al. 2001; Siddiqui et al. 2014). It is the precursor for intestinal microbial synthesis of vitamin B_{12}. The alternative name for vitamin B_{12}, cobalamin, highlights Co as structural component of vitamin B_{12} (Kräutler 2005; Moll and Davis 2017).Vitamin B_{12} is a co-factor of two important enzymes i.e. methylmalonyl–CoA mutase (MCM) and methionine synthase (MS). MS is involved in the synthesis of methionine by the transfer of one carbon from folic acid (methylated) to homocysteine, as well as for the biosynthesis of nucleic acids (essential for cell formation and functioning), which requires the methyl group as their basic constituent (McDowell 1989). MCM is important in the Kreb's cycle by contributing to the synthesis of succinyl–CoA from methylmalonyl–CoA (Takahashi-Iñiguez et al. 2012). Generally, B_{12} deficiency results in poor growth performance, low blood hemoglobin level, reduced feed intake and anemia in fish (Lukaski 2004; Bogard et al. 2015). In addition, B_{12} deficiency decreases immune response in rats (Partearroyo et al. 2013) and humans (Goyal 2015).

Worldwide Co abundance in earth crust and in waters is relatively low as compared to other essential minerals, and it is mostly present in the precipitated form as cobalt sulfate (Karadede-Akin and Ünlü 2007; Swanner et al. 2014). Fish can drive large proportion of some minerals like calcium from water. However, concentration of many minerals including Co in water is not in an amount to fulfill the needs of fish, therefore must be supplied via feed (Robbins 1993; Mukherjee and Kaviraj 2009). The Co dietary requirement in fish varies with age, size, species and culture conditions (Wilson 1991). Generally, animal and plant based feed ingredients for feedstuffs in fish farming provide satisfactory quantities of Co (Storebakken et al.

2000; Jahan et al. 2000; Li and Robinson 2015). For instance, there is no need for Co supplementation in diets containing above 4% animal protein (Lall 2002). However, some freshwater fish require additional Co in their diet for optimum growth (Wilson 1991). Though Co is ranked as essential dietary mineral, knowledge about optimum dietary requirement of Co 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 water bodies, culturing fish in captivity requires fortified nutrition and adequate feeding (Oliva-Teles 2012). Bearing in mind the importance of Co as essential micro-mineral 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 purchased from the Mahseer Fish Seed Hatchery, Garyala, District Attock (Pakistan) and transported in aerated plastic bags to the Aquaculture

research station at Quaid-i-Azam University, Islamabad. After tempering, they were stocked in circular fiberglass tank (500L) having flow through system. Fish were acclimated for a week and provided with 40 % crude protein basal diet. Uniform-sized mahseer (average wet weight ± SEM: 1.35 ± 0.02 g) were selected and randomly distributed in 21 rectangular fish rearing fiberglass troughs (capacity: 45 L) at a stocking density of 1.5 g/L (about 30 fish/tank). The experiment was conducted using three tanks per treatment. Tanks were well equipped with heaters and an aeration system for maintaining water temperature (22.5 ˚C) and DO level (6.0 mg/L). After that, feeding trial started and the control group was fed a basal diet (without Co supplementation) while other six experimental groups (A-F) were fed diets containing increasing amounts of Co ranging from 0.5 to 3 mg/kg diet. At the beginning of the experiment, fish were fed three times a day (9:00, 13:00 and 16:00 h) at a daily rate of 8 % body weight. One month later, the daily rate was reduced to 4 % body weight, supplied twice a day (9:00 and 18:00 h) until the end of the trial. Before providing feed to the concerned groups, it was initially passed through a sieve with fine mesh (200-500 µm), while feed particle size was adjusted after one month according to the size of fry. Remaining feed was removed after every two 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 like temperature, dissolved oxygen and ammonia 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).

Diet preparation

Dietary source cobalt chloride hexahydrate (CAT# 255599; ACS Reagent grade) was purchased from Sigma, USA. Locally available dry ingredients were used to formulate 40 % crude protein basal and experimental diets. All the ingredients were finely ground and mixed in the proper ratio as shown in Table 1. Cobalt was added to mixed ingredients at several experimental doses (A-F: 0.5, 1, 1.5, 2, 2.5 and 3.0 mg/kg diet, respectively). A control diet without Co supplementation was also prepared. Feed pellets were prepared following standard methods, dried and saved in Ziploc bags as described previously (Amir et al. 2018). Fresh feed was prepared after every 15 days. Before providing feed to respective groups, it was initially passed through a sieve with fine mesh (150-250 μm), while feed particle size was adjusted after one month on the basis of the size of fry.

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 evaluating the growth performance. Standard formulas reported previously (Zhou et al. 2013; Munir et al. 2016), were adopted for determining growth performance:

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

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

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

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), dissected at low temperature (on ice pad) and muscle and liver were immediately collected and preserved in RNA later (Thermo scientific CAT# AM7020) for molecular based studies. Twenty-one fish/tank were additionally collected for whole body proximate composition. Due to the small size of fish, proximate analysis was performed on 3 pools of 7 fishes for each tank (9 samples/treatment).

Body proximate composition

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

Metal bioaccumulation

Atomic Absorption Spectrometry (AA240 FS) 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 to 250 ˚C until clear and transparent solution was obtained. Samples were cooled at room temperature, filtered through 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 fast sequential atomic absorption spectroscopy.

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 (Thermoscientific, 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 hr at 37 ºC in the presence of random hexamers. 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 ºC in liquid LB media supplemented with ampicillin to isolate plasmid DNA by using the Gene elute plasmid Mini-prep 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.

Quantitative RT-PCR

Total RNA (1 µg) isolated from tissue samples was reverse transcribed 1 hr at 37 °C with M-MLV RT (Promega, Madison, WI, USA). MyoD, myogenin and Hsp-70 mRNA levels were determined with Step OnePlus 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 sec 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 control by 8 % and 11.3 %, respectively.

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 positive correlation (R^2 = 0.99) with

dietary Co supplementation up to 2 mg/kg diet (D-group), having values significantly higher than control fish ($P<0.05$). However, the highest level of dietary Co (3 mg/kg, F-group) 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 muscle of *T. putitora* showed negative correlation (Mn, $R^2 = 0.907$; Fe, $R^2 = 0.993$) while Co showed positive correlation ($R^2 = 0.953$) with dietary Co supplementation (Fig. 1a-c).

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

Amongst fish, *T. putitora* MyoD exhibited higher similarity with sequences reported for *Ctenopharyngodon idella*, *Danio rerio* and *Cyprinus 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 mammalian orthologues: identity with *Homo sapiens* and *Mus musculus* was 82.1 and 82.9 %, respectively (Fig. 2). As seen in MyoD, *T. putitora* myogenin also exhibited higher similarity with *Danio 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 was found with *H. sapiens* and *M. musculus*,

respectively (Fig. 3). For *T. putitora* Hsp-70, we found 98.3 % similarity with amino acid sequences reported for *Danio rerio*, while for other fish species similarity ranged from 88.9 to 93.2 %. The similarity of *T. putitora* Hsp-70 with *H. sapiens* and *M. musculus* was 85.5 % and 90.6 %, respectively (Fig. 4).

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. 5a, 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. 5c).

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 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 control by 8 % and 11.3 %, respectively. According to our results, 2 mg/kg of is the optimum level of dietary Co supplementation for early rearing of *T. putitora*. This level is somewhat higher than 1 mg/kg diet, which was reported for gaining 40 % increase in survival rates of *Cyprinus carpio* hatchlings (Mukherjee and Kaviraj 2009), and 0.05 mg/kg dietary Co as reported for channel catfish (Wilson 1991), while lower than 2.5 and 5 mg/kg diet which are known to improve growth performance of seabass (*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 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 growth performance of *T. putitora*. Like our results, Mukherjee and Kaviraj (2011) also reported decreased in 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. 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 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 as for *T. putitora*, dietary Co chloride also increased crude fat content in *Cyprinus 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 standardization of recommended dietary allowance (RDA) of Co for fish. Indeed, micronutrient deficiency may result in increased accumulation of heavy metals from the environment (Golovanova 2008). In fish, 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, at high concentration may disrupt many enzymatic functions, causing toxicity (Javed 2013; Rai et al. 2015). In our experimental trial, iron accumulation shows 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 DCT1. 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 molecular level, the expression of growth and stress response genes through RT-qPCR was determined. Due to 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 *Danio 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 suggests 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 compared *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 positive correlation with weight gain up to 2 mg/kg dietary level of Co. Cobalt chloride is a bioactive compound which induce the expression of a series of hypoxia response genes such as hypoxia inducible factor (HIFα) by acting as 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 stimulation of myogenic regulating factors (MRFs) such as myogenic factor 5 (MyF5), MyoD, MRF4 and myogenin as well as increased skeletal muscle fibers 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 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 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 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 up regulation of Hsp-70 in the liver of *T. putitora* fed 3 mg/kg dietary Co compared to 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). Over expression of Hsp-70 in response to higher dietary Co levels may indicate the

stimulation of 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.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure captions

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 3 fishes

Fig. 2 Multiple alignment and phylogenetic tree of MyoD. The upper part of the figure shows alignment of the predicted amino acid sequence of *T. putitora* MyoD with those reported for *Mus musculus* (AAA39798.1), *Homo sapiens* (CAA40000.1), *Paralichthys olivaceus* (ABA70719.1), *Salmo salar* (CAH65602.1), *Oncorhynchus mykiss* (CAA53436.1), *Cyprinus carpio* (BAA33565.1), *Danio rerio* (AAK06755.1) and *Ctenopharyngodon idella* (AFL56774.1). The phylogenetic tree of MyoD alignment is shown in the lower part of the figure. Multiple alignment and phylogenetic tree were made with Clustal Omega (Sievers et al. 2011)

Fig. 3 Multiple alignment and phylogenetic tree of myogenin. The upper part of the figure shows alignment of the predicted amino acid sequence of *T. putitora* myogenin with those reported for *Danio rerio* (NP_571081.1), *Cyprinus carpio* (BAA33564.1), *Sparus aurata* (ABR22022.1), *Salmo salar* (NP_001117072.1), *Oncorhynchus mykiss* (NP_001118199.1), *Xenopus tropicalis* (NP_001016725.1*), Mus musculus* (NP_112466.1) and *Homo sapiens* (NP_002470.2). The phylogenetic tree of myogenin alignment is shown in the lower part of the figure. Multiple alignment and phylogenetic tree were made with Clustal Omega (Sievers et al. 2011)

Fig. 4 Multiple alignment and phylogenetic tree of Hsp-70. The upper part of the figure shows alignment of the predicted amino acid sequence of *T. putitora* Hsp-70 with those reported for *Sus scrofa* (NP_001116599.1), *Homo sapiens* (NP_002146.2), *Ictalurus punctatus* (AAA64872.1), *Ctenopharyngodon idella* (EU816595.1), *Hypophthalmichthys molitrix* (ACJ03595.1), *Gallus gallus* (NP_001006686.1), *Mus musculus* (AAC84168.1), *Paralichthys olivaceus* (AGZ01970.1) and *Danio rerio* (NP_571472.2). The phylogenetic tree of Hsp-70 alignment is shown in the lower part of the figure. Multiple alignment and phylogenetic tree were made with Clustal Omega (Sievers et al. 2011)

Fig. 5 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 were performed by RT-qPCR in muscle and liver samples of *T. putitora* fed 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 control (without dietary Co supplementation) is indicated with different letters ($P<0.05$)

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

Ingredients	Quantity(g/100g)
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
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

Table 2 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	464bp
	Reverse	TGCCATCAGAGCAGTTGGATC	
Myogenin	Forward	CCAGCGTTTTTACGAAGGCG	665bp
	Reverse	ACGTCAGAGACCTCAGGTTGG	
HSP-70	Forward	ATGGTCCTGGTGAAGATGAAG	396bp
	Reverse	GATGTCCTTCTTGTGCTTCCTC	

Table 3 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	99bp
		Reverse	GGCGTAACCCTCGTAGATGG	
MyoD	MH545701	Forward	CGCTTTCGAGACCCTCAAGAG	107bp
		Reverse	GCGCCTGCAGAGACTCAATG	
Myogenin	MH545702	Forward	GGACAAACCGTCTCCATCTTC	165bp
		Reverse	CCTCCTCTTCTCCCTCAAAGTG	
Hsp-70	MH545703	Forward	GATTGCTGAAGCCTATTCTGG	83bp
		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

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

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

Diet Groups	Metal concentrations (mg/g tissue of sample)		
	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

Figure 1

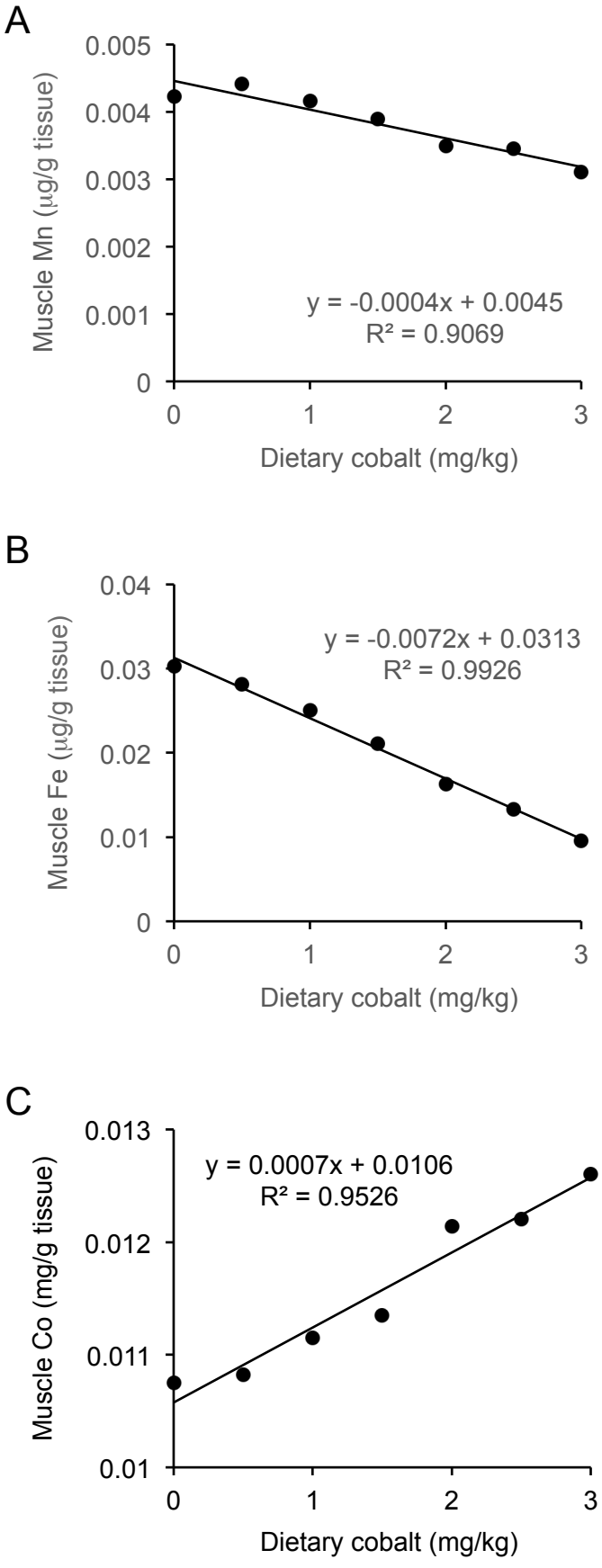

A
0.005
0.004
0.003
0.002
0.001
0
Muscle Mn (µg/g tissue)
y = -0.0004x + 0.0045
R² = 0.9069
0
1
2
3
Dietary cobalt (mg/kg)
B
0.04
0.03
0.02
0.01
0
Muscle Fe (µg/g tissue)
y = -0.0072x + 0.0313
R² = 0.9926
0
1
2
3
Dietary cobalt (mg/kg)
C
0.013
0.012
0.011
0.01
Muscle Co (mg/g tissue)
y = 0.0007x + 0.0106
R² = 0.9526
0
1
2
3
Dietary cobalt (mg/kg)

Figure 2

A

```
Mus                 MELLSPPLRDIDLTGPDGSLCSFETADDFYDDPCFDSPDLRFFEDLDPRLVHVGALLKPE   60
Homo                MELLSPPLRDVDLTAPDGSLCSFATTDDFYDDPCFDSPDLRFFEDLDPRLMHVGALLKPE   60
Paralichthys        MELS-------------DMSFPIPADDDFYDDPCFPPSDMHFFEDLDSRLVHVGLL-KPD   46
Salmo               MELS-------------DISFPITSADDFYDDPCFNTSDMHFFEDMDPRLVHVGLL-KPD   46
Oncorhynchus        MELP-------------DIPFPITSPDDFYDDPCFNTSDMHFFEDLDPRLVHVGLL-KPD   46
Cyprinus            MELS-------------DIPFPIPSADDFYDDPCFNTNDMHFFEDLDPRLVHVSLL-KPD   46
Danio               MELS-------------DIPFPIPSADDFYDDPCFNTNDMHFFEDLDPRLVHVSLL-KPD   46
Ctenopharyngodon    MELS-------------DIPFPISSADEFYDDPCFNTNDMHFFEDLDPRLVHVSLL-KPD   46
Tor                 -----------------------------------NTNDMHFFEDLDPRLVHVSLL-KPD   24
                                                        *::****:* **:**. * **:

Mus                 EHAHFSTA--------------VHPGPGAREDEHVRAPSGHHQAGRCLLWACKACKRKTT   106
Homo                EHSHFPAA--------------VHPAPGAREDEHVRAPSGHHQAGRCLLWACKACKRKTT   106
Paralichthys        DSSSLSSSSPSSSSSSPSSLLHLHHHAEVEDDEHVRAPSGHHQAGRCLLWACKACKWKTT   106
Salmo               DHH-------------------------HNEDEHIRAPSGHHQAGRCLLWACKACKRKTT   81
Oncorhynchus        DHH-------------------------HKEDEHIRAPSGHHQAGRCLLWACKACKRKTT   81
Cyprinus            EHH-------------------------HLEDEHVRAPSGHHQAGRCLLWACKACKRKTT   81
Danio               EHH-------------------------HIEDEHVRAPSGHHQAGRCLLWACKACKRKTT   81
Ctenopharyngodon    EHH-------------------------HIEDEHVRAPSGHHQAGRCLLWACKACKRKTT   81
Tor                 EHH-------------------------HIEDEHVRAPSGHHQAGRCLLWACKACKRKTT   59
                    :                             :***:********************* ***

Mus                 NADRRKAATMRERRRLSKVNEAFETLKRCTSSNPNQRLPKVEILRNAIRYIEGLQALLRD   166
Homo                NADRRKAATMRERRRLSKVNEAFETLKRCTSSNPNQRLPKVEILRNAIRYIEGLQALLRD   166
Paralichthys        NADRRKAATMRERRRLSKVNDAFETLKRCTSANPNQRLPKVEILRNAISYIESLQALLRG   166
Salmo               NTDRRKAATMRERRRLGKVNDAFENLKRCTSNNPNQRLPKVEILRNAISYIESLQSLLRG   141
Oncorhynchus        NADRRKAATMRERRRLSKVNDAFETLKRCTSTNPNQRLPKVDILRNAISYIESLQGLLRG   141
Cyprinus            NADRRKAATMRERRRLSKVNDAFETLKRCTSNNPNQRLPKVEILRNAISYIESLQALLRG   141
Danio               NADRRKAATMRERRRLSKVNDAFETLKRCTSTNPNQRLPKVEILRNAISYIESLQALLRS   141
Ctenopharyngodon    NADRRKAATMRERRRLSKVNDAFETLKRCTSTNPNQRLPKVEILRNAISYIESLQALLRS   141
Tor                 NADRRKAATMRERRRLSKVNDAFETLKRCTSTNPNQRLPKVEILRNAISYIESLQALLRS   119
                    *:**************.***:***.****** *********:****** ***.**.***.

Mus                 QDAAPPGAAAFYAPGPLPPGRGSEHYSGDSDASSPRSNCSDGMMDYSGPPSGPRRQNGYD   226
Homo                QDAAPPGAAAFYAPGPLPPGRGGEHYSGDSDASSPRSNCSDGMMDYSGPPSGARRRNCYE   226
Paralichthys        G-QDD----GFY--------PVLEHYSGDSDASSPRSNCSDGMTDFNGPTCQSTRRGSYE   213
Salmo               QD-GE----NYY--------PSLEHYNGDSDASSPRSNCSDGMMEYNA------------   176
Oncorhynchus        AGQEG----NYY--------PVMDHYSGDSDASSPRSNCSDGMMDFNGQSCPPRRRNKYD   189
Cyprinus            --QEE----NYY--------PVLEHYSGDSDASSPRSNCSDGMMDFMGPTCQSRRRNSYD   187
Danio               --QED----NYY--------PVLEHYSGDSDASSPRSNCSDGIMDFMGPTCQTRRRNSYD   187
Ctenopharyngodon    --QEE----NYY--------PVLEHYSGDSDASSPRSNCSDGMMDFMGPTCQSRRRNSYD   187
Tor                 --QEE----NYY--------PVLEHYSGDSDASSP------------------------   140
                              :*          :**.********

Mus                 T-AYYSEAVRE-SRPGKSAAVSSLDCLSSIVERISTDSPAAPALLLADAPPESPPGPPEG   284
Homo                G-AYYNEAPSE-PRPGKSAAVSSLDYLSSIVERISTESPAAPALLLADVPSESPPRRQEA   284
Paralichthys        SSSYFSQTPNGGQKSDRRSVVSSLDCLSSIVERISTDNSSLMPAVDG------PVSPPTD   267
Salmo               ------------------------------------------------------------   176
Oncorhynchus        S-TYFNEAPND-SRHKKNSVISSLDCLSNIVERITTDTSACPAVQDG-SEGSSPCSPGDG   246
Cyprinus            S-SYFNDTPNADARNTKSSVVSSLDCLSSIVERISTETPACPVLSVPEGHEGSPCSPQEG   246
Danio               S-SYFNDTPNADARNNKNSVVSSLDCLSSIVERISTETPACPVLSVPEGHEESPCSPHEG   246
Ctenopharyngodon    S-SYFNDTPNADARNNKSSVVSSLDCLSSIVERISTETPACPVLSVPEGHEGSPCSPQEG   246
Tor                 ------------------------------------------------------------   140

Mus                 ASLSD-TEQGTQTPSPDAAPQCPAGSNPNAIYQVL  318
Homo                AAPSEGESSGDPTQSPDAAPQCPAGANPNPIYQVL  319
Paralichthys        -QTGE---TAA--PGPLQVPSPTASQDPNLIYQVL  296
Salmo               -----------------------------------  176
Oncorhynchus        SIASE---NGA--PIPSPINCVPALHDPNTIYQVL  276
Cyprinus            SVLSE---TGA--PAPSPTTCPQQ-QARDPIYQVL  275
Danio               SVLSD---TGT--TAPSPTSCPQQ-QAQETIYQVL  275
Ctenopharyngodon    SVLSE---SGA--PAQSPTNCPQQ-QAQDPIYQVL  275
Tor                 -----------------------------------  140
```

B

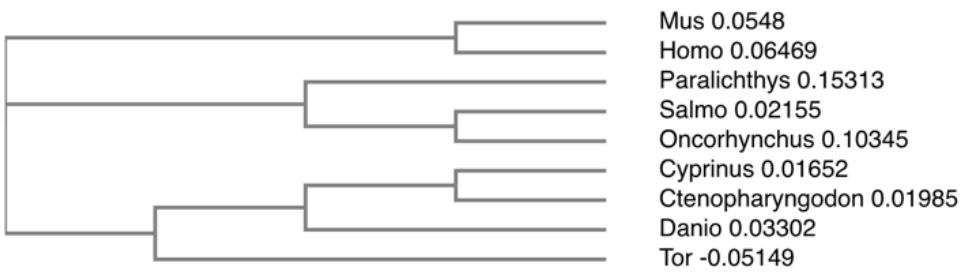


Figure 3

A

```
Danio            MELFETNPYFFNDQRFYEGADNFFQSRINGGFEQAGYQDRN-SMMGLCGDGRM---LTTT   56
Cyprinus         MELFETNPYFLADQRFYEGGDNFFQSRLTGGFDQTGYQDRS-SMMGLCGDGRL---LSNG   56
Tor              --------------------DNFFQSRLTGGFDQAGYQDRS-SMVGLCGDGRL---LSNG   36
Sparus           MELFETNPYFFPDQRSYEGGDSYFPSRLPGAYDQGAYQDRN-SMMGLCGSLSGGVGVGVT   59
Salmo            MELFETNPYFFPDQRFYEGGDNFYQSRLPGGYDQGGYQERGGSMMGLCGGLSGRVGVGLG   60
Oncorhynchus     MELFETNPYFFPDQRFYEGGDNFYQSRLPGGYDQGGYQERGGSMMGLCGGLSGGVGVGLG   60
Xenopus          MELFETSPYFFPDQRFYDN-DNYFSARLP-TYEQTGFQDRGTV--GICADGVL----LQG   52
Mus              MELYETSPYFYQEPHFYDG-ENYLPVHLQ-GFEPPGYERTELS--LSPEAR---------   47
Homo             MELYETSPYFYQEPRFYDG-ENYLPVHLQ-GFEPPGYERTELT--LSPEAP---------   47
                                     :.:   ::   ::  .::

Danio            VGLEDKPSPSSSLGLSMSPHQEQQHCPGQCLPWACKVCKRKSVTMDRRKAATLREKRRLK   116
Cyprinus         VGLEDKPSPSSSLGLSLSPHQEQQHCPGQCLPWACKVCKRKSVTMDRRKAATLREKRRLK   116
Tor              VGMEDKPSPSSSLGLSMSPHQEQQHCPGQCLPWACKVCKRKSVTMDRRKAATLREKRRLK   96
Sparus           G-TEEKASPSS-----LSPH-SEPHCPGQCLPWACKLCKRKTVTMDRRRAATMREKRRLK   112
Salmo            GGMEDKATPSG-----LSPH-PEPHCPGQCLPWACKLCKRKTVTMDRRKAATMREKRRLK   114
Oncorhynchus     GGMEDKATPSG-----LSPH-PEPHCPGQCLPWACKLCKRKTVTMDRRKAATMREKRRLK   114
Xenopus          SGIEDKVSPHPTVT-------QQEHCPGQCLPWACKVCKRKTVSMDRRRAATLREKRRLK   105
Mus              -----GPLEEKGLG-------TPEHCPGQCLPWACKVCKRKSVSVDRRRAATLREKRRLK   95
Homo             -----GPLEDKGLG-------TPEHCPGQCLPWACKVCKRKSVSVDRRRAATLREKRRLK   95
                                      ************:****:*::***:***:*******

Danio            KVNEAFEALKRSTLMNPNQRLPKVEILRSAIQYIERLQALVSSLNQQEHEQGNLHYRATA   176
Cyprinus         KVNEAFEALKRSTLMNPNQRLPKVEILRSAIQYIERLQALVSSLNQQEHEQGNLHYRSTA   176
Tor              KVNEAFEALKRSTLMNPNQRLPKVEILRSAIQYIERLQALVSSLNQQEHEQGNMHYRAAA   156
Sparus           KVNEAFDALKRSTLMNPNQRLPKVEILRSAIQYIERLQALVSSLNQQNTETGQQGLHYRP   172
Salmo            KVNEAFEALKRSTLMNPNQRLPKVEILRSAIQYIERLQALVSSLNQQENDQGTQGLHYRT   174
Oncorhynchus     KVNEAFEALKRSTLMNPNQRLPKVEILRSAIQYIERLQALVSSLNQQENDQGTQGLQYRT   174
Xenopus          KVNEAFEALKRSTLLNPNQRLPKVEILRSAIQYIERLQTLLASLNQQERDQRDLL--FIS   163
Mus              KVNEAFEALKRSTLLNPNQRLPKVEILRSAIQYIERLQALLSSLNQEERDLRY----RGG   151
Homo             KVNEAFEALKRSTLLNPNQRLPKVEILRSAIQYIERLQALLSSLNQEERDLRY----RGG   151
                 ******:*******:**********************:*::****:: :

Danio            AAPHTGVSSSSDQGSGSTCCSSPEWSSASDHCVPAYSSAHEDLLNDDSSEQSNLRSLTSI   236
Cyprinus         P---QAVSSSSDQGSGSTCCSSPEWSSASEQCAPAYSSTHEDLLNDDSSEQTNLRSLTSI   233
Tor              P---QGMSSSSDQGSGSTCCSSPEWSSTSEHCAPAYSSTHEDLLNDDSTEQ---------   204
Sparus           SATQPRVSSSSEPSSGSTCCSSPEWSSTPEQCTQSYSSED-LLSATDSPEQGNMRALTSI   231
Salmo            GPAQPRVSSSSEQGSGSTCCSSPEWSNTSDHCTQSYSNED-LLSA-DSPEQTNLRSLTSI   232
Oncorhynchus     GPAQPRVSSSSEQGSGSTCCSSPEWSNTSDHCAQSYSNED-LLSA-DSPEQTNLRSLTSI   232
Xenopus          NGSQRVVSS--ECGSSSSSC-SPEWNDS-DFS----GSQSDHLLSDDSSEQRDINSLSSI   215
Mus              GGPQPMVPS--ECNSHSASC-SPEWGNALEFG----PNPGDHLLAADPTDAHNLHSLTSI   204
Homo             GGPQPGVPS--ECSSHSASC-SPEWGSALEFS----ANPGDHLLTADPTDAHNLHSLTSI   204
                       : *  : .* *:.* ****..: :       .    *   *  :

Danio            VDSITGTEATPVAYSVDISK--    256
Cyprinus         VDSITGTEVTPVPYSVDISK--    253
Tor              ----------------------    204
Sparus           VNSISAADGAV---AFPMDIPK    250
Salmo            VDSITAAEGAPVAYPVPVDIPK    254
Oncorhynchus     VDSITAAEGAPLAYPVPVDIPK    254
Xenopus          VDSITSGEVSI-TYPEQ-HIQH    235
Mus              VDSITVEDMSV-AFPDE-TMPN    224
Homo             VDSITVEDVSV-AFPDE-TMPN    224
```

B

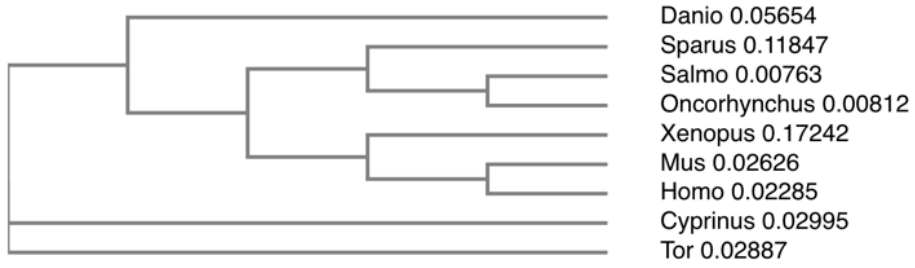


Figure 4

A

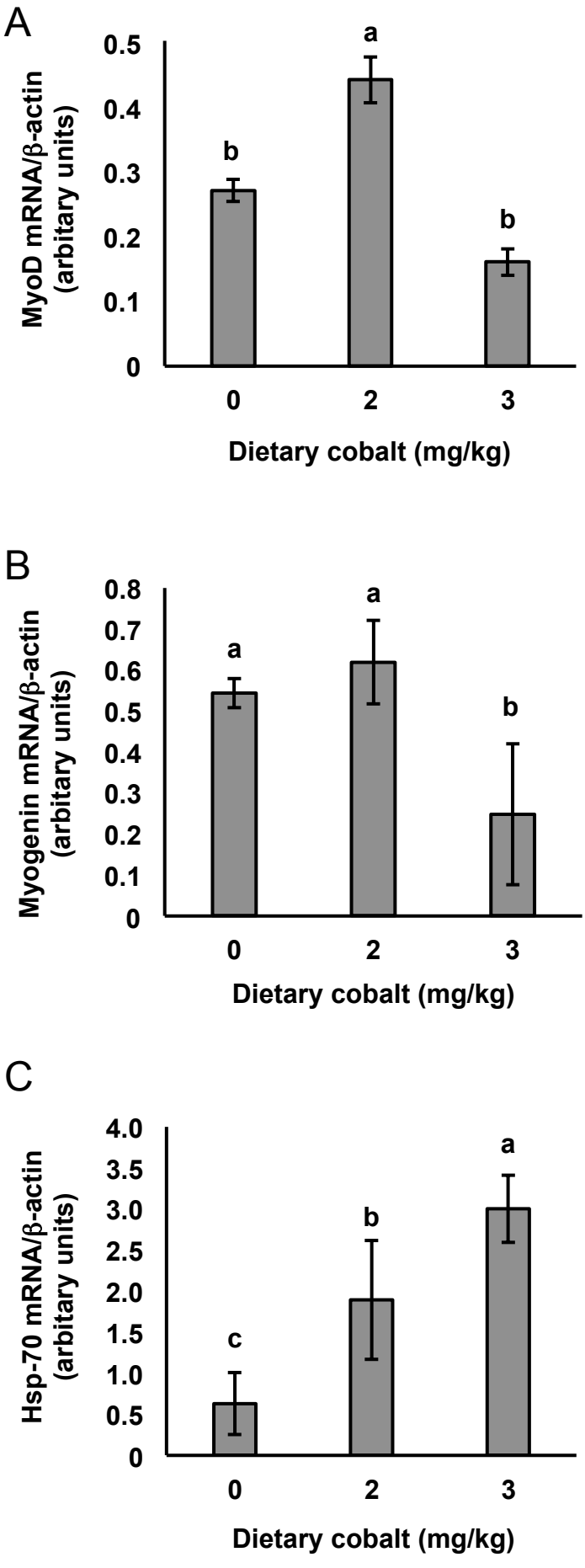
```
Sus                     ISSMVLSKMKETAEAYLGQPVRHAVITVPAYFNDSQRQATKDAGAIAGLNVLRIINEPTA   180
Homo                    ISSMVLSKMKETAEAYLGQPVKHAVITVPAYFNDSQRQATKDAGAIAGLNVLRIINEPTA   180
Ictalurus               ISSMVLVKMKEIAEAYLGKSINNAVITVPAYFNDSQRQRTKDAGTISGLNVLRIINEPTA   178
Ctenopharyngodon        ISSMVLTKMKEIAEAYLGKTVSNAVITVPAYFNDSQRQATKDAGTISGLNVLRIINEPTA   178
Hypophthalmichthys      ISSMVLTKMKEIAEAYLGKTVSNAVITVPAYFNDSQRQATKDAGTISGLNVLRIINEPTA   178
Gallus                  ISSMVLTKMKEIAEAYLGKKVETAVITVPAYFNDSQRQATKDAGTITGLNVMRIINEPTA   179
Mus                     ISSMVLTKMKEIAEAYLGHPVTNAVITVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTA   178
Paralichthys            ISSMVLVKMKEIAEAYLGQKVSNAVITVPAYFNDSQRQATKDAGVISGLNILRIINEPTA   180
Danio                   ISSMVLVKMKEIAEAYLGQKVTNAVITVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTA   180
Tor                     ----------EIAEAYLGQKVTNAVVTVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTA   50
                                  * ******: :  **:************ *****.*:***::********

Sus                     AAIAYGLDRRG--AGERNVLIFDLGGGTFDVSVLTIDAGVFEVKATAGDTHLGGEDFDNR   238
Homo                    AAIAYGLDRRG--AGERNVLIFDLGGGTFDVSVLSIDAGVFEVKATAGDTHLGGEDFDNR   238
Ictalurus               AAIAYGLDKKV--GSERNVLIFDLGGGTFDVSILTIEDGIFDLKSTAGDTHLGGEDFDNR   236
Ctenopharyngodon        AAIAYGLDKKV--GAERNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDTHLGGEDFDNR   236
Hypophthalmichthys      AAIAYGLDKKV--GAERNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDTHLGGEDFDNR   236
Gallus                  AAIAYGLDKKGTRAGEKNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDTHLGGEDFDNR   239
Mus                     AAIAYGLDRTG--KGERNVLIFDLGGGTFDVSILTIDDGIFEVKATAGDTHLGGEDFDNR   236
Paralichthys            AAIAYGLDKGK--RGERNVLIFDLGGGTFDVSILTIEDGIFEVKATAGDTHLGGEDFDNR   238
Danio                   AAIAYGLDKGK--SSERNVLIFDLGGGTFDVSILTIEDGIFEVKATAGDTHLGGEDFDNR   238
Tor                     AAIAYGLDKGK--ASERNVLIFDLGGGTFDVSILTIEDGIFEVKATAGDTHLGGEDFDNR   108
                        ********:     .*:****************:*:*: *:*::*:*****************

Sus                     LVNHFMEEFRRKHRKDLSRNKRALRRLRTACERAKRTLSSSTQATLEIDSLFEGVDFYTS   298
Homo                    LVNHFMEEFRRKHGKDLSGNKRALRRLRTACERAKRTLSSSTQATLEIDSLFEGVDFYTS   298
Ictalurus               MVNHFIAEFKRKHKKDISDNKRAVRRLATACERAKRTLSSSTQASIEIDSLYEGVDFYTS   296
Ctenopharyngodon        MVNHFITEFKRKHKKDISDNKRAVRRLRTACERAKRTLSSSTQASIEIDSLYEGIDFYTS   296
Hypophthalmichthys      MVNHFITEFKRKHKKDISDNKRAVRRLRTACERAKRTLSSSTQASIEIDSLYEGIDFYTS   296
Gallus                  MVNRFVEEFKGKHKRDNAGNKRAVRRLRTACERARRTLSSSTQASIEIDSLFEGIDFYTS   299
Mus                     LVSHFVEEFKRKHKKDISQNKRAVRRLRTACERAKRTLSSSTQASLEIDSLFEGIDFYTS   296
Paralichthys            MVSHFLEEFKRKYKKDISQNKRAVRRLRTACERAKRTLSSSTQASIEIDSLFEGIDFYTS   298
Danio                   MVNHFVEEFKRKHKKDISQNKRALRRLRTACERAKRTLSSSSQASIEIDSLYEGIDFYTS   298
Tor                     MVNHFVEEF-------------------------------------------------   117
                        :*.:*: **
```

B

Figure 5


A
MyoD mRNA/β-actin (arbitary units)
0.5
0.4
0.3
0.2
0.1
0
a
b
b
0
2
3
Dietary cobalt (mg/kg)
B
Myogenin mRNA/β-actin (arbitary units)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
a
b
0
2
3
Dietary cobalt (mg/kg)
C
Hsp-70 mRNA/β-actin (arbitary units)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
c
b
a
0
2
3
Dietary cobalt (mg/kg)