The optimum dietary organic and inorganic copper levels in marine fish, olive flounder, *Paralichthys olivaceus*, and freshwater fish, beluga sturgeon, *Huso huso*

치어기 해산어 넙치와 담수어 철갑상어에 있어서 최적의 사료내 유기 및 무기구리의 적정 함유량 규명

A thesis submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy

in the Department of Fisheries Biology, Graduate School,

Pukyong National University

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Advisor: Sungchul C. Bai

By

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주 심	공학박사	공 인 수	(인)
위 원	이학박사	**Craig Browdy**	(인)
위 원	수산학박사	김 강 웅	(인)
위 원	이학박사	한 경 민	(인)
위 원	영양학박사	배 승 철	(인)

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The optimum dietary organic and inorganic copper levels in marine fish, olive flounder, *Paralichthys olivaceus*, and freshwater fish, beluga sturgeon, *Huso huso*

Mahmoud Mohseni

Department of Fisheries Biology, Graduate School, Pukyong National University

Abstract

Four feeding trials were conducted to evaluate the optimum dietary organic (Mintrex®Cu, a chelated dietary copper source) and inorganic (copper sulfate, $CuSO_4.5H_2O$) copper levels on growth performance, nutrient utilization, whole-body proximate composition, immune responses, hematological parameters and tissue copper concentration in juvenile olive flounder, *Paralichthys olivaceus* (initial body weight 6.88 ± 0.05g), and beluga sturgeon, *Huso huso* (initial body weight 2.46 ± 0.09 g). In experiments I and II, broken-line regression analysis of weight gain (WG) suggested that the optimum dietary Cu levels in juvenile olive flounder, *P. olivaceus*, could be 8.44 mg Cu/kg diet and 9.05 mg Cu/kg diet in the form of Mintrex®Cu and copper sulfate, respectively. In experiments III and IV, broken-line regression analysis of WG indicated that the optimum dietary Cu levels for beluga sturgeon, *Huso huso,* could be 9.71 mg Cu/kg diet, when Mintrex®Cu is used as the copper source and 10.3 mg Cu/kg diet, when copper sulfate is used as the copper source.

Experiment I. The optimum dietary organic copper level in juvenile olive flounder, *Paralichthys olivaceus*

A 14-week feeding trial was conducted to evaluate the optimum dietary organic copper (Mintrex®Cu) in the juvenile olive flounder, *Paralichthys olivaceus*. Seven semi-purified diets were formulated to contain 0.89 ($Cu_{1.0}$), 5.56 ($CuM_{6.0}$), 10.1 (CuM_{10}), 14.9 (CuM_{15}), 19.0 (CuM_{19}), 42.4 (CuM_{42}) and 85.8 (CuM_{86}) mg Cu/kg diet on a dry matter basis. Fish averaging 6.86 ± 0.04 g (mean ± SD) were randomly distributed to each aquarium as triplicate groups of 15 fish each. At the end of 14 weeks of feeding trial, weight gain (WG) and specific growth rate (SGR) of fish fed CuM_{10} and CuM_{15} diets were significantly higher than those of fish fed $Cu_{1.0}$,

CuM_{19}, CuM_{42} and CuM_{86} ($P < 0.05$). Whole-body crude protein peaked at the dietary Cu supplementation level of 10.1 mg/kg diet and dropped. Whole-body and muscle crude lipid decreased while whole-body crude ash increased with dietary Cu content. Thiobarbituric acid-reactive substances (TBARS) were significantly lower while copper-zinc superoxide dismutase (SOD) activities were significantly higher in liver tissue of fish fed CuM_{10} and CuM_{15} diets than those of fish fed $Cu_{1.0}$, CuM_{19}, CuM_{42} and CuM_{86} diets. Tissue Cu concentrations increased with dietary Cu content. Copper accumulated most in liver, followed by intestine, kidney, gills and then muscle. Weight gain analyzed by broken-line regression suggested that the optimum dietary Cu level was 8.44 mg Cu/kg diet. Therefore, these results may indicate that the optimum dietary Cu levels could be greater than 8.44 mg Cu/kg diet but less than 10 mg Cu/kg diet in juvenile olive flounder, *P. olivaceus* when Mintrex®Cu was used as the dietary source of organic copper.

Experiment II. The optimum dietary inorganic copper level in juvenile olive flounder, *Paralichthys olivaceus*

A study was conducted to evaluate the optimum dietary inorganic copper (copper sulfate) in the juvenile olive flounder, *Paralichthys olivaceus*. Seven semi-purified diets containing 0.89 ($Cu_{1.0}$), 5.51 ($CuS_{6.0}$), 10.2 (CuS_{10}), 15.4 (CuS_{15}), 19.4 (CuS_{19}), 42.0 (CuS_{42}) and 86.3 (CuS_{86}) mg Cu/kg diet in the form of $CuSO_4.5H_2O$ were fed to fish averaging 6.89 ± 0.05g (mean ± SD) in triplicate groups for 14 weeks. Weight gain (WG) and specific growth rate (SGR) of fish fed CuS_{10} diet were significantly higher than those of fish fed $CuS_{1.0}$, $CuS_{6.0}$, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets ($P < 0.05$). Protein efficiency ratio (PER) of fish fed $CuS_{6.0}$, CuS_{10} and CuS_{15} diets were significantly higher than those of fish fed CuS_{42} and CuS_{86} diets. Crude lipid in muscle and whole-body decreased with increasing dietary Cu and was significantly lower in fish fed dietary Cu ≥15.4 mg/kg than in those fed Cu ≤5.5 mg Cu/kg diet. Results of copper-zinc superoxide dismutase activity and thiobarbituric acid-reactive substances level of liver from fish fed CuS_{10} diet were significantly better than those from fish fed $Cu_{1.0}$, $CuS_{6.0}$, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets ($P < 0.05$). Copper concentrations in tissue and whole-body from fish increased with dietary copper level, and these concentrations from fish fed CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diet were significantly higher than those from fish fed $Cu_{1.0}$ and $CuS_{6.0}$ diet. Broken-line analysis of WG suggested that the optimum dietary Cu level was 9.05 mg Cu/kg diet.

Therefore, these results may indicate that the optimum dietary Cu level could be higher than 9.05 mg Cu/kg diet but less than 10 mg Cu/kg diet in juvenile olive flounder, *P. olivaceus* when copper sulfate is used as the dietary source of inorganic copper.

Comparison of effects of dietary copper sources and levels on growth, enzyme activity and tissue copper concentration of juvenile olive flounder, *Paralichthys olivaceus*

A 2× 7 factorial design was used to compare the effects of dietary organic (Mintrex®Cu) and inorganic copper (copper sulfate) and levels in juvenile olive flounder, *Paralichthys olivaceus.* Three replicate groups of fish averaging 6.88 ± 0.05 g (mean ± SD) were fed one of the 13 experimental diets ($Cu_{1.0}$, $CuM_{6.0}$, CuM_{10}, CuM_{15}, CuM_{19}, CuM_{42}, CuM_{86}, $CuS_{6.0}$, CuS_{10}, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86}) that were prepared containing two different dietary copper sources (organic and inorganic Cu) at each of seven different levels of Cu 1, 6, 10, 15, 19, 42 and 86 mg Cu/kg diet. At the end of 14 weeks of the experimental period, there were significant dietary copper sources, levels and their interaction effects on weight gain (WG), specific growth rate (SGR), copper-zinc superoxide dismutase activity (Cu-Zn SOD), thiobarbituric acid-reactive substances (TBARS), hematological values (red blood cell, hematocrit and hemoglobin) and Cu content of tuissues (liver, intestine, gill and muscle) and whole boby. Growth performance, antioxidant status and hematological values of fish fed organic Cu were relatively greater than inorganic Cu ($P < 0.05$). Copper concentrations in fish tissues and whole-body increased with dietary copper level. WG and SGR of fish fed CuM_{10} diet were significantly higher than those of fish fed the other diets except for those of fish fed CuM_{15} diet. Results of Cu-Zn SOD and TBARS in liver of fish fed CuM_{10} diet were significantly better than those of fish fed other diets except for those of fish fed CuM_{15} and CuS_{10} diets and $CuM_{6.0}$, CuM_{15} and CuS_{10} diets, respectively. Also, fish fed high levels of copper from inorganic Cu showed significantly higher rates of accumulation in liver and intestine than from organic Cu. Broken-line regression analysis of WG suggested that the optimum dietary Cu levels in juvenile olive flounder, *P. olivaceus*, could be 8.44 mg/kg diet and 9.05 mg/kg diet in the form of organic Cu and inorganic Cu, respectively. This study indicates that Cu allowance in diets of olive flounder can be reduced when organic Cu replaces inorganic copper. Organic Cu supproted better performance, increased nutrient utilization, improved physiological responses and greater effectiveness than inorganic Cu.

Experiment III. The optimum dietary organic copper level in juvenile beluga sturgeon, *Huso huso*

A 12-week feeding trial was conducted to evaluate the optimum dietary organic copper (Mintrex®Cu) in juvenile beluga sturgeon, *Huso huso*. Eight semi-purified diets containing 1.1 ($Cu_{1.0}$), 3.6 ($CuM_{4.0}$), 6.8 ($CuM_{7.0}$), 9.5 (CuM_{10}), 12.8 (CuM_{13}), 24.9 (CuM_{25}), 49.3 (CuM_{49}) and 193.5 (CuM_{194}) mg Cu/kg diet in the form of Mintrex®Cu were fed to fish of initial body weight 2.43 ± 0.06 g and length 7.82 ± 0.68 cm (mean ± SD) in triplicate groups in a flow-through system. Weight gain (WG) and specific growth rate (SGR) of fish fed CuM_{10} and CuM_{13} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, CuM_{25}, CuM_{49} and CuM_{194} diets ($P < 0.05$). The lowest WG, SGR, feed efficiency (FE) and protein efficiency ratio (PER) were observed in fish fed CuM_{194} diet. Crude protein in muscle and whole-body peaked at the dietary Cu supplementation level of 9.5 mg/kg diet and dropped. Whole-body crude lipid of fish fed ≤24.9 was also significantly higher than those of fish fed ≥49.3 mg/kg diet. Thiobarbituric acid-reactive substances (TBARS) was significantly higher in liver tissue of fish fed $CuM_{7.0}$, CuM_{10} and CuM_{13} diets than those of fish fed $Cu_{1.0}$, $CuM_{3.0}$, CuM_{25}, CuM_{49} and CuM_{194} diets. Liver copper-zinc superoxide dismutase activity of fish fed CuM_{10} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{3.0}$, CuM_{49} and CuM_{194} diets. Muscle Cu-Zn SOD of fish fed CuM_{10} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, CuM_{25}, CuM_{49} and CuM_{194} diets. Glutamic oxaloacetic transaminase (GOT), Glutamic pyruvic transaminase (GPT) and Cu accumulation in tissues increased with dietary copper. While, lysozyme activity peaked at the dietary Cu supplementation level of 9.5 mg/kg diet and dropped. Broken-line analysis of WG suggested that the optimum dietary Cu level was 9.71 mg Cu/kg. Therefore, these results may indicate that the optimum dietary Cu level could be higher than 9.71 mg Cu/kg diet but less than 13 mg Cu/kg diet in juvenile juvenile beluga, *H. huso* when Mintrex®Cu is used as the dietary source of organic copper.

Experiment IV. The optimum dietary inorganic copper level in juvenile beluga sturgeon, *Huso huso*

A 12-week feeding trial was conducted to evaluate the optimum dietary inorganic copper (copper sulfate) in juvenile beluga sturgeon, *Huso huso*. Eight semi-purified diets containing 1.1 ($Cu_{1.0}$), 3.5 ($CuS_{4.0}$), 7.1 ($CuS_{7.0}$), 9.7 (CuS_{10}), 13.1 (CuS_{13}), 25.1 (CuS_{25}), 49.9 (CuS_{50}) and 195 (CuS_{195}) mg Cu/kg diet in the form of $CuSO_4.5H_2O$ were fed to fish of initial body weight 2.49 ± 0.12 g and length 7.85 ± 0.66 cm (mean±SD) in triplicate groups in a flow-through system. Weight gain (WG) and specific growth rate of fish fed CuS_{10} and CuS_{13} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, CuS_{25}, CuS_{50} and CuS_{195} diets ($P < 0.05$). Whole-body and muscle crude protein increased with dietary Cu up to the supplementation level of 13.1 mg/kg diet and then decreased. Whole-body lipid content was negatively correlated while whole-body ash was positively correlated with dietary copper concentration. Hepatic copper-zinc superoxide dismutase (Cu-Zn SOD) activity of fish fed CuS_{10} and CuS_{13} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$ and CuS_{195} diets. Hepatic thiobarbituric acid-reactive substances (TBARS) of fish fed CuS_{13} diet was significantly lower than those of fish fed the other diets except for that of fish fed CuS_{10} diet. Glutamic oxaloacetic transaminase (GOT), Glutamic pyruvic transaminase (GPT) and copper accumulation in tissues increased with dietary copper. Broken-line analysis of WG suggested that the optimum dietary Cu level was 10.3 mg Cu/kg diet. Therefore, these results may indicate that the optimum dietary Cu levels could be greater than 10.3 mg Cu/kg diet but less than 13 mg Cu/kg diet in juvenile beluga, *H. huso* when copper sulfate is used as the dietary source of inorganic copper.

Comparison of effects of dietary copper sources and levels on growth, enzyme activity and tissue copper concentration of juvenile beluga sturgeon, ***Huso huso***

A 2 × 8 factorial design was used to compare the effects of dietary organic (Mintrex®Cu) and inorganic copper (copper sulfate) and levels in juvenile beluga sturgeon, *Huso huso*. Three replicate groups of fish averaging 2.46 ± 0.09g (mean±SD) were fed one of the 15 experimental diets ($Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25}, CuM_{49}, CuM_{194}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25}, CuS_{50} and CuS_{195}) that were prepared containing two different dietary copper sources (organic and inorganic Cu) at each of eight different levels of Cu 1, 4, 7, 10, 13, 25, 49

and 194 mg Cu/kg diet. At the end of 12 weeks of the experimental period, there were significant dietary copper sources, levels and their interaction effects on weight gain (WG), feed efficiency (FE), protein efficiency ratio (PER), condition factor (CF), copper-zinc superoxide dismutase activity (Cu-Zn SOD), glutathione peroxidase (GPx) and thiobarbituric acid-reactive substances (TBARS), hematological values (Glutamic oxaloacetic transaminase and Glutamic pyruvic transaminase) and Cu content of tuissues (liver and muscle) and whole boby. There were also significant dietary Cu levels effects and interactive effects of Cu sources and levels on lysozyme activities. Growth performance, nutrient utilization, antioxidant status and hematological values of fish fed organic Cu were relatively greater than inorganic Cu ($P < 0.05$). Copper concentrations in fish tissues and whole-body increased with dietary copper level. There was a trend that fish fed diets with inorganic Cu had higher liver but lower whole-body and muscle copper concentrations than those of fish fed from organinc Cu. WG and specific growth rate (SGR) of fish fed CuM_{10} and CuM_{13} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, CuM_{25}, CuM_{49}, CuM_{194}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{25}, CuS_{50} and CuS_{195} diets. Hepatic Cu-Zn SOD activity of fish fed CuM_{10} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{49}, CuM_{195}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{25}, CuS_{50} and CuS_{195} diets. While, TBARS levels of fish fed CuM_{10} diet was significantly lower than those of fish fed other diets, except for those of fish fed CuS_{13} diet. Broken-line regression analysis of WG suggested that the optimum dietary Cu levels in juvenile beluga could be 9.71 mg/kg diet and 10.3 mg/kg diet in the form of organic Cu and inorganic Cu, respectively. This study showed that organic Cu is more bioavalible to juvenile beluga sturgeon than inorganic Cu and could use as a new copper source.

Keywords: Organic copper (Mintrex®Cu), Inorganic copper (copper sulfate), Body composition, Tissue, Enzyme, Olive flounder, *Paralichthys olivaceus*, Beluga sturgeon, *Huso huso*

치어기 해산어 넙치와 담수어 철갑상어에 있어서 최적의 사료내 유기 및 무기구리의 적정 함유량 규명

Mahmoud Mohseni

요약문

총 4 개의 실험은 치어기 넙치와 철갑상어에 있어서 사료내 유기구리 (Mintrex®Cu, 킬레이트 결합된 사료내 구리 공급원) 와 무기구리 (copper sulfate, $CuSO_4.5H_2O$)의 첨가에 따른 성장, 영양소 이용성, 전어체 조성, 면역반응, 혈액학적 분석, 조직내 구리 축적의 결과를 통해 최적의 요구량을 평가하기 위해 시행 되었다. 실험 I 과 II 에서 증체율에 대한 Broken-line 분석 결과 치어기 넙치 사료내 Mintrex®Cu 의 최적량은 8.44 mg Cu/kg 로 나타났으며, 사료내 copper sulfate 의 최적량은 9.05 mg u/kg 로 나타났다. 실험 III 과 IV 에서 증체율에 대한 Broken-line 분석 결과 치어기 철갑상어 사료내 Mintrex®Cu 의 최적량은 9.71 mg Cu/kg 로 나타났으며, 사료내 copper sulfate 의 최적량은 10.3 mg Cu/kg 인 것으로 나타났다.

Chapter 1. General Information

Korea is one of the world's major fishing nations, ranking 12th largest in marine fishery production and 6th in aquaculture. In 2004, total production volume was 2.5 million tons, of this total fisheries production in coastal and deep-sea water contributed to 63% of industry's total production, 37% came from aquaculture. Since the peak of overall fish catches in 1986, the Korean fishery industry's catches have declined as a result of overfishing in the Korean coastal. On the other hand, seafood consumption is increasing. Koreans consumed 4.2 million tons of seafood in 2005 (approx. 49 kg per person), among the highest in the world. The fishing industry is unable to meet domestic demand, and consequently has led to the development of fish farming and import of large amount of fish and fish products from other countries. Coastal aquaculture is the most active and predominant type of mariculture in Korea. Total license area for marine aquaculture is about 122.000 ha in sea water surface and 2.000 ha for land-based farming. It has rapidly developed over the last 30 years in the Korean sea. Major species changed during the past 10 years as seaweeds culture (Laver, Porphyra tenera, Sea mustard, Undaria pinnatifida) used to be the main species of mariculture during 1960s, so as shellfish farming (Mussels, Mytilus edulis, oyster, Crassostrea gigas, arkshell, Scapharca brogtonii) during 1970s. During the 1980s breeding and hatching technique has been set up to some marine fish, red sea bream, *Pagrus major*, olive flounder, *Paralichthys olivaceus,* as well as black rockfish, *Sebastes schlegeli*. Since then, marine fish farming has become the most rapidly growing and economically profitable industry, although a family-run business in Korea. During the 1990s, the production of flounder and black rockfish, *Sebastes schlegeli,* has sharply increased and led the live fish supplies to the seafood market. However, marine finfish farming is growing and gaining attention from government and industry, and it is becoming an important part of the Korean aquaculture industry today. Also, there is an increasing demand in the research sector for the diversification of artificial breeding for high value marine fish species.

Olive flounder, *P. olivaceus* is one of the most commercially important fish species in Korea. Its production is the top among the Korean mariculture finfish species. The aquaculture production of olive flounder reached 54.675 mt in 2009 (National Statistical Office 2010). As one of the important fin fish culture species in Korea, several studies have been conducted to estimate the dietary nutrient requirements in olive flounder including protein (Kim et al. 2002;

Kim et al. 2003), protein to energy ratio (Kim et al., 2004), fishmeal replacement (Kim et al. 2008; Kim et al. 2009) and vitamin C (Wang et al. 2002). However, reports on the copper requirement in olive flounder were lacking.

Sturgeons (Acipenseriformes) are one of the most ancient groups of the Osteichthyes with 25 species distributed in the temperate waters of the Northern Hemisphere, Eurasia and North America (Birstein 1993). Environmental pressures, such as water pollution, dam construction and development of adjacent watersheds for irrigation purposes, have been responsible for the loss of spawning grounds and have threatened populations for some time (Gisbert and Williot 2002). Aquaculture of sturgeon can help in the conservation of declined wild populations through restocking and by providing a consistent supply without exploiting wild population. The main species used in aquaculture production worldwide are white sturgeon, *Acipenser transmontanus,* Siberian sturgeon. *A. baerii*, Russian sturgeon, *A. gueldenstaedtii*, sterlet and bester and beluga, *Huso huso,* (Mims et al. 2002; Mohseni et al. 2006). Tasty meat and high nutritional value make sturgeon very desirable fish species special in European countries. Sturgeons can be found in the market in fresh, frozen, and especially smoked forms (Table 1). The world-famous caviar is produced from sturgeons. Sturgeons also have in its swim bladder a special material called ihtiocol used in glue production, and another substance in its sperm poaches that is used in the treatment of burns. In recent years, the intensive culture of certain sturgeon species has been developed as an alternative to other more traditional fish species such as salmonids and cyprinids (Garcia-Gallego et al. 1999).

Among sturgeons of the southern Caspian Sea, beluga, *Huso huso*, is a good candidate for aquaculture because of its market price, fast growth, reproduction in captivity, and accelerating decline as a result of overfishing (Mohseni et al. 2008). It is the largest sturgeon in the world and the largest European freshwater fish; it can reach up to five metres in length. This ancient fish has an elongated body shape and a flattened, slightly upturned snout, with the mouth located underneath. There are five rows of bony plates (or 'scutes') that run the length of the body, one along the back, one on each flank and two on the undersurface. The body is predominantly dark grey or greenish whilst the belly tends to be white. Reputed to be the most expensive fish in the world, it also produces the most prized caviar.

The sharp decline of natural beluga populations that has been observed in the past decades has prompted several countries to initiate juvenile production programmes for re-stocking (Khodorevskaya et al. 1997; Chebanov and Billard 2001) and for caviar and meat production purposes (Wade and Fadel 1997). Rearing of Acipenser sturgeons in ponds and tanks is a relatively new industry in Iran, and information on nutrient utilization and dietary requirements is limited (Moore et al. 1988; Gershanovich and Kiselev 1993; Ebrahimi 2006; Mohseni et al. 2007). From hatching until market size (4.6 kg), which takes approximately 15.17 months, 98% of the beluga is fed a commercial diet. Research studies aiming to evaluate dose response effects of growth-enhancing supplements in *H. huso* are of immediate concern for the Sturgeon Research Institute, Rasht, Iran, to reduce the grow-out period and costs.

Trace minerals function primarily as catalysts in enzyme systems within cells. The roles that trace minerals play in enzymatic reactions range from weak, ionic strength effects to highly specific associations known as metalloenzymes (Underwood 1971). Deficiencies and or imbalances of trace minerals can alter the activity of certain enzymes and function of specific organs thus impairing specific metabolic pathways as well as overall immune function.

Copper (Cu) is an essential element for all organisms including fish (Watanabe et al. 1997; Lorentzen et al. 1998; Shao et al. 2010). It functions in hematopoiesis and in numerous copper dependent enzymes including lysyl oxidase, cytochrome c oxidase, ferroxidase, tyrosinase (O'Dell, 1976). It is also important as a part of antioxidant enzymes (e.g. Cu-Zn SOD) (Lorentzen et al. 1998). Although dietary copper requirements have been studied in many aquatic species, i.e. rainbow trout and common carp (Ogino and Yang 1980; Knox et al. 1982), channel catfish (Murai et al. 1981; Gatlin and Wilson 1986), grass shrimp (Lee and Shiau 2002), hybrid tilapia (Shiau and Ning 2003), grouper (Lin et al. 2008), soft-shelled turtle (Wu and Huang 2008), abalone (Wang et al. 2009), crucian carp, *Carassius auratus gibelio* (Shao et al. 2010) and yellow catfish (Tan et al. 2011) there are no studies on dietary copper requirements of olive flounder and beluga sturgeon.

Knowledge of bioavailability of supplemental copper sources is critical in selection of a copper source in feed production (Spears et al. 2004; Luo et al. 2005; Shao et al. 2010). The most common form of copper used in feeds for growth promotion is the sulfate salt ($CuSO_4$). The nitrate salt of copper has been reported to be effective in feed production (Clearwater et al. 2002). Chelated forms of various elements have also been found to be effective for some aquatic

animals (Paripatananont and Lovell 1995, 1997; Apines-Amar et al. 2004). Chelated minerals are widely used in the livestock and poultry industries, yet research concerning these compounds with respect to aquatic species such as fish has been very limited (Apines-Amar et al. 2003; 2004). Mintrex®Cu is a chelated source of copper that contains a minimum of 17% copper and 78% hydroxy methionine analogue ((2-hydroxy-4-methylthio) butanoic acid, HMTBa). Since it has low hygroscopicity and is insoluble in neutral water, it should be a less reactive and less destructive form of copper when combined with vitamins in diets (Cromwell et al. 1998).

However, use of inorganic salts can result in poor bioavailability of the mineral, mainly due to the numerous nutrient and ingredient antagonisms that impair absorption (Underwood and Suttle 1999). Improved availability of Cu from chelated Cu sources compared with the commonly used Cu salts has been suggested (Downs et al. 2000; Yu et al. 2000; Guo et al. 2001). The increased availability of copper from a chelated source however may also make it toxic at lower levels and it was therefore of interest to evaluate its toxicity in fish. According to the available research, there is lack of information on Mintrex®Cu use with aquatic animals. In this research, we used juvenile olive flounder and beluga sturgeon as experimental animals, which were fed different dietary copper sources and levels. The objective was to determine the dietary copper requirement of those species and compare the effects of dietary copper sources and levels on growth performance, antioxidant activities and copper status of juvenile olive flounder and beluga sturgeon fed a semi-purified diet.

Chapter 2:

Evaluation of the optimum dietary organic and inorganic copper levels in juvenile olive flounder, *Paralichthys olivaceus*

Experiment I. The optimum dietary organic copper level in juvenile olive flounder, *Paralichthys olivaceus*

Abstract

A 14-week feeding trial was conducted to evaluate the optimum dietary organic copper (Mintrex®Cu) in the juvenile olive flounder, *Paralichthys olivaceus*. Seven semi-purified diets were formulated to contain 0.89 ($Cu_{1.0}$), 5.56 ($CuM_{6.0}$), 10.1 (CuM_{10}), 14.9 (CuM_{15}), 19.0 (CuM_{19}), 42.4 (CuM_{42}) and 85.8 (CuM_{86}) mg Cu/kg diet on a dry matter basis. Fish averaging 6.86 ± 0.04g (mean ± SD) were randomly distributed to each aquarium as triplicate groups of 15 fish each. At the end of 14 weeks of feeding trial, weight gain (WG) and specific growth rate (SGR) of fish fed CuM_{10} and CuM_{15} diets were significantly higher than those of fish fed $Cu_{1.0}$, CuM_{19}, CuM_{42} and CuM_{86} ($P < 0.05$). Whole-body crude protein peaked at the dietary Cu supplementation level of 10.1 mg/kg diet and dropped. Whole-body and muscle crude lipid decreased while whole-body crude ash increased with dietary Cu content. Thiobarbituric acid-reactive substances (TBARS) were significantly lower while copper-zinc superoxide dismutase (SOD) activities were significantly higher in liver tissue of fish fed CuM_{10} and CuM_{15} diets than those of fish fed $Cu_{1.0}$, CuM_{19}, CuM_{42} and CuM_{86} diets. Tissue Cu concentrations increased with dietary Cu content. Copper accumulated most in liver, followed by intestine, kidney, gills and then muscle. Weight gain analyzed by broken-line regression suggested that the optimum dietary Cu level was 8.44 mg Cu/kg diet. Therefore, these results may indicate that the optimum dietary Cu levels could be greater than 8.44 mg Cu/kg diet but less than 10 mg Cu/kg diet in juvenile olive flounder, *P. olivaceus* when Mintrex®Cu was used as the dietary source of organic copper.

Introduction

Copper (Cu) is an essential trace element for all organisms including fish (Lall 2002). It is a constituent of many enzymes such as cytochrome oxidase, superoxide dismutase, lysyl oxidase, dopamine hydroxylase and tyrosinase, and is essential for their activities (Watanabe et al. 1997). However, Cu may also be toxic at elevated concentrations and hence, its supplementation in fish feed is a balance between fulfilling Cu requirement and avoiding Cu toxicity. Toxic effects of excess dietary Cu include reduced growth (Clearwater et al. 2002), severe lesions in the gut (Handy 1996), and changes in intestinal cell proliferation and turnover (Berntssen et al. 1999; Lundebye et al. 1999). On the other hand, dietary Cu deficiency has been shown to lead to reduced appetite and growth and to cause anemia in animals such as hybrid tilapia and grouper (Shiau and Ning 2003; Lin et al. 2008).

Dietary Cu requirements have been reported in selected fish species. These include 3 mg/kg diet in rainbow trout, *Oncorhynchus mykiss*, and common carp, *Cyprinus carpio* (Ogino and Yang 1980); 5 mg/kg diet in channel catfish, *Ictalurus punctatus* (Gatlin and Wilson 1986); 5 mg/kg diet in Atlantic salmon, *Salmo salar* (Lall and Hines 1987) and 4 mg/kg diet in hybrid tilapia, *Oreochromis niloticus*×*Orechromis aureus* (Shiau and Ning 2003). Conversely, Cu toxicity has been experimentally produced in rainbow trout, *Salmo gairdneri*, fed 730 mg Cu/kg diet (Lanno et al. 1985). In general, teleosts have been reported to have a nutritional requirement of about 3-10 mg Cu/kg diet (Clearwater et al. 2002) while thresholds for excess dietary Cu toxicity in freshwater fish have been shown to be between 16 and 730 mg Cu/kg diet (Clearwater et al. 2002), although most of the available toxicity data are for salmonids (Handy 1996; Clearwater et al. 2002). Historically, zinc, copper and manganese have been supplemented in animal diets using inorganic salts such as oxides and sulfates. Copper sulfate ($CuSO_4$. $5H_2O$) is the most commonly used dietary Cu supplement. Copper in the form of copper sulfate has been shown to improve growth rate and feed efficiency in broilers (Choi and Paik 1989; Baker et al. 1991) and in pigs (Edmonds et al. 1985). However, use of inorganic salts can result in poor bioavailability of the mineral, mainly due to the numerous nutrient and ingredient antagonisms that impair absorption (Underwood and Suttle 1999). Improved availability of Cu from organic Cu complexes compared with the commonly used Cu salts has recently been suggested.

More bioavailability of Cu is probably due to better absorption, which enhances its efficiency (Downs et al. 2000; Yu et al. 2000; Guo et al. 2001). Baker and Ammerman (1995) reported that relative bioavailability estimate of organic Cu sources ranged from 88% to 147% of the response to cupric sulfate in poultry, swine, sheep and cattle. Nevertheless, traditional bioavailability assays, as well as more recently developed gene expression assays for mineral bioavailability demonstrate that not all organic trace minerals are equally bioavailable. These assays indicate that Mintrex® organic trace minerals, a chelate of two 2-hydroxy-4 (methylthio) butanoic acid (HMTBa) ligands per atom of trace mineral (zinc, copper or manganese), provide a highly bioavailable source of trace minerals. Chelates are the organic form of Cu and are usually considered for use in animal diet as alternatives to inorganic Cu source. Mintrex® Copper, one of the chelates, has been shown to have bioavailability comparable to the commonly used inorganic source of Cu for fattening chickens.

Olive flounder, *P. olivaceus*, is one of the commercially important marine aquaculture species in Korea, ranking first among the marine finfish aquaculture production in the country in 2009. Olive flounder is very popular and highly valued by Koreans for its good percent dress out and great taste. It is served in various ways; one of the most popular of these is the raw fish called hoe. Due to the importance of olive flounder in Korea, various studies have been conducted in this species (Wang et al. 2002; Choi et al. 2003; Choi et al. 2004; Kim et al. 2004; Kim et al. 2005; Sun et al. 2007; Yoo et al. 2007; Kim et al. 2008). However, information on Cu requirement in this species is scarce. Therefore, this study was conducted to determine the optimum dietary Cu level in olive flounder, *P. olivaceus*, using organic Cu (Mintrex®Cu) as the Cu source.

Materials and Methods

Diet Formulation and Preparation

Proximate composition of the basal diet used in this feeding trial is shown in Table 2. Seven semi-purified diets were formulated and prepared to contain 0, 5, 10, 15, 20, 40 and 80 mg Cu/kg diet using Mintrex®Cu, a chelate of copper and 2-hydroxy-4-methylthiobutanoic acid, an organic form of Cu, as the Cu source. However, the actual copper concentrations as determined by analysis were 0.89, 5.56, 10.1, 14.9, 19.0, 42.4 and 85.8 mg Cu/kg diet for $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10}, CuM_{15}, CuM_{19}, CuM_{42} and CuM_{86} diets, respectively. The premixture containing the Cu source was added to the diet to replace the equal amount of cellulose in the diet. Vitamin-free casein (United States Biochemical, Cleveland, OH, USA), fish oil (DHA + EPA enriched; refined fish oil, E-Wha oil Co. Ltd., Pusan, Korea), and corn starch (United States Biochemical, Cleveland, OH, USA) were used as the main dietary protein, lipid and carbohydrate sources, respectively. All diets were kept isoenergetic at 520 kcal/100 g diet as recommended by Kim et al. (2002). Lyophilized olive flounder muscle powder was added at 10 % to all diets to increase palatability and acceptance of the experimental diets. All dry ingredients were finely ground, weighed, mixed manually for 5 minutes and then transferred to a mixer for another 15-minute mixing. Fish oil was then added slowly while mixing was continued. All ingredients were mixed for another 10 minutes and then Mintrex®Cu mixed well with the other feed ingredients. Finally, distilled water was added to the mixture to form dough and dry pellets were made from this dough using a laboratory pelleting machine (Baokyong Commercial Co., Pusan, Korea). The pellets were air dried until the moisture was reduced to <10 g/kg diet. After processing, all the diets were broken up and sieved into the appropriate pellet size (4 mm diameter), packed into small bags and stored at -24°C until used.

Experimental Fish and Feeding Trial

The feeding trial was carried out at the Feeds and Foods Nutrition Research Center, Pukyong National University, Busan. Fish were transported to the experimental station and acclimated to the experimental conditions for two weeks before the feeding trial began. During this period, fish were fed copper-free diet to deplete the body Cu reserve. Fifteen fish of initial average weight 6.86 ± 0.04 g (mean ± SD) were randomly distributed into each of 21 aquaria.

Each aquarium was then randomly assigned to one of the three replicates of the 7 dietary treatments. The diets were fed to triplicate groups of fish at approximately 3% of wet body weight per day at the beginning and 2% of wet body weight per day at the end of the feeding trial. Fish were fed twice a day at 1000 and 1900 h with the daily ration divided into two portions for the feeding periods for 14 weeks. One hour before feeding, the feed was defrosted at room temperature and subsequently weighed and distributed in the tanks. Feeding was done slowly and fish ate the ration within 1-2 minutes after distribution of feed. Care was taken to ensure that no uneaten food remained in the tanks during feeding, thus leaching of Cu into water was negligible. Tanks were siphoned an hour before the morning feeding and water in the center tank was completely replaced one hour after feeding in the evening. The Cu concentration in rearing water was monitored regularly and remained less than 1.3 µg/L. Mortality was checked daily. Any dead fish were removed and not replaced during the experiment. Total fish weight in each aquarium was determined every 2 weeks, and the amount of diet fed to the fish was adjusted accordingly. The aquaria were thoroughly cleaned during the time fish were removed for weighing to minimize algae and fungal growth. Fish were starved 24 h before each weighing to avoid inclusion of ingested feed in the weight measurement as well as to reduce stress.

The feeding trial was conducted by using an indoor semi-recirculating system with rectangular aquaria receiving filtered seawater at the rate of 1.1 L/min through two separate biofilters to remove impurities and reduce ammonia concentration from the center tank. All the experimental aquaria were maintained at 14:10 (light: dark). The seawater temperature was maintained at 20 ± 1^{o}C by heaters in the center tank during the whole experimental period. Supplemental aerations were provided to maintain dissolved oxygen levels near saturation (8.8 ± 0.3 mg/L). The seawater pH, salinity, total ammonia-nitrogen and nitrites were 8.23 ± 0.13, 32.5 ± 0.7 ppt, 0.037 - 0.052 mg/L and 0.13 ± 0.09 mg/L, respectively. These values were within optimum ranges for normal growth and health of juvenile olive flounder (Wang et al. 2002).

Sample Collection and Analysis

At the beginning of the experiment, a pooled sample of 10 fish was taken for determining whole-body proximate composition and copper content. At the end of the feeding trial, fish were starved for 24 h and the total number and weight of fish in each aquarium was determined for calculation of weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER), and survival. Blood samples were obtained from the caudal vessels of three randomly selected fish per aquarium by using a heparinized syringe and pooled for blood analysis. After blood sampling, gill, muscle, liver, intestine and kidney tissues were obtained from the same fish, pooled and stored at -63^{o}C for tissue copper analysis. Three other fish were selected from each aquarium, killed and frozen at -63^{o}C for proximate composition and whole body Cu concentration analyses. Also, two fish were randomly sampled from each tank, then liver and muscle were removed from each fish and pooled for determining hepatosomatic index (HSI), Cu-Zn superoxide dismutase (SOD) activity, glutathione peroxidase (GPx) and thiobarbituric acid reaction substances (TBARS).

Red blood cell (RBC), hematocrit (packed cell volume; PCV), white blood cell (WBC), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated and analyzed. Hematocrit was determined using the microhematocrit method (Brown 1980) and hemoglobin (Hb) was measured by the cyanmethemoglobin procedure using Drabkin's reagent. An Hb standard prepared from human blood (Sigma Chemical, St Louis, MO, USA) was used. Proximate composition analyses of experimental diets and fish body were performed by the standard methods of AOAC (2000). Samples of diets and fish were dried to a constant weight at 105°C to determine moisture content. Ash was determined by incineration at 550°C; crude lipid by soxhlet extraction using Soxtec system 1046 (Foss, Hoganas, Sweden) and crude protein by Kjeldahl method (N × 6.25) after acid digestion.

Enzyme Assay

The activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were conducted in Tabriz University (Iran) according to Somi et al. (2009). To measure cytosolic enzyme activity, the liver samples were homogenized in 1.15% KCl solution. Glutathione peroxidase (GPx) activity was measured according to Paglia and Valentine (1967), using Randox (United Kingdom). Tissue superoxide dismutase (SOD) was assayed by a spectrophotometric method based on the inhibition of a superoxide-induced reduced nicotinamide adenine dinucleotide (NADH) oxidation according to Paoletti et al. (1986). The thiobarbituric acid reaction substances (TBARS) value was analyzed according to the method of Uchiyama and Mihara (1978). Absorbancy of the solution was measured at 530 nm and the concentration of the TBARS in the sample was measured by multiplying the optical density 5.2.

Copper Analysis

Copper contents of rearing water, diet, tissue, and initial and final whole-body were determined by digestion of samples in nitric acid (AOAC 2000). The concentrations of copper in the diluted digest solution were determined by using an Inductively Coupled Plasma Mass Spectrometer (Perkin-Elmer 3300, Waltham, MA).

Statistical Analysis

After confirming normality and homogeneity of variance, data were analysed by one-way ANOVA (SPSS, version 12, Chicago, IL) to test for the effects of the dietary treatments. When a significant treatment effect was observed, a Tukey's test for multiple comparisons was performed. Treatment effects were considered at $P < 0.05$ level of significance. Broken line model was used to estimate the optimum dietary copper level in olive flounder.

Results

Growth and Survival

Weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER) and survival of juvenile olive flounder, *P. olivaceus*, fed diets containing different levels of Mintrex®Cu are shown in Table 3. At the end of 14 weeks of feeding trial, weight gain (WG) and specific growth rate (SGR) of fish fed CuM_{10} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{19}, CuM_{42} and CuM_{86} diets ($P < 0.05$). Also, WG and SGR of fish fed Cu_1, $CuM_{6.0}$, CuM_{10}, CuM_{15} and CuM_{19} diets were significantly higher than those of fish fed CuM_{42} and CuM_{86} diets. There were no significant differences in WG and SGR among fish fed CuM_{10} and CuM_{15} diets or among those fed $CuM_{6.0}$ and CuM_{15} diets. There were no significant differences in FE of fish fed all the diets except for the FE of fish fed CuM_{86} diet, which was significantly lower than those of fish fed the other diets. Protein efficiency ratio of fish fed CuM_{15} diet was significantly higher than those of fish fed CuM_{42} and CuM_{86} diets. Furthermore, PER of fish fed CuM_{86} diet was significantly lower than those of fish fed other diets. There were no significant differences in PER among fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10}, CuM_{15} and CuM_{19} diets, or among those fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10}, CuM_{20} and CuM_{42} diets. There were no significant differences in survival of fish fed all the diets. Broken-line analysis of WG indicated that the optimum dietary Mintrex®Cu supplementation level could be 8.44 mg/kg diet in juvenile olive flounder, *P. olivaceus* (Figure 1).

Proximate Composition

Table 4 shows the proximate composition of whole body and muscle of juvenile olive flounder, *P. olivaceus*, fed the experimental diets. Whole-body crude protein content of fish fed CuM_{10} diet was significantly higher than that of fish fed CuM_{86} diet ($P < 0.05$). There were no significant differences in whole-body crude protein among fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10}, CuM_{15}, CuM_{19} and CuM_{42} diet or among those fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{15}, CuM_{19}, CuM_{42} and CuM_{86} diets. Whole-body crude lipid of fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10} and CuM_{15} diets were significantly higher than those of fish fed CuM_{19}, CuM_{42} and CuM_{86} diets. There were no significant differences in whole-body crude lipid among fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10} and CuM_{15} diets or among those fed CuM_{19}, CuM_{42} and CuM_{86} diets.

Whole-body ash content of fish fed CuM_{86} diet was significantly higher than those of fish fed $Cu_{1.0}$ and $CuM_{6.0}$ diets. Also, Whole-body ash of fish fed CuM_{19}, CuM_{42} and CuM_{86} diets were significantly higher than that of fish fed $Cu_{1.0}$ diet. There were no significant differences in whole-body ash among fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10} and CuM_{15} diets, among fish fed $CuM_{6.0}$, CuM_{10}, CuM_{15}, CuM_{19} and CuM_{42} diets or among those fed CuM_{10}, CuM_{15}, CuM_{19}, CuM_{42} and CuM_{86} diets. Muscle lipid content of fish fed $Cu_{1.0}$ diet was significantly higher than those of fish fed CuM_{15}, CuM_{19}, CuM_{42} and CuM_{86} diets. This parameter was also significantly higher for fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10} and CuM_{15} diets than for those fed CuM_{19}, CuM_{42} and CuM_{86} diets. There were no significant differences in muscle lipid among fish fed $Cu_{1.0}$, $CuM_{6.0}$ and CuM_{10} diets, among fish fed $CuM_{6.0}$, CuM_{10} and CuM_{15} diets or among those fed CuM_{19}, CuM_{42} and CuM_{86} diets. Furthermore, there were no significant differences in whole-body moisture or muscle protein among fish fed all the experimental diets.

Enzyme activity and Hepatosomatic Index

Hepatic thiobarbituric acid reactive substances (TBARS), copper-zinc superoxide dismutase (Cu-Zn SOD) activity and hepatosomatic index (HSI) are shown in Table 5. Hepatic thiobarbituric acid reactive substances (TBARS) of fish fed CuM_{10} diet were significantly lower than those of fish fed $Cu_{1.0}$, CuM_{19}, CuM_{42} and CuM_{86} diets ($P < 0.05$). This parameter was significantly higher for fish fed CuM_{42} and CuM_{86} diets than for those fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10}, CuM_{15} and CuM_{19} diets. There were no significant differences in TBARS among fish fed $CuM_{6.0}$ and M_{15} diets or among those fed CuM_{10} and CuM_{15} diets. Broken-line analysis of TBARS levels indicated that the optimum dietary Mintrex®Cu supplementation level could be 7.61 mg/kg diet in juvenile olive flounder (Figure 2). Copper-zinc superoxide dismutase (Cu-Zn SOD) activity of fish fed CuM_{10} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{19}, CuM_{42} and CuM_{86} diets. Copper-zinc superoxide dismutase activity of fish fed $CuM_{6.0}$, CuM_{10}, CuM_{15} and CuM_{19} diets were significantly higher than those of fish fed $Cu_{1.0}$, CuM_{42} and CuM_{86} diets. There were no significant differences in Cu-Zn SOD activity among fish fed CuM_{10} and CuM_{15} diets, among fish fed $CuM_{6.0}$ and CuM_{15} diets, among fish fed CuM_{42} and CuM_{86} diets or among those fed $Cu_{1.0}$ and CuM_{42} diets. Broken-line analysis of Cu-Zn SOD activity indicated that the optimum dietary Mintrex®Cu supplementation level could be 7.71 mg/kg diet in juvenile olive flounder, *P. olivaceus* (Figure 3).

Hepatosomatic index of fish fed $Cu_{1.0}$ diet was significantly higher than those of fish fed CuM_{19} and CuM_{86} diets. There were no significant differences in hepatosomatic index among fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10}, CuM_{15} and CuM_{42} diets, among fish fed $CuM_{6.0}$, CuM_{10}, CuM_{15}, CuM_{19} and CuM_{42} diets or among those fed CuM_{19} and CuM_{86} diets.

Hematological Parameters

Hematological characteristics of fish fed the experimental diets are shown in Table 6. Red blood cell (RBC), hematocrit (packed cell volume, PCV) and hemoglobin (Hb) increased to peak values obtained at the dietary Cu level of 10.1 mg/kg diet and dropped beyond this supplementation level. Red blood cell (RBC) counts of fish fed CuM_{10} and CuM_{15} diets were significantly higher than those of fish fed $Cu_{1.0}$, CuM_6, CuM_{19}, CuM_{42} and CuM_{86} diets ($P < 0.05$). Also, RBC of fish fed Cu_1, CuM_6, CuM_{10}, CuM_{15} and CuM_{19} diets were significantly higher than those of fish fed CuM_{86} diet. There were no significant differences in RBC among fish fed CuM_{10} and CuM_{15} diets, among fish fed $Cu_{1.0}$, CuM_6, CuM_{19} and CuM_{42} diets or among those fed CuM_{42} and CuM_{86} diets. Hematocrit of fish fed CuM_{10} diet was significantly higher than those of fish fed $Cu_{1.0}$ and CuM_{86} diets. There were no significant differences in PCV among fish fed CuM_6, CuM_{10}, CuM_{15}, CuM_{19}, CuM_{42} and CuM_{86} diets, among fish fed $Cu_{1.0}$, CuM_6, CuM_{15}, CuM_{19} and CuM_{42} diets or among those fed $Cu_{1.0}$, CuM_6, CuM_{19}, CuM_{42} and CuM_{86} diets. White blood cell (WBC) increased with dietary Cu supplementation. White blood cell of fish fed CuM_{86} diet was significantly higher than those of fish fed the other diets. Hemoglobin of fish fed CuM_{10} diet was significantly higher than those of fish fed $Cu_{1.0}$, CuM_{15}, CuM_{19}, CuM_{42} and CuM_{86} diets. Hemoglobin of fish fed CuM_{86} diet was significantly lower than those of fish fed $Cu_{1.0}$, CuM_6, CuM_{10} and CuM_{15} diets. There were no significant differences in hemoglobin among fish fed CuM_6 and CuM_{10} diets, among fish fed $Cu_{1.0}$, CuM_6, CuM_{15}, CuM_{19} and CuM_{42} diets or among those fed CuM_{19}, CuM_{42} and CuM_{86} diets. Although significant differences were recorded in mean corpuscular hemoglobin (MCH) no clear trend was found. There were no significant differences in mean corpuscular volume (MCV) or mean corpuscular hemoglobin concentration (MCHC) of fish fed all the experimental diets.

Copper Concentration

Table 7 shows the whole-body and tissues Cu concentrations of fish fed the experimental diets. Whole-body and tissue Cu concentrations increased with dietary Cu supplementation. Copper accumulated most in liver, followed by intestine, kidney, gills and then muscle. Whole-body and tissue Cu concentrations of fish fed CuM_{86} diet were significantly higher than those of fish fed the other diets ($P < 0.05$). Similarly, whole-body and muscle Cu concentrations of fish fed CuM_{42} and CuM_{86} diets were significantly higher than those of fish fed $Cu_{1.0}$, CuM_6, CuM_{10}, CuM_{15} and CuM_{19} diets. There were no significant differences in whole-body and muscle Cu among fish fed $Cu_{1.0}$, CuM_6 and CuM_{10} diets or among those fed CuM_{42} and CuM_{86} diets. Also, there were no significant differences in muscle Cu concentrations among fish fed CuM_{10} and CuM_{15} diets or among those fed CuM_{15} and CuM_{19} diets.

Gill copper concentrations of fish fed CuM_{42} and CuM_{86} diets were significantly higher than those of fish fed $Cu_{1.0}$, CuM_6, CuM_{10} and CuM_{15} diets. However, there were no significant differences in gill Cu contents among fish fed $Cu_{1.0}$, CuM_6 and CuM_{10} diets, among fish fed CuM_{10} and CuM_{15} diets, among fish fed CuM_{15} and CuM_{19} diets or among those fed CuM_{19} and CuM_{42} diets. Kidney Cu concentrations of fish fed $Cu_{1.0}$ and CuM_6 diets were significantly lower than those of fish fed CuM_{15}, CuM_{19}, CuM_{42} and CuM_{86} diets. There were no significant differences in kidney Cu contents among fish fed $Cu_{1.0}$ and CuM_6 diets or among those fed CuM_6 and CuM_{10} diets. Liver and intestine Cu contents of fish fed $Cu_{1.0}$ and CuM_6 diets were significantly lower than those of fish fed the other diets. For those other treatments, these parameters were significantly higher at each dietary Cu supplementation level than they were for the preceding one. A non significant increase in total copper concentrations was observed in the water from tanks in which fish were fed Mintrex Cu supplemented diets compared to the tanks in which fish were fed the control diet. Waterborne Cu level was less than 0.82 μg/L at the beginning and increased to 1.3 μg/L at the end of the feeding trial.

Discussion

Results of this study demonstrate that the olive flounder has a copper (Cu) requirement that cannot be met by Cu in the rearing water; thus, dietary supplementation is necessary. In the present study, lower weight gain (WG) and specific growth rate (SGR) were recorded in fish fed low or high levels of dietary Cu compared to fish fed diets containing 10.1-14.9 mg Cu/kg diet. Broken line analysis of weight gain indicated the optimum dietary Cu requirement in the form of Mintrex®Cu in juvenile olive flounder, *P. olivaceus*, weighing 6.82 ± 0.18 g (mean ± SD) to be 7.1 mg Cu/kg diet. This is a bit higher than values reported in several other species: 3 mg Cu/kg diet for rainbow trout, *Oncorhynchus mykiss*, and common carp, *Cyprinus carpio* (Ogino and Yang 1980), 1.5-5 mg Cu/kg diet for channel catfish, *Ictalurus punctatus* (Murai et al. 1981; Gatlin and Wilson 1986), 4 mg Cu/kg diet for tilapia, *Oreochromis niloticus*×*O. aureus* (Shiau and Ning 2003) and 4-6 mg Cu/kg diet for grouper, *Epinephelus malabaricus* (Lin et al. 2008). It appears that the optimum dietary Cu level in olive flounder could be higher than the requirement levels in these other species but variability of results could equally be attributed to other factors such as Cu source, fish weight and duration of feeding trial. But considering the overall performance of fish fed the optimum level of dietary Cu in this trial as compared to other treatment groups, it could be seen that Mintrex®Cu is a bioavailable source of dietary Cu.

It is possible that optimum dietary Cu levels enhance growth of olive flounder by stimulating activities of the enzymes involved in nutrient utilization. Similar results were found in rainbow trout, *O. mykiss*, and channel catfish (Ogino and Yang 1980; Knox et al. 1982, 1984; Gatlin and Wilson 1986). Growth reduction has been seen in both aquatic and terrestrial animals fed either Cu-deficient or excess copper diets (Koh et al. 1996; Shiau and Ning 2003; Shaw and Handy 2006; Lin et al. 2008; Wu and Huang 2008). Conversely, Wang et al. (2009) showed that the growth performance of juvenile abalone, *Haliotis discus*, was not significantly affected by graded levels of dietary Cu. Gatlin and Wilson (1986) also reported that the growth rates were similar for channel catfish fed diets with copper levels ranging from 0 to 40 mg Cu/kg for 13 weeks. The reduction in growth in olive flounder fed excess Cu diets is likely explained by reduced feed intake. Loss of appetite as the cause of reduced growth has also been reported in rainbow trout, *Salmo gairdneri* (Lanno et al. 1985) and Nile tilapia, *Oreochromis niloticus* (Shaw and Handy 2006) when exposed to excessive dietary copper.

Decreased growth could also be due to an increased expenditure of energy for sustaining normal metabolism at Cu levels below or above the optimum level, leaving less energy available for growth. Feed efficiency (FE), however, seemed not to have been seriously affected by dietary Cu concentrations less than 85.8 mg/kg diet within the experimental period, as only fish fed the diet containing 85.8 mg Cu/kg diet had significantly reduced FE ($P < 0.05$). Also, survival was not affected by dietary Cu supplementation up to this level. On the contrary, high levels of copper did not only depress growth but also impaired feed conversion in channel catfish, *I. punctatus*, and yellow catfish, *Pelteobagrus fulvidraco* (Tan et al. 2011).

Whole-body moisture and protein as well as muscle protein were not affected by dietary Cu supplementation except for the whole-body protein content of fish fed the diet containing 85.8 mg Cu/kg diet, which was significantly lower than that of fish fed the diet containing 10.1 mg Cu/kg diet. Whole-body and muscle lipid decreased while whole-body ash increased with dietary Cu supplementation. De-Boeck et al. (1997) reported decreased liver and muscle protein, lipid and glycogen content in common carp, *C. carpio*, exposed to waterborne Cu. Similar results were reported in abalone, *Haliotis discus hannai* (Wang et al. 2009). Handy et al. (1999) suggested that, compared to fish consuming 11 mg Cu/kg dry diet, lipid stores decreased markedly in rainbow trout, *O. mykiss*, after one month of exposure to 500 mg Cu/kg diet. Although no significant differences were found in muscle protein, there was a trend towards increased whole-body and muscle protein with dietary Cu up to peak values of 10.1 and 14.9 mg Cu/kg diet, respectively, followed by drops beyond these values. Similar to the findings of the above authors, whole-body and muscle lipid decreased with dietary Cu supplementation.

Thiobarbituric acid reaction substances (TBARS) analysis is one of the most popular and commonly used indicators of tissue peroxidation (Rosmini et al. 1996). Higher TBARS values observed in fish fed low and high dietary Cu than in those fed adequate Cu indicated that both insufficient and excess dietary Cu induced oxidative stress in olive flounder. Similar results have been reported in juvenile grouper, *Epinephelus malabaricus* (Lin et al. 2008). To guard against oxidative damage, organisms have developed a variety of antioxidant defenses that include metal sequestering proteins, by using compounds such as vitamin C and E, and specialized antioxidant enzymes.

One family of antioxidant enzymes, the superoxide dismutase (SOD), functions to remove damaging reactive oxygen species (ROS) from the cellular environment by catalyzing the dismutation of two superoxide radicals to hydrogen peroxide and oxygen (Lin et al. 2008). Copper is involved in the antioxidant system as it is an integral part of the enzymes Cu-Zn superoxide dismutase (SOD) and ceruloplasmin molecules. The present study showed that liver Cu-Zn SOD activity significantly increased with dietary Cu supplementation up to a peak value at dietary Cu supplementation level of 10.1 mg Cu/kg diet and subsequently dropped. It has been reported that inadequate and excess dietary Cu reduced Cu-Zn SOD activities and destroyed SOD, respectively in fish (Lin et al. 2008). Gatlin and Wilson (1986) reported that the activity of liver Cu-Zn SOD was significantly reduced in channel catfish fed diets containing 0-2.0 mg Cu/kg as compared to those fed 4 mg Cu/kg diet. In their study, liver Cu-Zn SOD activity was taken as the most sensitive indicator of copper status, and the dietary copper requirement of channel catfish was estimated to be 5 mg Cu/kg diet based on the activity of this enzyme. Depressed hepatic Cu-Zn SOD activity in olive flounder was observed when the dietary Cu was insufficient (≤5.56 mg Cu/kg diet) or in excess (≥19.0 mg Cu/kg diet). Free Cu has been reported to induce the oxidation and destruction of SOD in vitro (Cecconi et al. 2002). Increased free Cu concentration is associated with depressed ceruloplasmin activity in human (Harris 1993). Lin et al. (2008) reported that low plasma ceruloplasmin activity in juvenile grouper, *E. malabaricus*, may explain the decreased Cu-Zn SOD activity in fish fed high Cu concentration in the diet.

Hepatosomatic Index (HSI) provides an indication on status of energy reserve in an animal. In a poor environment, fish usually have a smaller liver (with less energy reserved in the liver). HSI has been reported to decrease in fish exposed to high concentrations of cadmium and zinc (Huang et al. 2010). Studies in fish and shrimp, however, have shown a positive correlation between liver Cu content and HSI (Lee and Shiau 2002; Shaw and Handy 2006; Tan et al. 2011). Although significant differences were recorded in HSI, no clear trends were found in this parameter in the present study. Nevertheless, fish fed the diet with the highest Cu supplementation level had a significantly reduced HSI than did those fed other diets except for those fed the diet containing 19 mg Cu/kg diet, suggesting an adverse effect of Cu on olive flounder at this concentration. It is possible that olive flounder liver shrinks after long-term exposure to high dietary Cu.

Hepatosomatic index decreased with dietary Cu levels in soft-shelled turtle, *Pelodiscus sinensis* (Wu and Huang 2008). In this trial, reduced HSI may also be a result of either reduced feed intake or increased metabolic expenditure for detoxification and maintenance of homeostasis.

Red blood cell, hematocrit and hemoglobin all increased with dietary Cu up to the supplementation level of 10.1 mg/kg diet and dropped. Other hematological characteristics such as mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration were less dependent on dietary Cu levels. It could be suggested that the optimum dietary Cu level could improve hematological characteristics of olive flounder. Copper is associated with the oxygen-carrying protein, haemocyanin, and dietary Cu deficiency has been shown to cause anemia in hybrid tilapia and grouper (Shiau and Ning 2003; Lin et al. 2008). Furthermore, low levels of hemoglobin content and total count of RBCs in rabbit fish, *Siganus rivulatus*, were attributed to water pollution by heavy metals (Bin-Dohaish 2008). In line with other reports (Berntssen et al. 1999; Shiau and Ning 2003), Cu concentrations in olive flounder tissues were found to be positively correlated with dietary Cu level.

Metal accumulation in fish tissues depends on exposure dose and time as well as other factors such as temperature, age of fish, interaction with other metals, water chemistry and metabolic activity of fish (Heath 1995). Copper accumulated most in liver and the order of accumulation in tissues was liver > intestine > kidney > gill > muscle. Similar patterns of Cu accumulation were also shown in other studies carried out with aquatic animals (Grosell et al. 2004; Kim and Kang 2004). Generally, uptake of metal in aquatic organisms can occur by two major routes. These involve gill, in the case of dissolved forms, and the digestive tissues, in the case of metals in food or sediment (Leland and Kuwabara 1985). In this study, Cu accumulation in the liver of olive flounder fed organic Cu was approximately 2.47-fold, 2.2-fold and 2.26-fold higher for fish fed 85.8, 42.4 and 19.0 mg Cu/kg diet, respectively than in the control group. Miller et al. (1993) reported that accumulation of Cu in liver of rainbow trout, *O. mykiss*, increased as the Cu concentration in the diet increased. These authors concluded that diet appeared to be dominant source of Cu in the liver compared to waterborne Cu.

Copper accumulation in olive flounder in the current study reflected the route of exposure with large increases in the Cu content of the liver and intestine, and this is consistent with previous studies on other species such as rainbow trout (Handy 1992; Handy et al. 1999; Kamunde et al. 2001; Campbell et al. 2002; Kamunde and Wood 2003), Atlantic salmon, *Salmo salar* (Berntssen et al. 1999; Lundebye et al. 1999) and grey mullet, *Chelon labrosus* (Baker et al. 1998). In general, when fish are fed elevated Cu concentrations for approximately a week or more, Cu will accumulate in highest concentrations in the intestinal tissues, liver and gall bladder (bile) and in lower concentrations in the gill, muscle and kidney (Baker et al. 1998; Clearwater et al. 2000; Kamunde et al. 2001). One goal of aquaculture is to produce fish fillets for human consumption. Hence, it is essential that muscle, the edible part of fish, meets all organoleptic and safety standards. Many authors have paid attention to Cu accumulation in such organs as intestine, kidney and liver, ignoring muscle. However, metal accumulation in muscle is very important for health of human consumers of fish (Cinier et al. 1999). All exposure concentrations had elevated copper concentrations compared to the controls. Muscle Cu concentrations of fish fed CuM_{42} and CuM_{86} diets were significantly higher than those of fish fed $Cu_{1.0}$, CuM_{6}, CuM_{10}, CuM_{15} and CuM_{19} diets. There were no significant differences in muscle Cu concentrations among fish fed CuM_{10} and CuM_{15} diets or among those fed CuM_{15} and CuM_{19} diets. De-Boeck et al. (1997) observed no significant Cu accumulation in muscle tissue of common carp, *C. carpio*, during 28 days of Cu exposure.

In conclusion, this study shows that olive flounder has a copper requirement that cannot be met by Cu in the rearing water; thus, dietary supplementation is necessary. Mintrex®Cu was found to be a bioavailable source of dietary Cu in this species. Growth performance, proximate composition and hematological characteristics improved at the optimum dietary Cu supplementation level. ANOVA test suggested that the optimum dietary Cu level in olive flounder could be 10.1 mg Cu/kg diet but broken line analysis of WG, Cu-Zn SOD and TBARS indicated levels 8.44, 7.71 and 7.61 mg Cu/kg diet, respectively, when Mintrex®Cu was used as the dietary source of organic copper.

Experiment II. The optimum dietary inorganic copper level in juvenile olive flounder, *Paralichthys olivaceus*

Abstract

A study was conducted to evaluate the optimum dietary inorganic copper (copper sulfate) in the juvenile olive flounder, *Paralichthys olivaceus*. Seven semi-purified diets containing 0.89 ($Cu_{1.0}$), 5.51 ($CuS_{6.0}$), 10.2 (CuS_{10}), 15.4 (CuS_{15}), 19.4 (CuS_{19}), 42.0 (CuS_{42}) and 86.3 (CuS_{86}) mg Cu/kg diet in the form of $CuSO_4.5H_2O$ were fed to fish averaging 6.89 ± 0.05g (mean ± SD) in triplicate groups for 14 weeks. Weight gain (WG) and specific growth rate (SGR) of fish fed CuS_{10} diet were significantly higher than those of fish fed $CuS_{1.0}$, $CuS_{6.0}$, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets ($P < 0.05$). Protein efficiency ratio (PER) of fish fed $CuS_{6.0}$, CuS_{10} and CuS_{15} diets were significantly higher than those of fish fed CuS_{42} and CuS_{86} diets. Crude lipid in muscle and whole-body decreased with increasing dietary Cu and was significantly lower in fish fed dietary Cu ≥15.4 mg/kg than in those fed Cu ≤5.5 mg Cu/kg diet. Results of copper-zinc superoxide dismutase activity and thiobarbituric acid-reactive substances level of liver from fish fed CuS_{10} diet were significantly better than those from fish fed $Cu_{1.0}$, $CuS_{6.0}$, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets ($P < 0.05$). Copper concentrations in tissue and whole-body from fish increased with dietary copper level, and these concentrations from fish fed CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diet were significantly higher than those from fish fed $Cu_{1.0}$ and $CuS_{6.0}$ diet. Broken-line analysis of WG suggested that the optimum dietary Cu level was 9.05 mg Cu/kg diet. Therefore, these results may indicate that the optimum dietary Cu level could be higher than 9.05 mg Cu/kg diet but less than 10 mg Cu/kg diet in juvenile olive flounder, *P. olivaceus* when copper sulfate is used as the dietary source of inorganic copper.

Introduction

Trace minerals such as zinc, copper, and manganese are crucial for a wide variety of physiological processes in all animals (Richards et al. 2010). Copper is essential for various health and performance-related functions in all animal species. Functions performed by zinc are often enhanced by copper-dependent enzymes (Underwood and Suttle 1999; Richards et al. 2010). For example, lysyl oxidase, the enzyme that crosslinks collagen subunits into mature protein forms to increase their strength, is copper-dependent (Rucker et al. 1998). Because of its role in collagen crosslinking, copper promotes skin, bone, tendon and intestinal strength (Rath et al. 1999; Richards et al. 2010). Also, copper plays key roles in managing oxidative stress. Reactive oxygen species (ROS) are a normal byproduct of cellular energy production, and a primary weapon of the innate immune response (Mayne 2003; Iqbal et al. 2004). Unfortunately, these ROS are damaging to cellular lipids, proteins and DNA and if left unchecked can induce a variety of undesirable consequences. The superoxide dismutase (SOD) enzymes form a first-line defense that converts oxygen radicals to hydrogen peroxide, which is a less toxic molecule (Hydrogen peroxide is then converted to water through the action of glutathione peroxidase, a selenium-containing enzyme). Decreases in SOD activity, for example in a mineral deficiency, can lead to increased amounts of lipid, protein and nucleic acid damage, which can induce cellular death (Richards et al. 2010).

Historically, zinc, copper and manganese have been supplemented in animal diets using inorganic salts such as oxides and sulfates. Copper sulfate ($CuSO_4.5H_2O$) is the most commonly used dietary Cu supplement. Copper in the form of copper sulfate has been shown to improve growth rate and feed efficiency in broilers (Choi and Paik 1989; Baker et al. 1991), in pigs (Edmonds et al. 1985) and in fish (Berntssen et al. 1999; Lin et al. 2008; Tan et al. 2011). Besides its use as a dietary copper supplement, copper sulfate ($CuSO_4.5H_2O$) is used in freshwater aquaculture to control external parasites, bacterial diseases and also as a fungicide.

Olive flounder, *P. olivaceus*, is regarded as a good candidate for marine-water aquaculture in Korea for its delicious flesh and high market value. However, information on Cu requirement in this species is scarce. Hence, this study was designed to evaluate the effects of dietary inorganic copper (copper sulfate) on growth, survival, carcass composition and immune responses in juvenile olive flounder, *P. olivaceus*, to determine the optimum dietary copper requirement for this species.

Materials and methods

Diet Formulation and Preparation

Proximate composition of the basal diet used in this feeding trial is shown in Table 2. Seven semi-purified diets were formulated and prepared to contain 0, 5, 10, 15, 20, 40 and 80 mg Cu/kg diet using copper sulfate ($CuSO_4.5H_2O$), an inorganic form of copper, as the copper source. However, the actual copper concentrations as determined by analysis were 0.89, 5.51, 10.2, 15.4, 19.4, 42.0 and 86.3 mg Cu/kg diet for $Cu_{1.0}$, $CuS_{6.0}$, CuS_{10}, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets, respectively. The premixture containing the copper sulfate ($CuSO_4.5H_2O$), source was added to the diet to replace an equal amount of cellulose in the diet. Diet formulation and preparation were conducted in the same manner as in chapter 2, experiment 1 "Effects of dietary organic copper (Mintrex®Cu) on growth, hematological parameters and accumulation in the juvenile olive flounder, *P. olivaceus*".

Experimental Fish and Feeding Trial

The feeding trial was carried out at the Feeds and Foods Nutrition Research Center, Pukyong National University, Busan. Fish were transported to the experimental station and acclimated to the experimental conditions for two weeks before the feeding trial began. During this period, fish were fed copper-free diet to deplete the body Cu reserve. Fifteen fish of initial average weight 6.89 ± 0.05g (mean ± SD) were randomly distributed into each of 21 aquaria. Each aquarium was then randomly assigned to one of three replicates of 7 dietary treatments. The diets were fed to triplicate groups of fish at approximately 3% of wet body weight per day at the beginning and 2% of wet body weight per day at the end of the feeding trial. Fish were fed twice a day at 1000 and 1900 h for 14 weeks. One hour before feeding, the feed was defrosted at room temperature and subsequently weighed and distributed in the tanks. Feeding was done slowly and fish ingested the ration within 1-2 minutes after distribution into the tanks. Care was taken to ensure that no uneaten food remained in the tanks during feeding, thus leaching of Cu into water was very low and negligible. Tanks were siphoned an hour before the morning feeding and water in the center tank was completely replaced one hour after feeding in the evening. The Cu concentration in rearing water was monitored regularly and remained less than 1.5 μg/L. Mortality was checked daily.

Any dead fish were removed and not replaced during the experiment. Total fish weight in each aquarium was determined every 2 weeks, and the amount of diet fed to the fish was adjusted accordingly. The aquaria were thoroughly cleaned during the time fish were removed for weighing to minimize algal and fungal growth. Fish were starved 24 h before each weighing to avoid inclusion of ingested feed in the weight measurement as well as to reduce stress. The feeding trial was conducted by using an indoor semi-recirculating system with rectangular aquaria receiving filtered seawater at the rate of 1.1 L/min through two separate biofilters to remove impurities and reduce ammonia concentration from the center tank. All the experimental aquaria were maintained at 14:10 (light: dark). The seawater temperature was maintained at 20±1^{o}C by heaters in the center tank during the whole experimental period. Supplemental aerations were provided to maintain dissolved oxygen levels near saturation (8.8 ± 0.3 mg/L). The seawater pH, salinity, total ammonia-nitrogen and nitrites were 8.23 ± 0.13, 32.5 ± 0.7 psu, 0.037 - 0.050 mg/L and 0.13 ± 0.08 mg/L, respectively. These values were within optimum ranges for normal growth and health of juvenile olive flounder (Wang et al. 2002).

Sample collection and analysis, enzyme assay, copper analysis and statistical analysis were conducted in the same manner as in chapter 2, experiment I "The optimum dietary organic copper level in juvenile olive flounder, *Paralichthys olivaceus*".

Results

Growth Performance

Weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER) and survival of juvenile olive flounder, *P. olivaceus*, fed diets containing different levels of copper sulfate are shown in Table 8. At the end of 14 weeks of feeding trial, weight gain and SGR of fish fed CuS_{10} diet were significantly higher than those of fish fed Cu_1, $CuS_{6.0}$, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets ($P < 0.05$). Also, WG and SGR of fish fed $Cu_{1.0}$, $CuS_{6.0}$, CuS_{10}, CuS_{15} and diets were significantly higher than those of fish fed CuS_{42} and CuS_{86} diets. There were no significant differences in WG and SGR among fish fed $Cu_{1.0}$, $CuS_{6.0}$ and CuS_{15} diets or between those fed $Cu_{1.0}$ and CuS_{19} diets. Weight gain and SGR of fish fed CuS_{86} diet were significantly lower than those of fish fed the other diets. There were no significant differences in FE of fish fed all the diets except for the FE of fish fed CuS_{86} diet, which was significantly lower than those of fish fed the other diets. Protein efficiency ratio of fish fed $CuS_{6.0}$, CuS_{10} and CuS_{15} diets were significantly higher than those of fish fed CuS_{42} and CuS_{86} diets. Furthermore, PER of fish fed CuS_{86} diet was significantly lower than those of fish fed the other diets. There were no significant differences in PER among fish fed $Cu_{1.0}$, $CuS_{6.0}$, CuS_{10}, CuS_{15} and CuS_{19} diets, or among those fed $Cu_{1.0}$, CuS_{19} and CuS_{42} diets. There were no significant differences in survival of fish fed all the diets. Broken-line regression analysis of WG suggested that the optimum dietary Cu level in juvenile olive flounder could be 9.05 mg Cu/kg diet (Figure 4).

Proximate Composition

Proximate composition of whole-body and muscle of juvenile olive flounder fed diets containing various levels of copper sulfate for 14 weeks is summarized in Table 9. Whole-body protein content of fish fed CuS_6 diet was significantly higher than those of fish fed CuS_{42} and CuS_{86} diets ($P < 0.05$). However there were no significant differences in whole-body protein among fish fed $Cu_{1.0}$, CuS_6, CuS_{10}, CuS_{15} and CuS_{19} diets or among those fed $Cu_{1.0}$, CuS_{10}, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets. Whole-body crude lipid content was negatively correlated with dietary copper sulfate concentrations. Whole-body crude lipid contents of fish fed $Cu_{1.0}$ and $CuS_{6.0}$ diets were significantly higher than those of fish fed CuS_{15}, CuS_{19}, CuS_{42} and Cu_{86} diets. However there were no significant differences in whole-body crude lipid content among fish fed $Cu_{1.0}$, $CuS_{6.0}$ and CuS_{10} diets, among fish fed CuS_{10} and CuS_{15} diets or among those fed CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets.

Whole-body crude ash content was positively correlated with dietary copper sulfate concentrations. Whole-body crude ash content of fish fed $Cu_{1.0}$ was significantly lower than those of fish fed the other diets. However there were no significant differences in whole-body ash content among fish fed $CuS_{6.0}$, CuS_{10}, CuS_{15} and CuS_{19} diets, among fish fed CuS_{10}, CuS_{15}, CuS_{19} and CuS_{42} diets or among fish fed CuS_{19}, CuS_{42} and CuS_{86} diets. Muscle crude lipid content of fish fed $Cu_{1.0}$ and $CuS_{6.0}$ diets were significantly higher than those of fish fed CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets. However there were no significant differences in muscle lipid content among fish fed $Cu_{1.0}$, $CuS_{6.0}$ and CuS_{10} diets, between fish fed CuS_{10} and CuS_{15} diets or between those fed CuS_{42} and CuS_{86} diets. There were no significant differences in whole-body moisture or muscle protein of fish fed all the experimental diets.

Enzyme Activity and Hepatosomatic Index

Hepatic thiobarbituric acid reactive substances (TBARS), copper-zinc superoxide dismutase (Cu-Zn SOD) activity and hepatosomatic index (HSI) are shown in Table 10. Hepatic TBARS of fish fed CuS_{10} diet were significantly lower than those of fish fed $Cu_{1.0}$, $CuS_{6.0}$, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets ($P < 0.05$). Hepatic TBARS decreased to a minimum value obtained for fish fed CuS_{10} diet and then increased again with dietary copper concentration. Broken-line regression analysis of TBARS suggested that the optimum dietary Cu level in juvenile olive flounder could be 7.72 mg Cu/kg diet (Figure 5). Hepatic Cu-Zn SOD activity of fish fed CuS_{10} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{6.0}$, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets. Also, hepatic Cu-Zn SOD of fish fed $CuS_{6.0}$, CuS_{10}, CuS_{15} and CuS_{19} diets were significantly higher than those of fish fed $Cu_{1.0}$, CuS_{42} and CuS_{86} diets. There were no significant differences in hepatic Cu-Zn SOD between fish fed $CuS_{6.0}$ and CuS_{15} diets, between fish fed $CuS_{6.0}$ and CuS_{19} diets, or between fish fed CuS_{42} and CuS_{86} diets. Broken-line regression analysis of Cu-Zn SOD suggested that the optimum dietary Cu level in juvenile olive flounder could be 7.88 mg Cu/kg diet (Figure 6). Hepatosomatic index (HSI) of fish fed $Cu_{1.0}$ diet was significantly higher than those of fish fed CuS_{19}, CuS_{42} and CuS_{86} diets. There were no significant differences in HSI among fish fed $Cu_{1.0}$, $CuS_{6.0}$, CuS_{10} and CuS_{15} diets, among fish fed $CuS_{6.0}$, CuS_{10}, CuS_{15} and CuS_{19} diets or among those fed $CuS_{6.0}$, CuS_{15}, CuS_{19} and CuS_{42} diets. Hepatosomatic index of fish fed CuS_{86} was significantly lower than those of fish fed the other diets.

Hematological Characteristics

Hematological characteristics of fish fed the experimental diets are shown in Table 11. Red blood cell (RBC) counts of fish fed $Cu_{1.0}$ and $CuS_{6.0}$ diets were significantly higher than those of fish fed CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets ($P < 0.05$). Red blood cells of fish fed $Cu_{1.0}$, $CuS_{6.0}$ and CuS_{10} diets were significantly higher than those of fish fed CuS_{19}, CuS_{42} and CuS_{86} diets. There were no significant differences in RBC among fish fed $Cu_{1.0}$, $CuS_{6.0}$ and CuS_{10} diets, between fish fed CuS_{10} and CuS_{15} diets, or among fish fed CuS_{19}, CuS_{42} and CuS_{86} diets. Hematocrit (packed cell volume, PCV) of fish fed $CuS_{6.0}$ and CuS_{10} diets were significantly higher than those of fish fed CuS_{42} and CuS_{86} diets. Also, PCV of fish fed $Cu_{1.0}$, $CuS_{6.0}$, CuS_{10}, CuS_{15} and CuS_{19} diets were significantly higher than that of fish fed CuS_{86} diet. There were no significant differences in PCV among fish fed $Cu_{1.0}$, $CuS_{6.0}$, CuS_{10}, CuS_{15} and CuS_{19} diets, among fish fed $Cu_{1.0}$, CuS_{15}, CuS_{19} and CuS_{42} diets or between those fed CuS_{42} and CuS_{86} diets.

White blood cell (WBC) count increased with dietary Cu supplementation. White blood cell count of fish fed CuS_{86} was significantly higher than those of fish fed the other diets. However, there were no significant differences in WBC between fish fed $Cu_{1.0}$ and $CuS_{6.0}$ diets, between fish fed CuS_{10} and CuS_{15} diets or between fish fed CuS_{15} and CuS_{19} diets. Hemoglobin (Hb) of fish fed $CuS_{6.0}$ diet was significantly higher than those of fish fed CuS_{19}, CuS_{42} and CuS_{86} diets. Conversely, Hb of fish fed CuS_{86} diet was significantly lower than those of fish fed $Cu_{1.0}$, $CuS_{6.0}$, CuS_{10} and CuS_{15} diets. There were no significant differences in this parameter among fish fed $Cu_{1.0}$, $CuS_{6.0}$, CuS_{10} and CuS_{15} diets, among fish fed $Cu_{1.0}$, Cu_{10}, CuS_{15} and CuS_{19} diets or among those fed CuS_{10}, CuS_{15}, CuS_{19} and CuS_{42} diets. Mean corpuscular hemoglobin concentration (MCHC) of fish fed $Cu_{1.0}$ was significantly higher than those of fish fed CuS_{15} and CuS_{19} diets. There were no significant differences in MCHC among fish fed $Cu_{1.0}$, $CuS_{6.0}$, CuS_{10}, CuS_{42} and CuS_{86} diets or among fish fed $CuS_{6.0}$, CuS_{10}, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets. Also there were no significant differences in mean corpuscular volume (MCV) or mean corpuscular hemoglobin (MCH) among all treatments.

Copper Concentration

Table 12 shows the whole-body and tissue Cu concentrations of fish fed the experimental diets. Copper concentrations in gill, liver, kidney, intestine, muscle and whole-body of fish fed the experimental diets for 14 weeks were generally dose-dependent, increasing with increase in dietary copper concentration. Whole-body, kidney, liver and intestine Cu concentrations of fish fed CuS_{86} diet were significantly higher than those of fish fed the other diets ($P < 0.05$). Similarly, whole-body, kidney, muscle, liver and intestine Cu concentrations of fish fed CuS_{42} diet were significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{6.0}$, CuS_{10}, CuS_{15} and CuS_{19} diets. Conversely, whole-body, kidney, gill, liver and intestine Cu concentrations of fish fed $Cu_{1.0}$, $CuS_{6.0}$ and CuS_{10} diets were significantly lower than those of fish fed Cu_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets. There were no significant differences in whole-body and muscle Cu among fish fed $Cu_{1.0}$, $CuS_{6.0}$ and CuS_{10} diets. Also, there were no significant differences in kidney, gill and intestine Cu concentrations among fish fed $Cu_{1.0}$ and $CuS_{6.0}$ diets or among fish fed $CuS_{6.0}$ and CuS_{10} diets. Gill copper concentrations of fish fed CuS_{86} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{6.0}$, CuS_{10}, CuS_{15} and CuS_{19} diets. Liver of olive flounder is a more important storage tissue than other tissues, and the order of Cu accumulation in tissues was liver >intestine >kidney >gill >muscle.

A non significant increase in total copper concentrations was observed in the water from tanks in which fish were fed copper sulfate supplemented diets compared to the tanks in which fish were fed the control diet. Waterborne Cu level was less than 0.82 μg/L at the beginning and increased to 1.5 μg/L at the end of the feeding trial.

Discussion

Results of this study showed that the growth response of juvenile olive flounder was significantly affected by the supplementation of dietary copper sulfate. Growth performance improved with dietary copper supplementation up to a peak value and dropped beyond that. Fish fed high concentrations (≥42.0 mg Cu/kg diet) of dietary copper had growth retardation compared to fish fed the basal diet and diets containing ≤19.4 mg Cu/kg diet. Based on the broken-line regression analysis of WG, 9.05 mg Cu/kg diet seemed optimal for best growth performance in this species. This is a bit higher than 3-6 mg/kg diet for finfish based on growth performance or biochemical parameters (Ogino and Yang 1980; Murai et al. 1981; Gatlin and Wilson 1986; Lall and Hines 1987; Lorentzen et al. 1998; Shiau and Ning 2003; Lin et al. 2008).

Growth of fish fed the optimum level of dietary copper in this trial was improved compared to that of fish fed sub-optimal levels. Besides growth performance, signs of copper deficiency in this experiment included slightly reduced mineralization and an increase in body lipid content. These signs have also been observed by Andrews et al. (1973) and Ogino and Yang (1980), in channel catfish and common carp. In this study, growth reduction was recorded in olive flounder fed either insufficient or excess dietary copper, in agreement with results in other aquatic animals (Shaw and Handy 2006; Lin et al. 2008; Wu and Huang 2008; Tan et al. 2011). The reduction in growth in olive flounder fed excess Cu is most likely explained by reduced feed intake. Loss of appetite as the cause of reduced growth has also been reported in rainbow trout (Lanno et al. 1985) and Nile tilapia, *Oreochromis niloticus* (Shaw & Handy 2006) when exposed to excessive dietary copper. The poorest FE was observed for fish fed the highest level of dietary copper in olive flounder. Similarly, high levels of copper depressed growth and impaired feed conversion in channel catfish (Murai et al. 1981; Tan et al. 2011). It appears that the optimum dietary copper sulfate level in olive flounder could be higher than the requirement levels in the other species mentioned above but variability of results could equally be attributed to several factors such as age, stage of development, diet composition, duration of the experiment, health and rearing condition (Roy and Lall 2003). Whole-body moisture as well as whole-body and muscle protein contents of olive flounder appear not to be greatly influenced by dietary copper supplementation, as only the whole-body protein contents of fish fed CuS_{42} and CuS_{86} diets were significantly lower than that of fish fed CuS_6 diet.

Similar to our results only the whole-body protein content of Atlantic salmon, *Salmo salar* L., fed the highest dietary copper was significantly lower than that of fish fed the control diet (Berntssen et al. 1999). Hamre et al. (2004) reported only slightly reduced levels of plasma protein in salmon-fed diets containing high levels of copper. Whole-body and muscle lipid decreased with dietary copper supplementation. This is in agreement with the previous studies in aquatic and terrestrial animals (Berntssen et al. 1999; Wang et al. 2009). Berntssen et al. (1999) observed a negative correlation between dietary Cu concentrations and energy stores in Atlantic salmon fed practical diets. Similar results were reported in abalone, *Haliotis discus hannai* (Wang et al. 2009). Cheng et al. (2008) reported that dietary copper supplementation, regardless of source, significantly reduced 12th rib backfat and kidney fat in lambs. The decrease in backfat depth by dietary copper supplementation was also observed in goat kids (Solaiman et al. 2006) and finishing steers (Engle et al. $2000_{a,b}$). It has been documented that TNF-α (tumor necrosis factor) is produced in adipose tissues (Hotamisligil and Spiegelman 1994) and has been shown to cause an elevated rate of lipolysis in adipocytes (Kawakami et al. 1987; Green et al. 1994). Cheng et al. (2008) speculated that the higher plasma TNF-α concentration in Cu-supplemented lambs might increase the lipolysis of adipose tissue, resulting in the observed reduction in backfat and kidney fat and the tendency for increasing plasma nonesterified fatty acids. Whole-body ash content on the other hand increased with dietary Cu content.

Thiobarbituric acid reactive substances (TBARS) analysis is one of the most popular and commonly used indicators of tissue peroxidation (Rosmini et al. 1996). Higher TBARS values were observed in fish fed low and high dietary Cu than in those fed adequate Cu indicated that both insufficient and excess dietary Cu induced oxidative stress in olive flounder. Similar results have been reported in juvenile grouper, *Epinephelus malabaricus* (Lin et al. 2008). Oral Cu exposure is known to cause oxidative stress as observed from an increased hepatic lipid peroxidation and decreased α-tocopherol levels in grey mullet fed 2400 mg Cu/kg (Baker et al. 1998). Furthermore, negative effects of high dietary Cu on plasma α-tocopherol levels and lipid turnover have been shown in Atlantic salmon (Berntssen et al. 2000). One family of antioxidant enzymes, the superoxide dismutase (SOD), functions to remove damaging reactive oxygen species (ROS) from the cellular environment by catalyzing the dismutation of two superoxide radicals to hydrogen peroxide and oxygen (Fattman et al. 2003; Lin et al. 2008).

Superoxide dismutase as an anti-oxidant enzyme plays an important role in the protection of cells from free radical damage (Fang et al. 2002). This enzyme, as one of the copper-dependent enzymes, has been shown to be an excellent indicator of copper nutrition in rat and cat (Paynter et al. 1979; Doong et al. 1983; Wang et al. 2009). Gatlin and Wilson (1986) also reported that the activity of liver Cu-Zn SOD was significantly reduced in channel catfish fed diets containing 0-2.0 mg Cu/kg as compared to those fed 4 mg Cu/kg diet. In their study, hepatic Cu-Zn SOD activity was taken as the most sensitive indicator of copper status, and the dietary copper requirement of channel catfish was estimated to be 5 mg Cu/kg based on the activity of this enzyme. In the present study, depressed hepatic Cu-Zn SOD activity in olive flounder was observed when the dietary Cu was insufficient (≤5.56 mg Cu/kg diet) or in excess (≥19.0 mg Cu/kg diet). Superoxide dismutase value analyzed by broken-line regression suggested that the optimum dietary Cu level could be 7.88 mg Cu/kg diet.

Hepatosomatic Index (HSI) provides an indication on status of energy reserve in an animal. In a poor environment, fish usually have a smaller liver (with less energy reserved in the liver). Hepatosomatic index has been reported to decrease in fish exposed to high concentrations of cadmium and zinc (Huang et al. 2010). Studies in fish and shrimp, however, have shown a positive correlation between liver Cu content and HSI (Shaw and Handy 2006; Tan et al. 2011). Nevertheless, fish fed the diet with the highest Cu supplementation level had a significantly reduced HSI than did those fed other diets, suggesting an adverse effect of Cu on olive flounder at this concentration. It is possible that olive flounder liver shrinks after long-term exposure to high dietary Cu. HIS decreased with dietary Cu levels in soft-shelled turtle, *Pelodiscus sinensis* (Wu and Huang 2008). In this trial, reduced HSI may also be a result of either reduced feed intake or increased metabolic expenditure for detoxification and maintenance of homeostasis.

As hematology has been used to assess the health status of most animals, several studies have shown hematological changes in fish exposed to numerous stress agents, including those relating to current aquaculture practices (Affonso et al. 2002). Copper and pH stress are known to induce changes in the blood parameters of fish (Wang et al. 1998; Cerqueira and Fernandes 2002). In the present study, red blood cell, hematocrit and hemoglobin were all reduced at the highest dietary copper supplementation. Conversely, white blood cell increased with dietary copper supplementation.

Other hematological characteristics such as mean corpuscular volume mean corpuscular hemoglobin characteristic and mean corpuscular hemoglobin concentration were less dependent on dietary Cu levels. Copper is associated with the oxygen-carrying protein, haemocyanin, and dietary Cu deficiency has been shown to cause anemia in hybrid tilapia and grouper (Shiau and Ning 2003; Lin et al. 2008). However, excess dietary Cu levels could adversely affect hematological characteristics of olive flounder. Low levels of hemoglobin content and total count of RBCs in rabbit fish, *Siganus rivulatus*, were attributed to water pollution by heavy metals (Bin-Dohaish 2008). In line with other reports, copper accumulation in tissues of olive flounder increased with the copper concentration of the diet, the exposure medium (Berntssen et al. 1999; Shiau and Ning 2003).

Metal accumulation in fish tissues depends on exposure dose and time as well as other factors such as water chemistry and metabolic activity of the fish (Heath 1995; Fırat and Kargın 2010). It is well known that the gill tissue is the initial site of accumulation of water-borne metals. Also, Fırat and Kargın (2010) reported that Cu accumulation increased in gill of *Cyprinus carpio*. However, accumulation of dietary copper seems to differ from that of water-borne copper. Copper concentration in the gill tissue of olive flounder was lower than those in the liver, intestine and kidney in this study. The Cu accumulation pattern reflected the route of exposure with large increases in the Cu content of the liver and intestine, and is consistent with previous reports on teleost fish (e.g. Nile tilapia, Shaw and Handy 2006; rainbow trout, Handy et al. 1999; Kamunde and Wood 2003; Atlantic salmon, Berntssen et al. 1999). Also, tissue Cu concentration did not plateau in olive flounder in the current study. Increased copper concentrations following metal exposure have also been found in tissue of crabs, *Carcinus maenas* (Weeks et al. 1993) and Pachygrapsus marmoratus (Legras et al. 2000).

In summary, this study indicated that dietary copper is essential for maintaining normal physiology, growth and tissue mineralization of juvenile olive flounder, *P. olivaceus*. Broken-line analysis of TBARS, SOD and WG suggested the optimum dietary Cu levels in olive flounder to be 7.72, 7.88 and 9.05 mg Cu/kg diet, respectively. These results may indicate that the optimum dietary Cu levels in the form of copper sulfate could be higher than 7.72 Cu/kg diet but lower than 9.05 mg Cu/kg diet in juvenile olive flounder, *P. olivaceus*.

Comparison of effects of dietary copper sources and levels on growth, enzyme activity and tissue copper concentration of juvenile olive flounder, *Paralichthys olivaceus*

Abstract

A 2× 7 factorial design was used to compare the effects of dietary organic (Mintrex®Cu) and inorganic copper (copper sulfate) and levels in juvenile olive flounder, *Paralichthys olivaceus*. Three replicate groups of fish averaging 6.88 ± 0.05 g (mean ± SD) were fed one of the 13 experimental diets ($Cu_{1.0}$, $CuM_{6.0}$, CuM_{10}, CuM_{15}, CuM_{19}, CuM_{42}, CuM_{86}, $CuS_{6.0}$, CuS_{10}, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86}) that were prepared containing two different dietary copper sources (organic and inorganic Cu) at each of seven different levels of Cu 1, 6, 10, 15, 19, 42 and 86 mg Cu/kg diet. At the end of 14 weeks of the experimental period, there were significant dietary copper sources, levels and their interaction effects on weight gain (WG), specific growth rate (SGR), copper-zinc superoxide dismutase activity (Cu-Zn SOD), thiobarbituric acid-reactive substances (TBARS), hematological values (red blood cell, hematocrit and hemoglobin) and Cu content of tuissues (liver, intestine, gill and muscle) and whole boby. Growth performance, antioxidant status and hematological values of fish fed organic Cu were relatively greater than inorganic Cu ($P < 0.05$). Copper concentrations in fish tissues and whole-body increased with dietary copper level. WG and SGR of fish fed CuM_{10} diet were significantly higher than those of fish fed the other diets except for those of fish fed CuM_{15} diet. Results of Cu-Zn SOD and TBARS in liver of fish fed CuM_{10} diet were significantly better than those of fish fed other diets except for those of fish fed CuM_{15} and CuS_{10} diets and $CuM_{6.0}$, CuM_{15} and CuS_{10} diets, respectively. Also, fish fed high levels of copper from inorganic Cu showed significantly higher rates of accumulation in liver and intestine than from organic Cu. Broken-line regression analysis of WG suggested that the optimum dietary Cu levels in juvenile olive flounder, *P. olivaceus*, could be 8.44 mg/kg diet and 9.05 mg/kg diet in the form of organic Cu and inorganic Cu, respectively. This study indicates that Cu allowance in diets of olive flounder can be reduced when organic Cu replaces inorganic copper. Organic Cu supproted better performance, increased nutrient utilization, improved physiological responses and greater effectiveness than inorganic Cu.

Introduction

Copper (Cu) is an essential mineral, and serves as a co-factor in many enzyme systems in the body. It plays an important role in metabolism and its concentration is well regulated. The essentiality of copper for poultry and livestock is well documented. Teleost fish have a nutritional requirement of about 3-10 mg Cu/kg dry weight (DW) feed, depending on the species, feeding regime and life stage (Clearwater et al. 2002; Shaw and Handy 2006). Dietary Cu requirements have been quantified in several fish species, including rainbow trout, *Salmo gairdneri*, and common carp, *Cyprinus carpio* (Ogino and Yang 1980), channel catfish, *Ictalurus punctatus* (Gatlin and Wilson 1986), hybrid tilapia, *Oreochromis niloticus*×*O. aureus* (Shiau and Ning 2003) and juvenile grouper, *Epinephelus malabaricus* (Lin et al. 2008; 2010). Historically, zinc, copper and manganese have been supplemented in animal diets using inorganic salts such as oxides and sulfates. Copper sulfate ($CuSO_4.5H_2O$) is the most commonly used dietary Cu supplement. Copper in the form of copper sulfate has been shown to improve growth rate and feed efficiency in broilers (Choi and Paik 1989; Baker et al. 1991) and in pigs (Edmonds et al. 1985). Besides its use as a dietary copper supplement, copper sulfate ($CuSO_4.5H_2O$) is used in freshwater aquaculture to control external parasites, bacterial diseases and also as a fungicide. However, use of inorganic salts can result in poor bioavailability of the mineral, mainly due to the numerous nutrient and ingredient antagonisms that impair absorption (Underwood and Suttle 1999). Mintrex®Cu is a chelated source of copper that contains a minimum of 17% copper and 78% hydroxy methionine analogue ((2-hydroxy-4-methylthio) butanoic acid, HMTBa). Mintrex®Cu has been shown to be a bioavailable copper source, comparable to a traditionally used inorganic source of copper in chickens (Das et al. 2010). Mintrex®Cu shows the same pattern as copper sulfate in increasing liver copper content in chickens for fattening when added to a basal diet in supplementations between 10 and 500 mg/kg diet. These findings are taken as a demonstration of bioavailability. However, use of inorganic salts can result in poor bioavailability of the mineral, mainly due to the numerous nutrient and ingredient antagonisms that impair absorption (Underwood and Suttle 1999). Improved availability of Cu from chelated Cu sources compared with the commonly used Cu salts has been suggested (Downs et al. 2000; Yu et al. 2000; Guo et al. 2001). Baker and Ammerman (1995) reported that relative bioavailability estimate of organic Cu sources ranged from 88% to 147% of the response to cupric sulfate in poultry, swine, sheep and cattle.

Olive flounder, *Paralichthys olivaceus*, is one of the most economically important fish species farmed in eastern Asia including the Republic of Korea, Japan and China. The Korean aquaculture production of olive flounder reached 54.675 metric tons in 2009 (National Statistical Office 2010). Due to its high nutritional value and profitable economic returns, olive flounder has become an important aquaculture species in Asia. Several studies have been conducted on olive flounder nutrition. However, the requirement dietary copper levels have not been determined in this species. Thus, this study was designed to investigate the influence of different levels of copper from organic Cu (Mintrex®Cu) and inorganic Cu (copper sulfate) based on growth performance, nutrient utilization, enzyme activity and copper concentration in tissues and whole body of olive flounder, *P. olivaceus*.

Materials and Methods

Diet Formulation and Preparation

Proximate composition of the basal diet used in this feeding trial is shown in Table 2. Thirteen experimental diets were formulated and prepared containing equivalent in the form of copper (Mintrex®Cu, a chelate of copper and 2-hydroxy-4-methylthiobutanoic acid) or copper sulfate ($CuSO_4.5H_2O$), as the Cu source. Three replicate groups of fish averaging 6.88 ± 0.05 g (mean ± SD) were fed one of the 13 experimental diets for 14 weeks. Six of the diets contained Mintrex®Cu at 5, 10, 15, 20, 40 and 80 mg Cu/kg diet, providing the actual dietary copper concentrations of 5.56, 10.11, 14.86, 18.96, 42.41 and 85.68 Cu/kg diet ($CuM_{6.0}$, CuM_{10}, CuM_{15}, CuM_{19}, CuM_{42} and CuM_{86}), respectively. Six other diets contained $CuSO_4.5H_2O$ at the same concentrations as in the previous six, giving Cu concentrations of 5.51, 10.20, 15.4, 19.44, 42.0 and 86.36 mg Cu/kg diet ($CuS_{6.0}$, CuS_{10}, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86}), respectively, while the remaining diet, which served as the control diet, was not supplemented with dietary Cu. Control diet without supplementation of Cu contains 0.86 mg Cu/kg diet. In diets supplemented with an organic and inorganic source, an equivalent amount of cellulose was removed. Diet formulation and preparation, sample collection and analysis, enzyme assay and copper analysis were conducted in the same manner as in chapter 2, Experiment I & II. "Effects of dietary organic/inorganic copper on growth, hematological parameters and tissue copper concentaation in the juvenile olive flounder, *P. olivaceus*".

Statistical analysis

After confirming the normality and homogeneity of variance, two-way ANOVA test was performed to test whether there was any interaction between dietary copper source and dose. When we found the interaction, all data were analyzed by one-way ANOVA to test for the effects of the dietary treatments. When a significant treatment effect was observed, a Tukey's test for multiple comparisons was performed. Treatment effects were considered at $P < 0.05$ level of significance. All statistical analyses were carried out by SAS version 9.0 software (SAS Institute, Cary, NC, USA).

Results

Growth Performance

Weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER) and survival of juvenile olive flounder, *P. olivaceus*, fed diets containing different levels of organic and inorganic copper are shown in Table 13 and 14. There were dietary significant copper source effects on FW, WG and SGR ($P < 0.05$). Furthermore, there were significant copper level and interaction effects on FW, WG and SGR content. Also, there were significant copper source and levels effects on PER. However, there were no significant their interaction effects on PER for fish fed all diets. There were significant copper level effects on FE ($P < 0.05$). While, there were neither significant copper sources nor interaction effects on FE. Survival did not show any significant differences among fish fed all the experimental diets. At the end of 14 weeks of feeding trial, organic Cu supplementation enhanced fish growth performance and feed utilization; meanwhile, the exposure to high copper reduced them. Moreover, fish groups fed organic or inorganic Cu at 10 mg Cu/kg concentration exhibited better growth than those were fed Cu $\leq$ 6 and $\geq$15 mg/kg diet. Weight gain and SGR of fish fed $\geq$15 mg/kg diet was also significantly higher than those of fish fed other diets, except for that of fish fed 10 mg Cu/kg diet.

At the end of 14 weeks of feeding trial, final weight of fish fed CuM_{10} diet was significantly higher than those of fish fed other diets, except fish fed CuM_{15} and CuS_{10} diets ($P < 0.05$). Final weight of fish fed $CuM_{6.0}$, CuM_{15} and CuS_{10} were also significantly higher than those of fish fed Cu_1, CuM_{19}, CuM_{42}, CuM_{86}, $CuS_{6.0}$, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets. However, there were no significant differences in final weight among fish fed $Cu_{1.0}$, CuM_{19}, $CuS_{6.0}$ and CuS_{15} diets, among fish fed Cu_1, CuM_{19}, $CuS_{6.0}$, CuS_{15}, and CuS_{19} diets and between those fed CuM_{42} and CuS_{19} diets. Weight gain (WG) and specific growth rate (SGR) were higher ($P < 0.05$) in fish fed Mintrex®Cu supplemented diet with 10.1 mg Cu/kg diet than those in fish fed other diets, except fish fed CuM_{15} diet. Also, WG and SGR of fish fed $CuM_{6.0}$, CuM_{15} and CuS_{10} were significantly higher than those of fish fed $Cu_{1.0}$, CuM_{19}, CuM_{42}, CuM_{86}, $CuS_{6.0}$, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets. Weight gain and SGR of fish fed CuS_{86} diet was significantly lower than those of fish fed other diets. However, there was no significant difference in WG and SGR between fish fed CuM_{10} and CuM_{15}, among fish fed $CuM_{6.0}$, CuM_{15} and CuS_{10} diets or among those fed $Cu_{1.0}$, CuM_{19}, $CuS_{6.0}$, CuS_{15} and CuS_{19} diets.

Feed efficiency (FE) of fish fed CuM_{86} and CuS_{86} diets was significantly lower than those of fish fed other diets. However, there was no significant difference in FE among fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10}, CuM_{15}, CuM_{19}, CuM_{42}, $CuS_{6.0}$, CuS_{10}, CuS_{15}, CuS_{19} and CuS_{42} diets ($P > 0.05$). Protein efficiency ratio (PER) of fish fed CuM_{15} diet was significantly higher than did fish fed $Cu_{1.0}$, CuM_{42}, CuM_{86}, CuS_{19}, CuS_{42} and CuS_{86} diets. However, there was no significant difference in PER among fish fed $CuM_{6.0}$, CuM_{10}, CuM_{15}, CuM_{19}, $CuS_{6.0}$, CuS_{10} and CuS_{15} diets, among fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10}, CuM_{19}, $CuS_{6.0}$, CuS_{10}, CuS_{15} and CuS_{19} diets or among those fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{42}, $CuS_{6.0}$, CuS_{19} and CuS_{42} diets. There were no significant differences in survival of fish fed all the diets.

Proximate Composition

Proximate composition of whole body and muscle (% dry matter) of juvenile olive flounder fed diets containing various levels of organic and inorganic copper for 14 weeks is summarized in Table 15 and 16. At the end of the experimental period, there were significant copper sources effects ($P < 0.05$) on whole body protein, lipid, ash, as well as muscle protein and lipid content. Furthermore, there were also significant copper levels effects on whole body lipid, ash and muscle lipid content. There were significant their interaction effects on whole body and muscle lipid content ($P < 0.05$). However, there were no interaction effects on whole-body moisture, protein and ash content ash and muscle protein content for fish fed all diets. Dietary copper concentrations were negatively correlated with whole-body protein and lipid content, but positively correlated with ash content.

Whole-body protein content of fish fed $CuM_{6.0}$, CuM_{10} and $CuS_{6.0}$ diets were significantly higher than those of fish fed CuM_{42}, CuM_{86} and CuS_{86} diets. However there were no significant differences in whole body protein among fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10}, CuM_{15}, CuM_{19}, $CuS_{6.0}$, CuS_{10}, CuS_{15}, CuS_{19} and CuS_{42} or among those fed $Cu_{1.0}$, CuM_{15}, CuM_{19}, CuM_{42}, CuM_{86}, CuS_{10}, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets. Whole-body crude lipid contents of fish fed CuM_{42}, CuM_{86}, CuS_{19}, CuS_{42} and CuS_{86} diets were significantly lower than those of fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10}, CuM_{15}, CuM_{19}, $CuS_{6.0}$ and CuS_{10} diets. However there were no significant differences in whole-body lipid content among fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10}, CuM_{15} and $CuS_{6.0}$ or among those fed $CuM_{6.0}$, CuM_{10}, CuM_{15}, $CuS_{6.0}$ and CuS_{10} diet.

Whole-body ash content of fish fed CuS_{86} was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10}, CuM_{15}, CuM_{19}, $CuS_{6.0}$, CuS_{10} and CuS_{15} diets. However there were no significant differences in whole-body ash content among fish fed $Cu_{1.0}$, $CuM_{6.0}$ and CuM_{10} diets, among fish fed $CuM_{6.0}$, CuM_{10}, CuM_{15}, CuM_{19}, $CuS_{6.0}$, CuS_{10} and CuS_{15} diets or among those fed CuM_{15}, CuM_{19}, CuM_{42}, CuM_{86}, $CuS_{6.0}$, CuS_{10}, CuS_{15}, CuS_{19} and CuS_{42} diets. Muscle crude lipid content of fish fed $Cu_{1.0}$ and $CuS_{6.0}$ diets were significantly higher than those of fish fed CuM_{15}, CuM_{19}, CuM_{42}, CuM_{86}, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets. However, there were no significant differences in muscle lipid content among fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10}, $CuS_{6.0}$ and CuS_{10} diets, among those fed $CuM_{6.0}$, CuM_{10}, CuM_{15}, CuS_{10} and CuS_{15} diets. There were no significant differences in whole body moisture and muscle protein content of fish fed all the experimental diets.

Enzyme Activity and Hepatosomatic Index

Hepatic thiobarbituric acid reactive substances (TBARS), copper-zinc superoxide dismutase (Cu-Zn SOD) activity and hepatosomatic index (HSI) are shown in Table 17 and 18. All parameters were significantly affected by the copper sources and levels. The decrease in hepatic thiobarbituric acid reactive substances (TBARS) and hepatosomatic index (%HSI), but increased Cu-Zn superoxide dismutase (Cu-Zn SOD) activity became statistically significant in fish fed copper sources after 14 weeks of dietary Cu exposure, also results indicates that organic Cu showed better physiological responses and more effective than did inorganic Cu. TBARS were significantly lower while Cu-Zn SOD activities were significantly higher in liver tissue of fish fed 10 mg Cu/Kg diet from either organic or inorganic copper sources than those of fish fed 1, 15, 19, 42 and 86 mg Cu/Kg diets. %HSI was negatively correlated with dietary copper concentrations. The lowest %HSI was obtained with the diet containing 86 mg Cu/Kg diets.

Hepatic TBARS of fish fed CuM_{10} diet were significantly lower than those of fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{19}, CuM_{42}, CuM_{86}, $CuS_{6.0}$, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets ($P < 0.05$). Hepatic TBARS of fish fed CuS_{86} diet was significantly higher than those of fish fed other diets except fish fed CuM_{86} diets. However, there were no significant differences in hepatic TBARS among fish fed CuM_{10}, CuM_{15} and CuS_{10} diets, or among those fed $Cu_{1.0}$, CuM_{19}, $CuS_{6.0}$ and CuS_{19} diets.

Hepatic Cu-Zn SOD activity of fish fed CuM_{10} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{15}, CuM_{19}, CuM_{42}, CuM_{86}, $CuS_{6.0}$, CuS_{10}, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets. Also, hepatic Cu-Zn SOD of fish fed CuM_{15} and CuS_{10} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{19}, CuM_{42}, CuM_{86}, $CuS_{6.0}$, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets. Hepatosomatic index of fish fed CuS_{86} was significantly lower than those of fish fed the other diets, except fish fed CuM_{86} and CuS_{42} diets. There were no significant differences in %HSI among fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10}, CuM_{15}, CuM_{42}, $CuS_{6.0}$, CuS_{10} and CuS_{15} diets or among those fed $CuM_{6.0}$, CuM_{10}, CuM_{19}, CuM_{42}, $CuS_{6.0}$, CuS_{10}, CuS_{15}, CuS_{19} and CuM_{42} diets.

Hematological Parameters

Hematological characteristics of fish fed the experimental diets are shown in Table 19 and 20. At the end of the experimental period, there were significant copper sources and levels effects ($P < 0.05$) on red blood cell (RBC), hematocrit (packed cell volume, PCV), hemoglobin (Hb) and white blood cell (WBC) count. Furthermore, there were significant their interaction effects on RBC and Hb for fish fed all diets. Red blood cell (RBC) counts of fish fed CuM_{10} and CuM_{15} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{19}, CuM_{42}, CuM_{86}, $CuS_{6.0}$, CuS_{10}, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets ($P < 0.05$). Red blood cells of fish fed CuM_{19} diet was significantly higher than those of fish fed CuM_{42}, CuM_{86}, CuS_{10}, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets. There were no significant differences in RBC among fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{19} and $CuS_{6.0}$ diets, among fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{42}, $CuS_{6.0}$ and CuS_{10} diets or among those fed CuM_{86}, CuS_{19}, CuS_{42} and CuS_{86} diets.

Hematocrit (packed cell volume, PCV) of fish fed CuM_{10} diet was significantly higher than those of fish fed $Cu_{1.0}$, CuM_{42}, CuM_{86}, CuS_{42} and CuS_{86} diets. Also, PCV of fish fed CuS_{86} diet was significantly lower than that of fish fed $CuM_{6.0}$, CuM_{10}, CuM_{15}, CuM_{19}, CuM_{42}, $CuS_{6.0}$, CuS_{10}, CuS_{15} and CuS_{19} diets. There were no significant differences in PCV among fish fed $CuM_{6.0}$, CuM_{10}, CuM_{15}, CuM_{19}, $CuS_{6.0}$, CuS_{10}, CuS_{15} and CuS_{19} diets, among fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{15}, CuM_{19}, CuM_{42}, $CuS_{6.0}$, CuS_{10}, CuS_{15} and CuS_{19} diets or among those fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{19}, CuM_{42}, CuS_{10}, CuS_{15}, CuS_{19} and CuS_{42} diets.

Hemoglobin (Hb) of fish fed CuM_{10} diet was significantly higher than those of fish fed CuM_{15}, CuM_{19}, CuM_{42}, CuM_{86}, $CuS_{6.0}$, CuS_{10}, CuS_{15}, CuS_{19}, CuS_{42} and CuM_{86} diets. Conversely, Hb of fish fed CuS_{86} diet was significantly lower than those of fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10}, CuM_{15} and $CuS_{6.0}$ diets. There were no significant differences in this parameter among fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{15} CuM_{19}, CuM_{42}, $CuS_{6.0}$ and CuS_{10} diets, among fish fed CuM_{15}, CuM_{19}, CuM_{42}, CuM_{86}, $CuS_{6.0}$, CuS_{10}, CuS_{15}, CuS_{19} and CuS_{42} diets or among those fed CuM_{19}, CuM_{42}, CuM_{86}, CuS_{10}, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets. White blood cell (WBC) count increased with dietary Cu supplementation. White blood cell count of fish fed CuS_{86} was significantly higher than those of fish fed the other diets, except fish fed CuM_{86} diet. However, there were no significant differences in WBC between fish fed CuM_{86} and CuS_{42} diets, among fish fed CuM_{19}, CuM_{42}, CuS_{15} and CuS_{19} diets or among fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10} and $CuS_{6.0}$ diets. Mean corpuscular volume (MCV) of fish fed CuS_{19} was significantly higher than those of fish fed $Cu_{1.0}$, CuM_{10} and CuM_{15} diets. There were no significant differences in MCV among fish fed $CuM_{6.0}$, CuM_{19}, CuM_{42}, CuM_{86}, $CuS_{6.0}$, CuS_{10}, CuS_{15}, CuS_{19}, CuS_{42} and CuS_{86} diets or among fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{19}, CuM_{42}, CuM_{86}, $CuS_{6.0}$, CuS_{10}, CuS_{15}, CuS_{42} and CuS_{86} diets. There were no significant differences in mean corpuscular hemoglobin (MCH) of fish in all treatments except for MCH of fish fed $Cu_{1.0}$ and $CuM_{6.0}$ which was significantly higher than those of fish fed CuM_{15} diet. Fish fed increasing either of the dietary copper sources, had lower numeric values of mean corpuscular hemoglobin concentration (MCHC) than control diet, also there were no significant differences in MCHC in all treatments except for MCHC of fish fed $Cu_{1.0}$ which was significantly higher than those of fish fed CuS_{19} diets.

Copper Concentration

Table 21 and 22 shows the whole-body and tissue Cu concentrations of fish fed the experimental diets. There were significant copper sources effects on whole body and tissues (liver, intestine, kidney, gill and muscle) copper concentration. There were also significant copper levels effects on whole body and tissues Cu content. Furthermore, there were significant copper sources and levels interaction effects on whole body, liver, intestine, gill and muscle copper concentration. However, there were no significant their interaction effects on kidney Cu content for fish fed all diets.

Copper content in the liver of fish fed by from either dietary organic or inorganic copper sources showed a statistically significant rise, also, the results showed that hepatic and intestine Cu accumulations rate were higher in fish fed diets with inorganic Cu than in fish fed diets with organic copper. Copper concentrations in gill, liver, kidney, intestine, muscle and whole-body of fish fed the experimental diets for 14 weeks were generally dose-dependent, increasing with increase in dietary copper concentration. Liver of olive flounder is a more important storage tissue than other tissues, and the order of Cu accumulation in tissues was liver> intestine> kidney> gill> muscle.

Whole-body, gill, liver and intestine Cu concentrations of fish fed CuS_{86} diet were significantly higher than those of fish fed the other diets ($P < 0.05$). Liver Cu concentration of fish fed CuM_{86} diet was significantly higher than those of fish fed other diets, except fish fed CuS_{42} diet. Conversely, liver Cu concentration of fish fed $Cu_{1.0}$, $CuM_{6.0}$ and $CuS_{6.0}$ diets were significantly lower than those of fish fed other diets. Intestine Cu concentration of fish fed CuM_{42} and CuS_{86} diets were also significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10}, CuM_{15}, CuM_{19}, CuM_{42}, $CuS_{6.0}$, CuS_{10}, CuS_{15} and CuS_{19} diets. There were no significant differences in intestine Cu concentration among fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10}, $CuS_{6.0}$ and CuS_{10} diets, among fish fed CuM_{10}, CuM_{15}, $CuS_{6.0}$ and CuS_{10} diets or among those fed CuM_{10}, CuM_{15}, CuM_{19}, CuS_{10} and CuS_{15} diets.

Kidney Cu concentration of fish fed CuM_{86} and CuS_{86} diets were significantly higher than those of fish fed other diets. Copper content of kidney tissue of fish fed CuS_{42} was significantly higher than those of fish fed containing <42 mg/Cu kg diet of both copper sources. There were no significant differences in this parameter among fish fed $Cu_{1.0}$, $CuM_{6.0}$ and $CuS_{6.0}$ diets, or among those fed $CuM_{6.0}$, CuM_{10}, $CuS_{6.0}$ and CuS_{10} diets.

Gill Cu concentration of fish fed CuS_{86} diets was significantly higher than those of fish fed other diets. Gill Cu content of fish fed CuM_{86} and CuS_{42} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10}, CuM_{15}, CuM_{19}, CuM_{42}, $CuS_{6.0}$ and CuS_{10} diets. There were no significant differences in Cu concentration of gill among fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10} and $CuS_{6.0}$ diets or among those fed CuM_{10}, CuM_{15}, $CuS_{6.0}$ and CuS_{10} diets. Muscle Cu concentration of fish fed CuS_{86} diet was significantly higher than those of fish fed the other diets, except fish fed CuM_{82} diet.

There were no significant differences in muscle Cu concentration among fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10}, $CuS_{6.0}$ and CuS_{10} diets, among fish fed CuM_{10}, CuM_{15}, $CuS_{6.0}$ and CuS_{10} diets, among fish fed CuM_{15}, CuM_{19}, CuS_{10} and CuS_{15} diets or among those fed CuM_{42}, CuM_{86} and CuS_{42} diets. Although there were no significant differences among fish fed 5-10 mg Mintrex®Cu and copper sulfate/kg diet in the 14th week and remained relatively stable, there was a trend toward increased whole body copper with copper supplementation up to 15 mg/kg diet in fish fed organic and inorganic copper; beyond this level whole body copper significantly increased.

Copper content of whole body of fish fed CuS_{86} was significantly higher than those of fish fed other diets. Also, whole body Cu concentration of fish fed CuS_{42} was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10}, CuM_{15}, CuM_{19}, CuM_{42}, CuM_{86}, $CuS_{6.0}$, CuS_{10}, CuS_{15} and CuS_{19} diets. There were no significant differences in whole body Cu content among fish fed $Cu_{1.0}$, $CuM_{6.0}$, CuM_{10} and $CuS_{6.0}$ diets, among fish fed $CuM_{6.0}$, CuM_{10}, $CuS_{6.0}$ and CuS_{10} diets, or among those fed CuM_{42}, CuM_{86} and CuS_{19} diets.

A non significant increase in total Cu concentrations was observed in the water from tanks in which fish were fed organic and inorganic Cu supplemented diets compared to the tanks in which fish were fed the control diet. Waterborne Cu level was less than 0.82 μg/L at the beginning and increased to 1.5 μg/L at the end of the feeding trial.

Discussion

This study is the first report on the optimum dietary copper levels in olive flounder. Copper concentration in the rearing water was within the range in the wild. Although Cu concentrations in tanks of fish fed the copper-supplemented diets were higher than that in the tanks of fish fed the control diet, the differences were not statistically significant. It is therefore unlikely that the leaching of Cu from feed and/or faeces to water had an effect on growth or whole-body Cu contents in the present study. Berntssen et al (1999) and Lin et al. (2008) did not find significant differences in waterborne copper in Atlantic salmon, *Salmo salar*, fry and juvenile grouper, *Epinephelus malabaricus*, reared on 5-1750 mg Cu/kg diet for 3 months and 0.11-20.05 mg Cu/kg diet for 8 weeks, respectively. In the present study, growth performance improved with dietary copper supplementation up to a peak value and dropped beyond that of both copper sources. Satoh et al. (1983) and Shao et al. (2010) reported common carp and crucian carp, *Carassius auratus gibelio,* fed diets based and un-supplemented with copper had reduced growth. It was obvious that the WG and SGR were higher in fish fed Mintrex®Cu supplemented diet with 10.1 mg Cu/kg diet than those in fish fed other diets, except fish fed CuM_{15} diet ($P < 0.05$). Highest WG of fish fed Mintrex®Cu obtained (302%) compares fish fed copper sulfate ($CuSO_4.5H_2O$), in which 285% WG was observed in similar sized fish fed a nutritionally adequate diet for 14 weeks. This finding indicates Mintrex®Cu has the ability to improve performance in olive flounder over copper sulfate ($CuSO_4.5H_2O$) supplementation due to its better absorption. Apines-Amar et al. (2003) and Apines-Amar et al. (2004) indicated that trace elements chelated with amino acids, which include Cu, zinc (Zn) and manganese (Mn) seem to be more available for mineral deposition than those from inorganic compounds in rainbow trout. It should be noted that in the two rainbow trout studies the authors were able to ignore the interaction of Cu with other trace elements, i.e. Zn and Mn. This is because organic minerals are generally considered less sensitive to the inhibitory action of other compounds (e.g., phytate and fiber) due to the different absorption pathway (Ashmead 1992; Lin et al. 2010). Another potential benefit of stable chelated organic minerals for aquatic species is reduced solubility in water (Guo et al. 2001).

However, growth performance of fish fed organic Cu was significantly higher than fish fed inorganic copper sources. In olive flounder studies, however, growth was depressed in fish fed diets with ≥19 mg Cu/kg (~2.3× adequate) of fish organic Cu and ≥15 mg Cu/kg (~1.7× adequate) of fish inorganic Cu sources. Also, these differential levels required for growth reduction suggest that olive flounder have a lower toxicity threshold for inorganic than organic copper.

In this study, the highest inorganic copper concentrations (CuS_{86}) in feed markedly decreased feed efficiency. Similarly, high levels of inorganic copper depressed growth and impaired feed conversion in channel catfish and yellow cat fish, *Pelteobagrus fulvidraco* (Murai et al. 1981; Tan et al. 2011). Moreover, Lundebye et al. (1999) suggested that decreased growth in Atlantic salmon due to increased metabolic costs of intestinal cellular changes and Cu excretion. The physiological changes permitting metal detoxification and homeostasis cost energy and reduced growth caused by exposure to Cu has been attributed to metabolic costs associated with metal detoxification (Marr et al. 1996). In the case of olive flounder, the decreased growth rate and feed efficiency are probably due to an increased expenditure of energy for sustaining normal metabolism, leaving less energy available for growth. It is possible that high dietary Cu concentration enhance growth of olive flounder by stimulating activities of the enzymes involved in nutrient utilization. Olive flounder fed Cu-deficient or excess dietary Cu showed reduced PER of Cu exposure. Reduced PER has been seen in both aquatic and terrestrial animals fed either Cu-deficient or excess Cu diets (Koh et al. 1996; Tan et al. 2011). This phenomenon was also observed in our study. Overall, there was no effect of copper source or levels on mortality. This is in agreement with Berntssen et al (1999) who also did not observe significant mortality in Atlantic salmon (*Salmo salar* L.) reared on diets containing high copper.

In this study, dietary copper source or dietary copper concentration significantly influenced body composition of olive flounder. Whole body protein and lipid content negatively correlated with dietary copper. Tissue deposition of lipid, protein and copper is dependent on feed intake, metabolic use and intestinal absorption, and these factors can all be influenced by elevated dietary Cu concentrations (Berntssen et al. 1999). It was observed that the muscle crude lipid in olive flounder was significantly reduced in response to increasing dietary copper, in which fish were fed with the Mintrex®Cu (≥42 mg Cu/kg) and copper sulfate (≥19 mg Cu/kg) supplemented diets.

Zhao et al. (2011) reported that tilapia juvenile, *Oreochromis niloticus* × *O. aureus,* carcass composition was influenced by various levels of dietary zinc sources, zinc sulphate ($ZnSO_4$) or zinc methionine (ZnMet), indicating that Zn may participate in the synthesis and degradation of proteins and lipids. Inadequate zinc supply may also result in the impaired digestibility of protein and carbohydrates, increased moisture content, and depressed protein and lipid concentrations (Satoh et al. 1983).

Our data suggest that the fish fed with diets supplemented with copper sulfate seemed to more easily decrease whole-body lipid content than the fish fed with diets supplemented with Mintrex®Cu. Cheng et al. (2008) reported that dietary copper supplementation, regardless of source and level, significantly reduced rib backfat and kidney fat in lambs. The decrease in backfat depth by dietary copper supplementation was also observed in goat kids (Solaiman et al. 2006) and finishing steers (Engle and Spears 2000_b). However, there is no similar report in fish, so it would be interesting to study the relationship between dietary copper and lipid metabolism in fish. In general, body fat accumulation may be considered the net result of balance among dietary absorbed fat, endogenous fat synthesis (lipogenesis) and fat catabolism via β-oxidation (lipolysis). Lower body fat deposition may be attributed to increased fat catabolism and diminished endogenous fatty acid synthesis or to both processes. Effect of Cu on abdominal fat content can be explained by the way that Cu may alter lipogenesis or lipolysis in the body and decreased abdominal fat (Mondal et al. 2007).

Copper has a number of biological functions in animals including fish (Fattman et al. 2003). Copper is central to many enzymatic reactions, oxidative stress caused by hepatic Cu accumulation or deficiency must also be considered in order to determine optimal dietary levels of Cu. Ceruloplasmin is the major copper transport protein to deliver copper to cells and is an enzyme which possesses oxidase activity (Lin et al. 2008). To protect against oxidative damage, organisms have developed a variety of antioxidant defenses that include metal sequestering proteins, by using compounds such as vitamin C and E, and specialized antioxidant enzymes (Shao et al. 2010). Depressed hepatic Cu-Zn SOD activity in olive flounder was observed when the dietary Cu was insufficient (<6 mg Cu/kg diet) and in excess (19 mg Cu/kg diet) regardless of copper sources.

The results of Mintrex®Cu in this study also showed higher Cu-Zn SOD activity in liver of olive flounder as compared with the copper sulfate sources. Arthington et al. (2003) reported that plasma ceruloplasmin concentrations were higher ($P < 0.05$) for copper-supplemented heifers, independent of copper source, when heifers were fed with molasses-based diets. Additionally, the change in plasma ceruloplasmin tended ($P = 0.08$) to be greater for heifers fed with tribasic Cu chloride (TBCC) supplemented corn-based diets (Arthington and Spears 2007). Gatlin and Wilson (1986) reported that liver Cu-Zn SOD in channel catfish were reduced when dietary copper level reached 8 mg/kg, but recovered when copper level increased to 10 mg/kg, and similar results were found in grouper (Lin et al. 2008). The hepatic TBARS values showed an inverse trend with the performance of hepatic Cu-Zn SOD activity. The high hepatic TBARS values in fish fed the Cu-deficient and excess Cu diet than that of fish fed adequate copper independent of copper source, might be due to low activity of Cu-Zn SOD.

Reports for fish and shrimp have shown a positive correlation between liver Cu content and HSI (Lee and Shiau 2002; Shaw and Handy 2006; Tan et al. 2011). However, our study contradicted these findings. In this study, fish fed the Cu supplementation up to 86 mg/kg from Mintrex®Cu or 42 mg/kg from copper sulfate, possessed significantly lower HSI% clearly demonstrated an adverse effect of Cu on olive flounder at these concentrations. Duration of the trials could also be a discrepancy factor, as the olive flounder in the present study were fed diets for 14 weeks, which is about twice that of the studies on shrimp (Lee and Shiau 2002) and Nile tilapia, *Oreochromis niloticus* (Shaw and Handy 2006). It is possible that olive flounder livers shrink after long-term exposure to high dietary Cu. However, the results in our study are similar to those in soft-shelled turtle, *Pelodiscus sinensis* (Wu & Huang 2008). In fact, in the trial, reduced HSI may be a result of either reduced feed intake or increased metabolic expenditure for detoxification and maintenance of homeostasis among these treatments.

Generally, Blood parameters of fish are suitable biomarkers for evaluating the potential risk of chemicals (Roche & Boge 1996). Past investigators have also identified changes in several hematological variables as indicators of metal exposure (Cyriac et al. 1989). However, the present findings indicate that in the olive flounder, sub-chronic dietary exposure to Cu has significant effect on blood parameters and serum chemistry.

Red blood cell, hematocrit and hemoglobin all increased with dietary Cu up to the supplementation level of 10 mg/kg diet and dropped, with either of the dietary copper source. Furthermore, fish fed diet supplemented with copper sulfate (inorganic Cu, i.e. $CuSO_4$) showed lower value of RBC, PCV and Hb compared to organic Cu sources (Mintrex®Cu). Haemoglobin and PCV ranges reported here for olive flounder (10-13 g/dl and 18-24%, respectively) are similar to previous reports for olive flounder (Choi et al. 2003). Shiau and Ning (2003) give values for Hb and PCV ranging from 5.2 to 6.3 g/dl and 26-36%, respectively for Cu diets containing 1-5 mg Cu/kg feed. Copper is associated with the oxygen-carrying protein, haemocyanin, and dietary Cu deficiency has been shown to cause anemia in hybrid tilapia and grouper (Shiau and Ning 2003; Lin et al. 2008). Furthermore, low levels of hemoglobin content and total count of RBCs in rabbit fish, *Siganus rivulatus*, were attributed to water pollution by heavy metals (Bin-Dohaish 2008). Altwood et al. (2003) give PCV in the range of 23-30% depending on diet formulation and water temperature. The time-dependent increase in PCV from 20.2% in initial fish to about 23.6% after 14 weeks in this study, regardless of Cu exposure and without anaemia, is typical of normal growth effects on haematology in fish and has been reported during dietary Cu studies in rainbow trout (Handy et al. 1999). Gatlin and Wilson (1986) showed that Hb, PCV and RBC values were not affected in catfish fed 40 mg/kg dietary copper. Furthermore, Handy et al. (1999) examined blood chemistry in rainbow trout exposed to dietary copper and found no significant change in PCV, Hb, RBCs and serum mineral. Generally, metal exposure can result in gill damage, which in turn can affect blood parameters (Pelgrom et al. 1995).

Our results evidenced that organic forms of trace minerals improve production and health responses of dairy olive flounder relative to inorganic trace minerals (according the blood parameters as well as enzyme assay). Supplementation of Cu during times of oxidative stress may reduce oxidative damage to white blood cells (WBC) and increase disease resistance. Trace mineral supplementation may reduce oxidative damage to cells and metabolites. Free radical causes to increased disease susceptibility during times of increased oxidative stress, such as occurs around calving (Miller et al. 1993).

White blood cells are particularly sensitive to oxidative damage due to high concentration of unsaturated fatty acids (Spears and Weiss 2008). In present study, fish fed diets supplemented with copper sulfate showed highest WBC compared to Mintrex®Cu. Epidemiological and disease challenge studies also suggest that trace mineral supplementation may improve disease resistance.

The profile of Cu distribution in olive flounder is dependent on the exposure periods and Cu concentrations. In line with other reports (Berntssen et al. 1999; Shiau and Ning 2003), Cu concentration in olive flounder tissues was found to be positively correlated with dietary Cu level. In the present study, the fact that olive flounder had high deposition and accumulation of copper in tissues and good growth performance with high survival indicates that juvenile olive flounder have a tolerance to dietary copper at least not less than 40 mg/kg diet. There were significant effects of copper source, concentration and their interaction on day 42 and 84 days on body composition as well as whole body and tissues copper concentration, suggesting that olive flounder could accumulate excess Cu in tissues.

The Cu accumulation in olive flounder also reflected the route of exposure with large increases in the Cu content of the liver and intestine is consistent with previous studies on temperate species such as rainbow trout (Handy 1992; Handy et al. 1999; Kamunde et al. 2001; Campbell et al. 2002; Kamunde and Wood 2003), Atlantic salmon (Berntssen et al. 1999; Lundebye et al. 1999), and even marine teleosts such as grey mullet (Baker et al. 1998). In the present experiment, intestine copper concentrations were increased with elevated dietary copper, independent of copper source. Also, there was a trend that fish fed diets with Mintrex®Cu had relatively lower liver and intestine copper concentrations than those fed diets ≥ 20 mg/kg with equal levels of copper from copper-sulfate. High levels of Cu accumulation in the liver might cause toxic oxidative stress for fish (Lin and Shiau 2007). Also, these results indicate that the intestine can play an important role in regulating the uptake of dietary Cu in some species of fish, but this varies between species. Of these, liver copper concentration is regarded as the most sensitive in evaluating copper status (Apines-Amar et al. 2003; Shao et al. 2010). Lin et al. (2010) indicated that Cu chelated with amino acids, hepatic Cu accumulation rate was higher in juvenile grouper, *Epinephelus malabaricus*, fed diets with inorganic Cu than in fish fed diets with organic Cu.

This phenomenon was also reported in pig (Coffey et al. 1994), cow (Du et al. 1996) and ewe (Eckert et al. 1999). In contrast, Apines-Amar et al. (2004) reported that bone ($P < 0.01$) and liver ($P < 0.05$) copper contents were higher in rainbow trout fed with amino acid chelate trace elements than fish fed with trace elements from inorganic salts. This lower accumulation rate of Cu in the liver in organic Cu-fed fish also provides additional evidence for why organic Cu had a higher toxicity threshold for olive flounder than inorganic Cu in this study. The different metabolic pathways of organic and inorganic Cu, however, might influence the Cu storage site (Wang and Lovell 1997).

Mintrex®Cu shows the same pattern as copper sulfate in increasing tissues copper content in our study. However, no significant differences were observed in Cu concentrations in fish fed equal levels of copper from Mintrex®Cu or copper-sulfate in other tissues like muscle, gill and kidney. Similar patterns of Cu accumulation were also shown in other studies carried out with aquatic animals (Grosell et al. 2002; Kim and Kang 2004). Kegley and Spears (1994) also reported that Cu status of calves fed Cu-sulphate did not differ from calves fed Cu-lysine. Maage and Julshamn (1993) suggested that trace element contents in salmon fillets were not increased by dietary supplementation, while Lorentzen et al. (1998) found that muscles could be enriched with selenium if given in a protein-bound form.

In conclusion, this study indicates that dose and source of Cu-salt had an important role in growth, body composition, enzyme activities and copper concentration in plasma and tissues of olive flounder, *paralichthys olivaceus*. Organic Cu supproted better performance, increased nutrient utilization, improved physiological responses and greater effectiveness than inorganic Cu. Use of organic copper will have advantages environmentally because less copper will go into the aquacultural system. Broken-line regression analysis of WG suggested that the optimum dietary Cu levels in juvenile olive flounder, *P. olivaceus*, could be 8.44 mg/kg diet and 9.05 mg/kg diet in the form of organic Cu and inorganic Cu, respectively.

Chapter 3:

Evaluation of the optimum dietary organic and inorganic copper levels in juvenile beluga sturgeon, *Huso huso*

Experiment III. The optimum dietary organic copper level in juvenile beluga sturgeon, *Huso huso*

Abastract

A 12-week feeding trial was conducted to evaluate the optimum dietary organic copper (Mintrex®Cu) levels in juvenile beluga sturgeon, *Huso huso*. Eight semi-purified diets containing 1.1 ($Cu_{1.0}$), 3.6 ($CuM_{4.0}$), 6.8 ($CuM_{7.0}$), 9.5 (CuM_{10}), 12.8 (CuM_{13}), 24.9 (CuM_{25}), 49.3 (CuM_{49}) and 193.5 (CuM_{194}) mg Cu/kg diet in the form of Mintrex®Cu were fed to fish of initial body weight 2.43 ± 0.06 g and length 7.82 ± 0.68 cm (mean ± SD) in triplicate groups in a flow-through system. Weight gain (WG) and specific growth rate (SGR) of fish fed CuM_{10} and CuM_{13} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, CuM_{25}, CuM_{49} and CuM_{194} diets ($P < 0.05$). The lowest WG, SGR, feed efficiency (FE) and protein efficiency ratio (PER) were observed in fish fed CuM_{194} diet. Crude protein in muscle and whole-body peaked at the dietary Cu supplementation level of 9.5 mg/kg diet and dropped at higer levels. Whole-body crude lipid of fish fed ≤24.9 was also significantly higher than those of fish fed ≥49.3 mg/kg diet. Thiobarbituric acid-reactive substances (TBARS) was significantly higher in liver tissue of fish fed $CuM_{7.0}$, CuM_{10} and CuM_{13} diets than those of fish fed $Cu_{1.0}$, $CuM_{3.0}$, CuM_{25}, CuM_{49} and CuM_{194} diets. Liver copper-zinc superoxide dismutase activity of fish fed CuM_{10} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{3.0}$, CuM_{49} and CuM_{194} diets. Muscle Cu-Zn SOD of fish fed CuM_{10} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, CuM_{25}, CuM_{49} and CuM_{194} diets. Glutamic oxaloacetic transaminase (GOT), Glutamic pyruvic transaminase (GPT) and Cu accumulation in tissues increased with dietary copper. While, lysozyme activity peaked at the dietary Cu supplementation level of 9.5 mg/kg diet and dropped at higer levels. Broken-line analysis of WG suggested that the optimum dietary Cu level was 9.71 mg Cu/kg. Therefore, these results may indicate that the optimum dietary Cu level could be higher than 9.71 mg Cu/kg diet but less than 13 mg Cu/kg diet in juvenile beluga sturgeon, when Mintrex®Cu is used as the dietary source of organic copper.

Introduction

Trace minerals are an essential component of fish nutrition which lags in inquiry when compared to research devoted to other nutrients. Although fish can absorb minerals across the gill membrane and intestinal mucosa, diet is considered to be the major source of minerals for fish (Watanabe et al. 1997). Copper functions in hematopoiesis and numerous copper dependent enzymes including lysyl oxidase, cytochrome c oxidase, ferroxidase, tyrosinase (O'Dell 1976). It is also important as a part of antioxidant enzymes (e.g. Cu-Zn SOD) (Lorentzen et al. 1998). Although dietary copper requirements have been studied in many aquatic species, i.e. common carp, (Ogino and Yang 1980), rainbow trout, *Oncorhynchus mykiss* (Julshamn et al. 1988), hybrid tilapia (Shiau and Ning 2003), channel catfish, *Ictalurus punctatus* (Murai et al. 1981; Gatlin and Wilson 1986), Atlantic salmon, *Salmo salar* L. (Lorentzen et al. 1998), grass shrimp, *Penaeus monodon* (Lee and Shiau 2002), grouper, *Epinephelus malabaricus* (Lin et al. 2008; 2010), soft-shelled turtle, *Pelodiscus sinensis* (Wu and Huang 2008), yellow catfish, *Pelteobagrus fulvidraco* (Tan et al. 2011) and abalone, *Haliotis discus hannai Ino* (Wang et al. 2009) there are no studies on dietary copper requirements in beluga sturgeon, *Huso huso*. The most commonly used Cu for supplementation in animal diets is inorganic Cu in the form of Cu-sulphate pentahydrate ($CuSO_4.5H_2O$) due to cost and commercial availability. Chelated forms of various elements have also been found to be effective for some aquatic animals (Paripatananont and Lovell 1995; 1997; Apines-Amar et al. 2004). Chick (Guo et al. 2001; Mondal et al. 2007) and lamb (Eckert et al. 1999; Senthilkumar et al. 2009) have been reported to utilize organic Cu better than inorganic Cu, which was evident from tissue storage or plasma ceruloplasmin activity. Pork (Coffey et al. 1994) and cattle (Kegley and Spears 1994; Ward et al. 1996; Du et al. 1996), however, were reported as having similar utilization of both forms of Cu.

In recent years the intensive culture of certain sturgeon species has been developed as an alternative to other more traditional fish species such as salmonids and cyprinids. The Beluga sturgeon, *Huso huso*, is an increasingly important aquaculture species in Russia, Eastern Europe, Turkey, Japan and Iran because of the dwindling natural sources for its caviar and meat. There is a lack of research with regard to the utilization of organic Cu in sturgeon fish. The purpose of the present study was to estimate the optimum dietary Cu level for beluga using an organic copper source.

Materials and Methods

Diet Formulation and Preparation

Proximate composition of the basal diet used in this feeding trial is shown in Table 23. Eight semi-purified diets were formulated and prepared to contain 0, 3, 6, 9, 12, 24, 48 and 192 mg Cu/kg diet using Mintrex®Cu, a chelate of copper and 2-hydroxy-4-methylthiobutanoic acid, an organic form of Cu, as the Cu source. However, the actual copper concentrations as determined by analysis were 1.1, 3.6, 6.8, 9.5, 12.8, 24.9, 49.3 and 193.5 mg Cu/kg diet for $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25}, CuM_{49} and CuM_{194} diets, respectively. The premixture containing the Cu source was added to the diet to replace the equal amount of cellulose in the diet. Vitamin-free Casein (United States Biochemical, Cleveland, OH, USA) was used as the main dietary protein sources; equal ratio of fish oil (refined fish oil, Khazar oil Co. Ltd., Anzali, Iran), plus corn oil (1: 1) as the lipid source; wheat flour, dextrin, and corn starch (United States Biochemical, Cleveland, OH, USA) as the carbohydrate sources, respectively. Anchovy fish meal was added at 5% to all diets to increase palatability and acceptance of the experimental diets. Diets were formulated to be similar to a sturgeon semi-purified diet with 12.9% crude lipid, 8.8% ash and 43.4% crude protein (Mohseni et al. 2005 & 2006). Diets were prepared by mixing the dry ingredients in an electric mixer, followed by the addition of oil and distilled water. This mixture was formed into dough. Finally, the last mixture was passed through a screw extrusion press with a CPM meat grinder (California Pellet Mill Co., San Francisco, CA, USA) using a 4 mm diameter module (Biomar Commercial Co., French). Pellets were dried in an air-convection drier at 30^{o}C (until the moisture content was reduced to less than 10%). After processing, all the diets were broken up and sieved into the appropriate pellet size (4 mm diameter) and packed into small bags and stored at -24°C until used.

Experimental Fish and Feeding Trial

The feeding trial was carried out at the Nutrition Research Department at International Sturgeon Research Institute, Guilan-Iran. Fish were transported from wild spawners reproduced artificially at the Shahid Behashty Hatchery, Guilan-Iran to the Institute and acclimated to the experimental conditions for two weeks before the feeding trial began. Prior to the start of the feeding trial, fish were fed with the basal diet for 10 days to adjust to the semi-purified (copper-free) diet to deplete the body Cu reserve.

At the beginning of the feeding trial, fish were fasted for 18 h and anaesthetized in clove powder, *Syzygium aromaticum* (200 mg/L). A total of 360 fingerling fish averaging initial weight and length 2.43 ± 0.06 g and 7.65 ± 0.63 cm (mean ± SD) respectively, were carefully selected from the stock tanks. Fifteen fish were randomly selected and weighed individually into each of 24 circular fiberglass tanks (diameter: 105 cm; height: 51 cm) in a flow-through system. Each tank was then randomly assigned to one of three replicates of the 8 diets. Fish were fed (Mohseni et al. 2003 & 2006) one of the experimental diets four times daily (0200, 0800, 1400 and 2000 h) at approximately 2.5% of wet body weight per day at the beginning and 2% wet body weight per day at the end of the feeding trial). The feed was defrosted at room temperature one hour before feeding, weighed and distributed in the tanks. The amounts of diets fed were adjusted in the fourth week of the experiment; and the duration of the feeding trial was 12 weeks. Fish in each tank were anesthetized, counted and weighed individually every two weeks. Fish were not fed on the day of weighing to avoid inclusion of ingested feed in the weight measurement as well as to reduce stress. The tanks were thoroughly cleaned during the time fish were removed for weighing to minimize algae and fungal growth. Feeding was done slowly and fish ingested the ration within 2-4 minutes after distribution into the tanks care was taken to ensure that no uneaten food remained in the tanks during feeding, thus leaching of Cu into water was negligible. Water was supplied from Sepidroud River and the water flow rate through the tanks was 4.75 l/min. Oxygen concentration was 7.8 ± 0.09 mg/l, average water temperature was 21.5 ± 0.6^{o}C, pH 7.3± 0.05, CO_2 6.1 mg/l, alkalinity 157.7 mg/l, total hardness 360-400 mg/l and NH_4 0.1 mg/l and a natural photoperiod (approximately, 12L: 12D) was in effect throughout the experimental period.

Sample Collection and Analysis

At the beginning of the experiment, a pooled sample of 15 fish was taken for determining whole-body proximate composition and copper content. At the termination of the feeding trial, fish were starved for 24 h and the total number and weight of fish in each tank was determined for calculation of weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER), condition factor (CF) and survival. After obtaining the final total weight of fish in each tank, three or four fish per tank were randomly selected, dissected to obtain liver and white muscle samples and hepatosomatic index (HSI) were calculated.

Three fish were randomly sampled from each tank, then liver and muscle were removed from each fish and pooled for determining Cu-Zn superoxide dismutase (Cu-Zn SOD) activity, glutathione peroxidase (GPx) and thiobarbituric acid reaction substances (TBARS) levels. Three other fish were selected from each tank and frozen at -63ºC for proximate composition and whole body Cu concentration analyses. Proximate composition analyses of experimental diets and fish body were performed by the standard methods of AOAC (1995). Samples of diets and fish were dried to a constant weight at 105°C to determine moisture content. Ash was determined by incineration at 550°C; crude lipid by soxhlet extraction using Soxtec system 1046 (Foss, Hoganas, Sweden) and crude protein by Kjeldahl method (N × 6.25) after acid digestion.

Blood samples were obtained from the caudal vessels of three randomly selected fish per tank by using a heparinized syringe and pooled to evaluate immunophysiological indices. The extracted blood was divided in two sets of Eppendorf tubes. One set contained heparin for hematology studies and the other one (non-heparinized) were centrifuged at 3000 rpm for 10 min in order to measure biochemical and immune indices. All sera were stored at -80^{o}C until analyzed. Before the blood samplings, fish were starved for 24 h. Hematocrit (PCV) values were determined using microhematocrit heparinized capillary tubes. The amount of hemoglobin (Hb) was measured according to the cyanmethemoglobin method. White blood cell (WBC) was counted in a Neubauer hemocytometer. To estimate the differential leucocyte count, blood smeare were prepared, air-dried; fixed in methanol and stained using Giemsa (Merck, Germany) (Zorriehzahra et al. 2010). Leucocytes in blood smears were categorized into lymphocytes, neutrophils, eosinophils and monocytes. The remaining blood was centrifuged (6000 rpm for 10 min at 4°C), and the serum was frozen (-20°C) until required for analysis. Frozen serum samples were analyzed for glucose (Sigma Diagnostic Kits No. 510-A), total serum protein was evaluated using the biuret reaction (Doumas et al. 1981; Soltani et al. 2010). Ca^{2+} and mg^{2+} values were determined using colorimetric method using an autoanalyzer (Technicon RA-1000, USA) according to (Kazemi et al. 2006; Táati et al. 2011). Lysozyme levels were determined based on the method of Ellis (1990). Enzyme activities in the serum were measured with a temperature-controlled spectrophotometer (DR/4000U, Germany). The assays were run in triplicate. The activities of the enzymes GOT and GPT were measured according to Reitman and Frankel (1957). After blood sampling, gill, muscle, liver, intestine and kidney tissues were obtained from the same fish, pooled and stored at -63°C for tissue copper analysis.

Enzyme Assay

The activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were conducted in Tabriz University (Iran) according to Somi et al. (2009). To measure cytosolic enzyme activity, the liver samples were homogenized in 1.15% KCl solution. Tissue superoxide dismutase (SOD) was assayed by a spectrophotometric method based on the inhibition of a superoxide-induced reduced nicotinamide adenine dinucleotide (NADH) oxidation according to Paoletti et al. (1986). Activities of hepatic and muscle glutathione peroxidase (GPx) were measured according to the method of Noguchi et al. (1973). The thiobarbituric acid reaction substances (TBARS) value was analyzed according to the method of Uchiyama and Mihara (1978). Absorbancy of the solution was measured at 530 nm and the concentration of the TBARS in the sample was measured by multiplying the optical density 5.2.

Copper Analysis

Copper contents of rearing water, diet, tissue, and initial and final whole-body were determined by digestion of samples in nitric acid (AOAC 2000). The concentrations of copper in the diluted digest solution were determined by using an Inductively Coupled Plasma Mass Spectrometer (Perkin-Elmer 3300, Waltham, MA).

Statistical Analysis

After confirming normality and homogeneity of variance, data were analysed by one-way ANOVA (SPSS software, version 15, Chicago, IL) to test for the effects of the dietary treatments. When a significant treatment effect was observed, a Tukey's test for multiple comparisons was performed. Treatment effects were considered at $P < 0.05$ level of significance. Broken line model was used to estimate the optimum dietary copper level in beluga sturgeon.

Results

Growth Performance (Mintrex®Cu)

After 12 weeks of dietary exposure, individual weight was significantly reduced ($P < 0.05$) in beluga sturgeon fed diets containing ≥12.8 mg Cu/kg and insufficient copper diet. Final wet weight (Growth) was highest in fish fed the diet with 9.5 and 12.8 mg Cu/kg diet, intermediate in fish fed the diets with 24.9 and 6.8 mg Cu/kg diet, and lowest in fish fed the diet with 193.5 mg Cu/kg diet in the 12 week (Table 24). All values in each of the three groups were significantly different from the values of three other groups. Weight gain (WG) and specific growth rate (SGR) were significantly improved by the elevation of dietary copper level from 3.3-12.8 mg/kg, but decreased with further increment. Weight gain and SGR of fish fed CuM_{10} and CuM_{13} were significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, CuM_{25}, CuM_{49} and CuM_{194} diets. There were no significant differences in WG among fish fed $CuM_{7.0}$, CuM_{10} and CuM_{13} diets, between fish fed $CuM_{7.0}$ and CuM_{25} diets or among those fed $Cu_{1.0}$, $CuM_{4.0}$, CuM_{49} and CuM_{194} diets ($P > 0.05$). Feed efficiency (FE) of fish fed CuM_{194} was significantly lower than those of fish fed other groups, exept fish fed $Cu_{1.0}$ diet. Whereas fish fed $CuM_{7.0}$ was significantly higher feed efficiency than that of fish $Cu_{1.0}$, CuM_{25}, CuM_{49} and CuM_{194} diets. There were no significant differences in FE among fish fed $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10} and CuM_{13} diets, among fish fed $CuM_{4.0}$, CuM_{10}, CuM_{13} and CuM_{25} diets or between those fed $Cu_{1.0}$ and CuM_{49} diets. Protein efficiency ratio (PER) of fish fed $CuM_{4.0}$, $CuM_{7.0}$ and CuM_{10} diets were significantly higher than those of fish fed $Cu_{1.0}$, CuM_{49} and CuM_{194} diets. However, there were no significant differences in PER among fish fed $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13} and CuM_{25} diets, among fish fed $Cu_{1.0}$, CuM_{13}, CuM_{25} and CuM_{49} diets or among those fed $Cu_{1.0}$, CuM_{49} and CuM_{194} diets. There were no significant differences in survival of fish in all treatments. Broken-line analysis of WG indicated that the optimum dietary Mintrex®Cu supplementation level could be 9.71 mg/kg diet in juvenile beluga sturgeon, *H. huso* (Figure 7).

Significant differences in the condition factors (CF) and hepatosomatic indices (HSI) among treatment groups were found, with the lowest values in sturgeon fed CuM_{194} diet. Hepatosomatic indices of fish fed $CuM_{7.0}$ was significantly higher than those of fish fed CuM_{13}, CuM_{25}, CuM_{49} and CuM_{194} diets. However, there were no significant differences in HSI among fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$ and CuM_{10} diets or among those fed CuM_{10}, CuM_{13}, CuM_{25} and CuM_{49} diets.

Condition factors of fish fed CuM_{10} was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{13}, CuM_{25}, CuM_{50} and CuM_{194} diets. However, there were no significant differences in CF among fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{13}, CuM_{25} and CuM_{49} diets.

Proximate Composition (Mintrex®Cu)

Table 25 shows the proximate composition of whole body and muscle of beluga sturgeon, *Huso huso*, fed the experimental diets. Whole body lipid contents were negatively correlated with dietary Mintrex®Cu concentrations while ash and moisture contents were positively correlated with dietary Cu concentrations ($P < 0.05$). Whole-body moisture was significantly higher in fish fed CuM_{194} than in those from the other treatments. However, there were no significant differences in whole body moisture among fish from $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25} and CuM_{49} diets ($P > 0.05$). Whole body protein of fish fed CuM_{194} was significantly lower than those of fish fed other diets. Whole body protein of fish fed CuM_{49} was also significantly lower than those of fish fed ≤24.9 mg Cu/kg diet, but significantly higher than fish fed CuM_{194} diets. Also, fish fed the diet containing $CuM_{7.0}$ and CuM_{10} diets was significantly higher whole body protein than those of fish fed $Cu_{1.0}$, CuM_{25}, CuM_{49} and CuM_{194} diets. There were no significant differences in whole body protein among fish fed $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10} and CuM_{13} diets, among fish fed $CuM_{4.0}$, CuM_{13} and CuM_{25} diets or between those fed $Cu_{1.0}$ and CuM_{25} diets. Whole-body lipid content of fish fed CuM_{194} was significantly lower than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25} and CuM_{49} diets. Lipid content of whole body of fish fed CuM_{49} was significantly lower than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13} and CuM_{25} diets. Also, there were no significant differences in whole-body lipid content among fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13} and CuM_{25} diets.

Whole body ash content of fish fed CuM_{49} and CuM_{194} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13} and CuM_{25} diets. There were no significant differences in ash content of whole body ash among fish fed $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13} and CuM_{25} diets or among those fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10} and CuM_{13} diets. Muscle moisture contents of fish fed CuM_{10} and CuM_{13} diets were significantly lower than those of fish fed CuM_{49} and CuM_{194} diets.

There were no significant differences in muscle moisture content among fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{25}, CuM_{49} and CuM_{194} diets or among those fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13} and CuM_{25} diets.

Muscle protein content of fish fed CuM_{10} was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, CuM_{49} and CuM_{194} diets. Protein content of muscle tissue of fish fed CuM_{194} was significantly lower than those of fish fed the other diets. There were no significant differences in muscle protein content among fish fed $CuM_{7.0}$, CuM_{10}, CuM_{13} and CuM_{25} diets or among those fed $Cu_{1.0}$, $CuM_{4.0}$ and CuM_{49} diets. Muscle lipid content of fish fed CuM_{194} was significantly lower than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13} and CuM_{25} diets. Muscle lipid content of fish fed CuM_{13} and CuM_{25} were also significantly higher than those of fish fed $CuM_{4.0}$, CuM_{49} and CuM_{194} diets. There were no significant differences in lipid content of muscle among fish fed $Cu_{1.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13} and CuM_{25} diets, among fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$ and CuM_{10} diets or among those fed $Cu_{1.0}$, $CuM_{4.0}$ and CuM_{49} diets.

Enzyme Assay (Mintrex® Cu)

The activities of hepatic and muscle copper-zinc superoxide dismutase (Cu-Zn SOD) and glutathione peroxidase (GPx) activity of juvenile beluga fed diets containing various levels of Mintrex®Cu supplementation for 12 weeks, increased significantly with the increase of dietary Cu and reached a peak (Table 26), then showed a decreasing trend ($P < 0.05$). Hepatic Cu-Zn SOD content, of fish fed CuM_{10} was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, CuM_{49} and CuM_{194} diets. While, hepatic Cu-Zn SOD content, of fish fed $Cu_{1.0}$, $CuM_{4.0}$ and CuM_{194} diets were significantly lower than those of fish fed other diets. However, there were no significant differences in this parameter among fish fed $CuM_{7.0}$, CuM_{10}, CuM_{13} and CuM_{25} diets or among those fed $CuM_{7.0}$, CuM_{13}, CuM_{25} and CuM_{49} diets. Broken-line analysis of Cu-Zn SOD indicated that the optimum dietary Mintrex®Cu supplementation level could be 9.28 mg/kg diet in juvenile beluga sturgeon (Figure 8). Hepatic glutathione peroxidase (GPx) activity was significantly reduced in beluga fed diets containing high levels of Mintrex®Cu (CuM_{194}) supplemented compared to fish fed diets containing $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13} and CuM_{25} diets.

Hepatic GPx of fish fed $Cu_{1.0}$ and $CuM_{4.0}$ diets was also significantly lower than those of fish fed other diets. There were no significant differences in hepatic GPx among fish fed $CuM_{7.0}$, CuM_{13}, CuM_{25} and CuM_{49} diets or among fish fed $CuM_{7.0}$, CuM_{49} and CuM_{194} diets and between those fed $Cu_{1.0}$ and $CuM_{4.0}$ diets. Hepatic thiobarbituric acid reactive substances (TBARS) of juvenile beluga sturgeon fed at $CuM_{7.0}$, CuM_{10} and CuM_{13} diets were significantly lower than those of fish fed at ≤3.6 mg Cu/kg and those fed to ≥24.9 mg Cu/kg diets.

Hepatic TBARS of fish fed CuM_{194} diet was also significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25} and CuM_{49} diets. However, there were no significant differences in this parameter between fish fed at $Cu_{1.0}$ and CuM_{49} diets, among fish fed $CuM_{4.0}$ and CuM_{25} diets or among those fed CuM_{25} and CuM_{49} diets. Broken-line analysis of TBARS indicated that the optimum dietary Mintrex®Cu supplementation level could be 8.73 mg/kg diet in juvenile beluga sturgeon (Figure 9).

Muscle Cu-Zn SOD increased and decreased after peak values which were attained in fish fed at the dietary Cu supplementation level of 9.5 mg/kg diet and dropped. However, this parameter was significantly higher in fish fed at CuM_{10} than those of fish fed at $Cu_{1.0}$, $CuM_{4.0}$, CuM_{25}, CuM_{49} and CuM_{194} diets. Muscle Cu-Zn SOD of fish fed $Cu_{1.0}$ diet was significantly lower than those of all the fed ones. There were no significant differences in muscle Cu-Zn SOD among fish fed $CuM_{7.0}$, CuM_{10} and CuM_{13} diets, among fish fed $CuM_{7.0}$, CuM_{13} and CuM_{25} diets and among those fed $CuM_{7.0}$, CuM_{25} and CuM_{49} diets. Muscle GPx of juvenile beluga fed $Cu_{1.0}$ and CuM_{194} diets were significantly lower than those of fish fed $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25} and CuM_{49} diets. However, there were no significant differences in this parameter among fish fed $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25} and CuM_{49} diets.

Hematological Parameters (Mintrex®Cu)

Hematological characteristics of beluga sturgeon, on the 12th week's exposure at different levels of Mintrex®Cu are shown in Table 27. The activities of hematocrit (packed cell volume, PCV), of beluga were found to increase to an optimal at a dietary Cu level of 9.5 mg Cu/kg diet and then declined gradually thereafter. Hematocrit content of fish fed CuM_{10} was significantly higher than those of fish fed $Cu_{1.0}$, CuM_{49} and CuM_{194} diets ($P < 0.05$).

There were no significant differences in PCV among fish fed $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13} and CuM_{25} diets or among those fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{13} and CuM_{25} diets. The activities of hemoglobin (Hb) presented a similar pattern as seen for PCV of beluga sturgeon. The peak values of these parameters were observed at around 9.5 mg Cu/kg diet. Hemoglobin content of fish fed CuM_{10} was significantly higher than those of fish fed CuM_{49} and CuM_{194} diets. There were no significant differences in Hb content among fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13} and CuM_{25} diets, among fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{13}, CuM_{25} and CuM_{49} diets or among those fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{25}, CuM_{49} and CuM_{194} diets.

Glucose content of fish fed CuM_{10} was significantly lower than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$ and $CuM_{7.0}$ diets. There were no significant differences in glucose content among fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{49} and CuM_{194} diets, among fish fed $CuM_{4.0}$, $CuM_{7.0}$, CuM_{25}, CuM_{49} and CuM_{194} diets or among those fed $CuM_{7.0}$, CuM_{13}, CuM_{25}, CuM_{49} and CuM_{194} diets. There were no significant differences in total protein content of serum in all treatments.

Lysozyme concentration of fish fed CuM_{10} was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{13}, CuM_{25}, CuM_{49} and CuM_{195} diets. Lysozyme activity of serum of fish fed $Cu_{1.0}$, $CuM_{4.0}$ and CuM_{194} were significantly lower than those of fish fed $CuM_{7.0}$, CuM_{10}, CuM_{13}, and CuM_{25} diets. There were no significant differences in lysozyme activity between fish fed $CuM_{7.0}$ and CuM_{13} diets, among fish fed CuM_{13}, CuM_{25} and CuM_{49} diets or among those fed $Cu_{1.0}$, $CuM_{4.0}$ and CuM_{194} diets. Cholesterol content of fish fed CuM_{194} was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25} and CuM_{49} diets. Cholesterol content of serum of fish fed CuM_{25} was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10} and CuM_{13} diets. There were no significant differences in cholesterol content among fish fed $CuM_{7.0}$, CuM_{10} and CuM_{13} diets, between fish fed CuM_{25} and CuM_{49} diets or among those fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$ and CuM_{10} diets.

Peroxidase content of serum of fish fed CuM_{194} was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10} and CuM_{13} diets. There were no significant differences in peroxidase content among fish fed CuM_{194}, CuM_{49} and CuM_{25} diets, among fish fed CuM_{49}, CuM_{25} and CuM_{13} diets or among those fed $CuM_{7.0}$, CuM_{10} and CuM_{13} diets.

Copper exposure increased the activities of the enzymes Glutamic oxaloacetic transaminase (GOT) and Glutamic pyruvic transaminase (GPT) serum concentrations with increasing dose. After 12 weeks of exposure, GOT concentrations increased significantly in the ≥49.3 mg Cu/kg diet than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10} and CuM_{13} diets. There were no significant differences GOT serum concentration between fish fed CuM_{25}, CuM_{49} and CuM_{194} diets or among those fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10} and CuM_{13} diets. Serum GPT concentrations in the was significantly elevated at ≥49.3 mg Cu/kg diet than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10} and CuM_{13} diets. There were no significant differences GPT serum between fish fed CuM_{49} and CuM_{194} diets, among fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13} and CuM_{25} diets or between those fed CuM_{25} and CuM_{49} diets.

The serum calcium concentration was unaffected in the exposure groups compared to the control diet. There were no significant differences in serum magnesium concentrations of fish in all treatments except for magnesium concentrations of fish fed $Cu_{1.0}$ which was significantly lower than those of fish fed CuM_{49} and CuM_{194} diets.

Copper Concentration (Mintrex®Cu)

Copper concentration in gill, liver, kidney, intestine, muscle, and whole-body of fish fed the experimental diets for 12 weeks was generally dose-dependent, increasing with increase in dietary copper concentration (Table 28). Copper accumulated mostly in the liver, followed by the intestine, gill, muscle and kidney in that order. Liver of beluga sturgeon is a more important storage tissue than other tissues, and the order of Cu accumulation in tissues was liver > intestine >gill > muscle > kidney. During the first 84 days, Cu concentration increased sharply reaching 1.7 to 1.8-fold higher (43.3-45.3 mg Cu/kg dry weight) than in the control group. The lower value (26.9 mg/kg) were found in beluga sturgeon fed diet with 3.3 mg Cu/kg, the intermediate value (36.5 mg/kg) in beluga fed the diet with 12.8 mg Cu/kg, and the highest liver Cu concentrations (49.0 mg/kg) in beluga fed the diet with the highest dietary Cu (193.5 mg Cu/kg diet), second highest (43.3 mg Cu/kg diet) in fish from 49.3 mg Cu/kg. The differences in liver Cu concentrations among the four groups were statistically significant ($P < 0.05$).

Copper content of intestine tissue of fish fed CuM_{194} and CuM_{49} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13} and CuM_{25} diets in the 12th weeks of dietary Cu exposure. Copper content of intestine tissue of fish fed CuM_{25} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, and CuM_{10} diets. Also, intestine copper concentration of fish fed CuM_{13} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$ and $CuM_{7.0}$ diets. There were no significant differences in intestine copper content among fish fed $Cu_{1.0}$, $CuM_{4.0}$ and $CuM_{7.0}$ diets, between fish fed $CuM_{7.0}$ and CuM_{10} diets or between those fed CuM_{10} and CuM_{13} diets.

Gill copper concentration of fish of fish fed CuM_{49} and CuM_{194} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13} and CuM_{25} diets. Gill copper content of fish fed CuM_{25} was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10} and CuM_{13} diets. There were no significant differences in this parameter between fish fed CuM_{10} and CuM_{13} diets or between those fed $Cu_{1.0}$ and $CuM_{4.0}$ diets.

Muscle copper content of fish fed CuM_{194} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25} and CuM_{49} diets. Copper content of muscle tissue of fish fed CuM_{25} and CuM_{49} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10} and CuM_{13} diets. There were no significant differences in muscle copper content between fish fed $Cu_{1.0}$ and $CuM_{4.0}$ diets or among those fed $CuM_{7.0}$ and CuM_{10} diets.

Copper content of kidney tissue of fish fed CuM_{25}, CuM_{49} and CuM_{194} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$ and CuM_{10} diets. Also, kidney copper concentration of fish fed CuM_{13} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$ and $CuM_{7.0}$ diets. There were no significant differences in kidney copper content among fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$ and CuM_{10} diets or between fish fed CuM_{10} and CuM_{13} diets or among those fed CuM_{13}, CuM_{25}, CuM_{49} and CuM_{194} diets.

Copper plasma contents of fish fed CuM_{194} was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25} and CuM_{49} diets. Copper contents of plasma of fish fed CuM_{25} and CuM_{49} were significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10} and CuM_{13} diets. Also, plasma copper concentration of fish fed CuM_{13} was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$ and CuM_{10} diets. There were no significant differences in plasma copper content between fish fed $Cu_{1.0}$ and $CuM_{4.0}$ diets or between those fed $CuM_{7.0}$ and CuM_{10} diets.

Whole body copper content of fish fed CuM_{194} was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25} and CuM_{49} diets. Copper content of whole body of fish fed CuM_{49} was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10} and CuM_{13} diets. Also, whole body copper concentration of fish fed CuM_{25} was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$ and CuM_{10} diets. There were no significant differences in whole body Cu content among fish fed $Cu_{1.0}$, $CuM_{4.0}$ and $CuM_{7.0}$ diets, between fish fed $CuM_{7.0}$ and CuM_{10} diets or between those fed CuM_{10} and CuM_{13} diets.

A non significant increase in total Cu concentrations was observed in the water from tanks in which fish were fed Mintrex®Cu supplemented diets compared to the control diet. Waterborne Cu level was less than 0.001 ppm at the beginning and increased to 0.011 ppm at the end of the feeding trial.

Discussion

The results of this study indicate that juvenile beluga sturgeon, *Huso huso*, have a requirement of copper that cannot met by Cu in the rearing water. However, no mortality or no gross deficiency symptoms were observed except the poor growth, feed and protein efficiency in beluga sturgeon fed Cu-deficient diets in this study. Meanwhile, this study showed that WG and SGR of juvenile beluga sturgeon increased with the dietary copper levels up to a concentration of 9.5 mg/kg diet and dropped, when compared with growth rates reported for the same fish in other studies (Mohseni et al. 2006; 2008). Those results indicate that supplemental copper could improve growth performance of juvenile beluga, which is consistent with the results obtained on rainbow trout, *Oncorhynchus mykiss*, and common carp, *Cyprinus carpio* (Ogino & Yang 1980), channel catfish, *Ictalurus punctatus* (Gatlin & Wilson 1986) and grouper, *Epinephelus malabaricus* (Lin et al. 2008; 2010). Based on the broken-line regression of WG against dietary Cu concentration from 3.2 to 193.5 mg Cu/kg diet, 9.71 mg Cu/kg diet seemed optimal for the best growth performance for this species, weighing 2.82 ± 0.18 g (mean ± SD). Berntssen et al. (2000) concluded that the growth suppression observed in Atlantic salmon fry fed a diet containing 7.2 mg Cu/kg for 12 weeks, indicated a dietary copper requirement in juveniles slightly higher than that previously suggested in the literature. In this study, highest WG of fish obtained (1777) compares favorably with those of previous studies in our laboratory, in which 1674 WG was observed in similar sized fish fed (inorganic Cu; $CuSO_4.5H_2O$) a nutritionally adequate diet for 12 wks. Variability of results could equally be attributed to other factors such as Cu source, fish weight and duration of feeding trial.

Feed efficiency also followed a similar pattern to that observed with WG and SGR. When inadequate copper amounts were supplemented in the diets, rainbow trout, *Salmo gairdneri* (Lanno et al. 1985) and Nile tilapia, *Oreochromis niloticus* (Shaw and Handy 2006) were observed to experience anorexia. Fish size, basal diet composition, rearing system, water temperature and water Cu concentration were identical in this study. Feed utilization efficiency has close relationship with growth performance (He et al. 2009). In the present experiment, FE and PER enhanced with increasing dietary copper levels up to a certain point.
A similar trend is found in channel catfish (Gatlin & Wilson 1986), grouper (Lin et al. 2008) juvenile yellow catfish, *Pelteobagrus fulvidraco* (Tan et al. 2011).

Better utilization of the feed is depending on both digestive and absorptive capacity (Tan et al. 2011_b). Digestive enzymes play a key role in digesting nutrients for fish, of which the activity can directly reflect the digestive ability (Wen et al. 2009). Probably, in this study, trypsin, chymotrypsin, lipase and amylase activities in intestine enhanced with the increment of dietary copper levels. It has been reported that zinc participates in stabilizing lipase (Choi et al. 2005; Tan et al. 2011_b) and amylase in bacteria (Alexei et al. 2002). These findings suggested that copper may have something to do with intestinal enzyme activity in fish; however, the mechanism needs further investigation.

In this study, dietary copper levels significantly influenced body composition of beluga sturgeon. Whole-body and muscle crude protein increased with dietary Cu up to the supplementation level of 9.5 mg Cu/kg diet and then decreased at higher levels. Whole-body lipid content was negatively correlated while whole-body ash was positively correlated with dietary Cu concentration, which was consistent with rainbow trout and guppy (Knox et al. 1982; Shim & Ng 1988). The results may be because of a shift of water from the intracellular to the extracellular compartment (Knox et al. 1982). Copper promoting body protein deposition in the present study is probably because of its participation in the body protein synthesis (Vormann 2003). Tissue deposition of lipid and protein is dependent on feed intake, metabolic use and intestinal absorption, and these factors can all be influenced by elevated dietary Cu contents (Tan et al. 2011). Handy et al. (1999) suggested that, compared to fish consuming 11 mg Cu/kg dry diet, lipid stores decreased markedly in rainbow trout after ofadatne month of exposure with 500 mg Cu/kg diet. Lipid peroxidation in response to copper exposure has been reported in the freshwater crab Oziotelphusa senex senex (Reddy and Bhagyalakshmi 1994) and mollusks (Connors and Ringwood 2000). This effect is probably specific for copper, as increased lipid peroxidation was not observed in response to cadmium exposure (Romeo et al. 2000). Whether from copper-induced lipid peroxidation or from increased catabolism of fatty acids, free fatty acids increase in the mid gut gland and other tissues (Vosloo et al. 2002) of *P. warreni* exposed to sublethal copper. This is important in explaining the effect of copper on *P. warreni*.

The present study showed that liver Cu-Zn SOD and glutathione peroxidase (GPx) activites significantly increased with dietary Cu supplementation up to a peak value at dietary Cu supplementation level of 9.5 mg Cu/kg diet and subsequently dropped at higer levels. It has been reported that inadequate and excess dietary Cu reduced Cu-Zn SOD activities and destroyed SOD, respectively in fish (Lin et al. 2008). In humans, SOD has also been affected by dietary Cu (Uauy et al. 1985), and in rats, increased Cu-Zn SOD activity in the liver and heart was observed with the supplementation of chelated forms of Cu and Zn (Buzadzic et al. 2002). Being antioxidant enzymes, elevation in their expression/activity may effectively prevent several diseases related to oxidative stress. Organic minerals which are commonly used in cultured animal feeds are chelated with organic compounds, such as carbohydrates, hydrolyzed protein (peptides) and amino acids. If an element is chelated by a compound that can either release it in its ionic form at the site of absorption or readily be absorbed as an intact chelate, this chelation may greatly enhance the absorption of the element by preventing its conversion to insoluble chemical compounds in the intestine or by preventing its strong adsorption of insoluble colloids (Apines-Amar et al. 2003; Lin et al. 2010).

In fish, GPx are important enzymatic antioxidant defenses and the activities are induced in the fish suffered from oxidative stress. Glutathione plays an important role in modulating metal-induced lipid peroxidation through its role as a reducing substrate in oxidative reactions and its sequestering capacity of metals (Schlenk and Rice 1998). Transition metals such as Cu are reduced by glutathione peroxidase activity forming stable GS-Cu binding complexes which can lead to decreased total glutathione levels (Freedman et al. 1989). Alternatively, lipid peroxidation may cause an increased utilisation of GPx activity in conjugation reactions involved in metabolism of lipid hydroperoxides, resulting in decreased total glutathione peroxidase levels (Canesi et al. 1999). In the present study total glutathione levels were reduced in tissues with significant Cu accumulation and subsequent lipid peroxidation. Selenium deficiency caused reduction in weight gain, feed efficiency and GPx activity in rainbow trout, *Oncorhyncus mykiss* (Hilton et al. 1989), Atlantic salmon, *Salmo salar* (Bell et al. 1987) and channel catfish, *Ictulurus punctutus* (Gatlin and Wilson 1986; Wang and Lovell 1997). Higher hepatic TBARS values observed in fish fed low and high dietary Cu than in those fed adequate Cu indicated that both insufficient and excess dietary Cu induced oxidative stress in beluga sturgeon. Similar results have been reported in juvenile grouper, *E. malabaricus* (Lin et al. 2008).

The hepatic TBARS values showed an inverse trend with the performance of hepatic Cu-Zn SOD activity. The high hepatic TBARS values in fish fed both insufficient and excess dietary Cu might be due to low activity of Cu-Zn SOD (Lin et al. 2010). Moreover, free Cu has been reported to induce the oxidation and destruction of SOD in vitro (Cecconi et al. 2002). It is suggested, then, that excess Cu stored in the liver caused high oxidative stress and destroyed the enzyme activity. This further clarifies why the lower Cu accumulation rate in organic Cu may be favorable for fish fitness.

As hematology has been used to assess the health status of most animals, several studies have shown hematological changes in fish exposed to numerous stress agents, including those relating to current aquaculture practices (Affonso et al. 2002). Copper is known to induce changes in the blood parameters of fish (Cerqueira and Fernandes 2002). Hematocrit and hemoglobin all increased with dietary Cu up to the supplementation level of 9.5 mg/kg diet and dropped at higer levels. It could be suggested that the optimum dietary Cu level could improve hematological characteristics of beluga sturgeon. Copper is associated with the oxygen-carrying protein, haemocyanin, and dietary Cu deficiency has been shown to cause anemia in hybrid tilapia and grouper (Shiau and Ning 2003; Lin et al. 2008). Furthermore, low levels of Hb content and total count of RBCs in rabbit fish, *Siganus rivulatus*, were attributed to water pollution by heavy metals (Bin-Dohaish et al. 2004). Under the influence of different heavy metals or in the state of stress, damage of the liver, kidneys, heart and other tissues and organs may occur with concomitant liberation of transaminases into the circulation. Higher glucose content observed in fish fed low and high dietary Cu than in those fed adequate copper. Stress caused by cadmium/copper increases the glucose content in blood, because of intensive glycogenolysis, and the synthesis of glucose from extrahepatic tissue proteins and amino acids (Larson and Haux 1982). Glucose may also be released into the circulation in cadmium-induced hypoxia, which enhances the mobilization of catecholamines and processes of glycogenolysis. The non-specific immune response parameters measured in the present study, i.e. respiratory burst activity, lysozyme activity is commonly used for evaluating the effect of nutrients on the immunity of fish (Lin and Shiau 2005; Puangkaew et al. 2004). Lysozyme response in fish may be involved in the overall alarm response, acting as an acute-phase protein (Tort et al. 2003). In the present study, lysozyme activity increased with dietary Cu up to the supplementation level of 9.5 mg/kg diet and then decreased at higer levels.

Nevertheless, reductions in lysozyme have been recorded in gilthead seabream following crowding stress (Ortuno et al. 2001). Depending on certain conditions such as the duration of stress, serum lysozyme level can either increase or decrease on stress stimuli (Binuramesh et al. 2005). The lack of consistency in the lysozyme activity suggests that the influence of stressors remains controversial (Fevolden et al. 1992). High plasma cortisol levels produce immunosuppressive action on lysozyme (Cnaani and McLean 2009). Various hormonal pathways have been considered to be responsible for lower lysozyme activity in fish during stress (Caruso et al. 2002). Also, in this study, lysozyme activity was negatively correlated with TBARS values, suggesting that immunity in beluga was impaired due to oxidative stress induced by high Cu ingestion (Lin and Shiau 2007). However, they do suggest that immune function can be altered by changing the Cu concentratioin of cell membranes, which is a more likely explanation for the changes in immune responses seen here. The results of the present study show that beluga sturgeon held at high Cu concentration appear to be experiencing chronic stress, as indicated by the significant elevation in immune system. This dietary stress affected some physiological functions such as lipid distribution and immune activity. The fish showed no signs of disease but displayed symptoms of immunosuppression. Further experiments are required to study whether high Cu concentration affects the ability of fish to resist additional stressors, thus beingmore susceptible to stress and diseases.

In line with other reports (Berntssen et al. 1999; Shiau and Ning, 2003), Cu concentrations in beluga sturgeon tissues were found to be positively correlated with dietary Cu level. The liver is the major Cu storage organ in fish (Miller et al. 1993; Lorentzen et al. 1998; Shiau and Ning 2003; Lin et al. 2010). In this study also suggested that hepatic Cu accumulation rate was lower in fish fed diets with organic Cu than in fish fed diets with inorganic Cu. This phenomenon was also reported in grouper (Lin et al. 2010), pig (Coffey et al. 1994), cow (Du et al. 1996) and ewe (Eckert et al. 1999). High levels of Cu accumulation in the liver might cause toxic oxidative stress for fish (Lin and Shiau 2007; Lin et al. 2010). This lower accumulation rate of Cu in organic Cu fed fish also provides additional evidence for why organic Cu had a higher toxicity threshold for beluga than inorganic Cu in this study. One goal of aquaculture is to produce fish fillets for human consumption. Hence, it is of interest whether the nutritional value of fish fillets can be manipulated through the diet. The present study indicates that muscle copper content could not be increased through addition of adequate dietary organic copper form.

No significant differences were found in muscle Cu concentrations between fish fed 3.3-12.8 mg Cu/kg diets. DeBoeck et al. (1997) observed no significant Cu accumulation in muscle tissue of common carp, *C. carpio*, during 28 days of Cu exposure. Copper content in muscle ranged between 2 and 5 mg/kg DM for dietary supplies ranging from 180 to 240 mg/kg in the diet (Bradley et al. 1983). Copper from organic sources was markedly more available for accumulation in muscle of beluga sturgeon. In one study with Atlantic salmon, selenium from organic sources also had greater retention in muscle (Lorentzen et al. 1994). According to Waschulewski and Sunde (1988), being bound to an amino acid, as is selenium in selenomethionine, facilitates more efficient incorporation of selenium into animal tissue. They contend that selenium-bound amino acids can serve as analogs of amino acids for non-specific protein synthesis (Wang and Lovell 1997).

One reason for the higher bioavailability of organic copper than copper from the inorganic source to beluga sturgeon is enhanced absorption. Paripatananont and Lovell (1997) showed that net absorption of selenomethionine was higher than that of inorganic selenium (Na_2SeO_3) in a purified egg-white-based diet (90.8 versus 62.8%) and in a soybeanmeal-based diet (88.9 versus 69.7%) in channel catfish. Chelated Cu might be absorbed and transported intact to target tissues and be more available in metabolic processes than inorganic copper. The dietary Cu allowance of beluga sturgeon can be reduced by using of the organic Cu sources to replace conventional inorganic source. Use of organic copper will have advantages environmentally because less copper will go into the aquacultural system. Nevertheless, copper solubility and its excretion from cultured animals due to high ingestion continue to draw environmental concern and hence lend importance to organic mineral research.

In summary, copper plays a role in promoting growth, enhancing feed utilization efficiency, increasing protein and lipid deposition and intestinal enzyme activities in beluga sturgeon. In the present study, growth performance, hematology and immune responses were depressed by deficiency or excess dietary Cu concentration. Broken-line analysis of WG, Cu-Zn SOD and TBARS suggested that the optimum dietary Cu level for juvenile beluga sturgeon is about 9.71, 9.28 and 8.73 mg Cu/kg, respectively. The ratio of Cu tolerance over requirement may be as low as 5 fold (50 vs. 10 mg/kg) for juvenile beluga sturgeon, when Mintrex®Cu is used as the dietary source of organic copper.

Experiment IV. The optimum dietary inorganic copper level in juvenile beluga sturgeon, *Huso huso*

Abstract

A 12-week feeding trial was conducted to evaluate the optimum dietary inorganic copper (copper sulfate) levels in juvenile beluga sturgeon, *Huso huso*. Eight semi-purified diets containing 1.1 ($Cu_{1.0}$), 3.5 ($CuS_{4.0}$), 7.1 ($CuS_{7.0}$), 9.7 (CuS_{10}), 13.1 (CuS_{13}), 25.1 (CuS_{25}), 49.9 (CuS_{50}) and 195 (CuS_{195}) mg Cu/kg diet in the form of $CuSO_4.5H_2O$ were fed to fish of initial body weight 2.49 ± 0.12 g and length 7.85 ± 0.66 cm (mean±SD) in triplicate groups in a flow-through system. Weight gain (WG) and specific growth rate of fish fed CuS_{10} and CuS_{13} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, CuS_{25}, CuS_{50} and CuS_{195} diets ($P < 0.05$). Whole-body and muscle crude protein increased with dietary Cu up to the supplementation level of 13.1 mg/kg diet and then decreased at higer levels. Whole-body lipid content was negatively correlated while whole-body ash was positively correlated with dietary copper concentration. Hepatic copper-zinc superoxide dismutase (Cu-Zn SOD) activity of fish fed CuS_{10} and CuS_{13} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$ and CuS_{195} diets. Hepatic thiobarbituric acid-reactive substances (TBARS) of fish fed CuS_{13} diet was significantly lower than those of fish fed the other diets except for that of fish fed CuS_{10} diet. Glutamic oxaloacetic transaminase (GOT), Glutamic pyruvic transaminase (GPT) and copper accumulation in tissues increased with dietary copper. Broken-line analysis of WG suggested that the optimum dietary Cu level was 10.3 mg Cu/kg diet. Therefore, these results may indicate that the optimum dietary Cu levels could be greater than 10.3 mg Cu/kg diet but less than 13.1 mg Cu/kg diet in juvenile beluga, *H. huso* when copper sulfate is used as the dietary source of inorganic copper.

Introduction

Copper is an essential trace element for vertebrates, including fish, for a large number of biological processes, mainly as a co-factor for some enzymes, such as cytochrome oxidase, superoxide dismutase, lysyl oxidase, dopamine hydroxylase and tyrosinase (Tan et al. 2011_b). Under normal dietary conditions, copper deficiency is not common in humans or cultured animals since most dietary protein sources contain this mineral. However, under experimental conditions, a copper deficiency signs in fish diet include impaired growth, increased mortality, eye cataracts, anemia, short body dwarfism and low tissue Cu (Lall 2002; Wu and Huang 2008). The Cu requirement or toxicity in beluga has not been reported, whereas the Cu requirements include 3 mg/kg diet in rainbow trout and common carp (Ogino and Yang 1980); 5 mg/kg diet in channel catfish (Gatlin and Wilson 1986); 5 mg/kg diet in Atlantic salmon (Lall and Hines 1987); 4 mg/kg diet in hybrid tilapia, *Oreochromis niloticus*×*Orechromis aureus* (Shiau and Ning 2003) and 4-6 mg/kg diet in grouper, *Epinephelus malabaricus* (Lin et al. 2008). Dietary Cu deficiency has been shown to reduce the activities of liver Cu-Zn superoxide dismutase (SOD) and heart cytochrome oxidase in fish (Lall 2002). However, Cu is not only an essential but also a potentially toxic trace element, depending on its concentration. Copper is often supplemented in fish feeds that exceed its requirement level. For example, inorganic copper concentrations in commercial fish feed can reach maximum levels of to 34 mg Cu/kg diet. These concentrations exceed the dietary requirements of copper and may be on the borderline of toxic concentrations (Maage 1994). High levels of dietary Cu in fish cause a toxic syndrome which includes growth depression, increased mortality (Shiau and Ning 2003), oxidative stress (Berntssen et al. 2000) and reduced immune response (Lundebye et al. 1999; Berntssen et al. 1999). Therefore, supplementation of Cu to fish feed is a balance between fulfilling the Cu requirement and avoiding Cu toxicity (Tan et al. 2011).

There are five species of sturgeon in the Caspian Sea of the Iranian waters is home to five sturgeon species namely *Huso huso*, *Acipenser nudiventris*, *A. stellatus*, *A. gueldenstedtii* and *A. persicus,* which are of economic importance to Iran. The composite stocks of sturgeon species represent, economically, the most valuable fish resource of Iran. Confined to the Caspian Sea, the fishery has a high export value earning significant foreign exchange each year. Beluga is one of the commercially important fresh and brackish water aquaculture species in Iran. Its high commercial value makes it a promising aquaculture species in the future.

Information on the dietary requirements of most sturgeon species is scarce and there is a lack of suitable diets for sturgeon. Hence sturgeon culturists were initially forced to use commercial salmon feeds for sturgeon marketable size (Mohseni et al. 2006), Prolonged use of these feeds may lead to poor growth, scoliosis, lack of equilibrium and other nutritionally related deficiencies in sturgeon (Hung and Deng 2002). In line with the dearth of research in sturgeon fish, the optimum and the toxic dietary copper levels have not been studied in this species. Copper sulfate was chosen as the dietary Cu source because the Cu used in commercial diet of sturgeon is predominantly inorganic rather than the organic form. Hence, the objective of this study was to investigate the optimum and the minimum dose of toxic dietary copper levels based on growth, nutrient utilization, blood parameters, enzyme activity and tissue copper concentration in juvenile beluga, using inorganic copper (copper sulfate) as the copper source.

Materials and Methods

Diet Formulation and Preparation

Eight isoenergetic and isonitrogenous semi-purified diets were formulated and manufactured to contain 0, 3, 6, 9, 12, 24, 48 and 192 mg Cu/kg diet in the form of copper sulfate ($CuSO_4.5H_2O$). However, the actual copper concentrations as determined by analysis were 1.1, 3.5, 7.1, 9.7, 13.1, 25.1, 50 and 194.6 mg Cu/kg diet for $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25}, CuS_{50} and CuS_{195} diets, respectively. The control diet ($Cu_{1.0}$) without supplementation of Cu contained 1.1 mg Cu/kg diet (Table 23). The premixture containing the copper sulfate was added to the diet to replace the equal amount of cellulose in the diet.

Experimental Fish and Feeding Trial

At the beginning of the experiment, beluga fingerlings (360 fish with the similar size, averaging initial weight and length 2.49 ± 0.12 g and 7.85 ± 0.66 cm (mean ± SD) respectively, were starved for 18 h and anesthetized with 200 mg/L clove solution, *Syzyglum aromaticum,* and weighed individually. Fish were then randomly allocated in 24 fiberglass tanks 500-L (15 fish per tank) circular fiberglass tanks (diameter: 105 cm; height: 51 cm) in a flow-through system. Each tank was then randomly assigned to one of the three replicates of the 8 dietary treatments for 12 weeks. The mean (± SD) and ranges of measured water quality parameters during the study period were: water temperature ranged was (21.3 ± 0.1^oC), dissolved oxygen level ranged was 7.8 ± 0.05 mg/L provided with continuous aeration, pH 7.3 ± 0.02, CO_2 6.1 mg/l, alkalinity 157.7 mg/l, total hardness 360-400 mg/l and NH_4 0.1 mg/l during the whole experimental period.

Diet formulation and preparation, feeding trial, sample collection and analysis, enzyme assay, copper analysis and statistical analysis were conducted in the same manner as in chapter 3, experiment III "The optimum dietary organic copper level in juvenile beluga sturgeon, *Huso huso*"

Results

Growth and Survival (copper sulfate)

Weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER) and survival of juvenile beluga sturgeon, *H. huso*, fed diets containing different levels of inorganic copper are shown in Table 29. After 12 weeks of dietary exposure, final individual weight increased with dietary Cu up to the supplementation level of 13.1 mg/kg diet and then decreased. Final wet weight (Growth) was highest in fish fed the diet with 13.1 mg Cu/kg diet, intermediate in fish fed the diet 7.1 mg Cu/kg diet, and lowest in fish fed the diet with 195 mg Cu/kg diet. All values in each of the three groups were significantly different from the values of three other groups ($P < 0.05$). Weight gain and SGR were significantly improved by the elevation of dietary copper level from 3.5-13.1 mg/kg, but decreased with further increment. Weight gain and SGR of fish fed CuS_{10} and CuS_{13} were significantly higher than those of fish fed Cu_1, CuS_4, CuS_{25}, CuS_{50} and CuS_{195} diets. There were no significant differences in WG among fish fed $CuS_{7.0}$, CuS_{10} and CuS_{13} diets or among those fed Cu_1, CuS_4, CuS_{25}, CuS_{50} and CuS_{195} diets. Also, there were no significant differences in SGR among fish fed $CuS_{7.0}$, CuS_{10} and CuS_{13} diets, among fish fed CuS_4, $CuS_{7.0}$ and CuS_{25} diets or among those fed Cu_1, CuS_4, CuS_{25}, CuS_{50} and CuS_{195} diets. Feed efficiency of fish fed CuS_{195} was significantly lower than those of fish fed Cu_1, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets. However, there were no significant differences in FE among fish fed $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets. There were no significant differences in survival of fish in all treatments. Protein efficiency ratio (PER) of fish fed CuS_{195} was significantly lower than those of fish fed Cu_1, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets. However, there were no significant differences in PER among fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets. Broken-line analysis of WG indicated that the optimum dietary inorganic Cu supplementation level could be 10.3 mg/kg diet in juvenile beluga sturgeon, *H. huso* (Figure 10).

Significant differences in the condition factors (CF) among treatment groups were found, with the lowest values in beluga sturgeon fed CuS_{195} diet. Condition factor of fish fed CuS_{13} was significantly higher than those of fish fed other diets. However, there were no significant differences in CF among fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10} and CuS_{25} diets or among those fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10} and CuS_{50} diets.

The lowest hepatosomatic indices (HSI) was observed in fish fed the diet containing the highest Cu content. Hepatosomatic indices of fish fed $CuS_{4.0}$ and $CuS_{7.0}$ were also significantly higher than those of fish fed CuS_{50} and CuS_{195} diets. There were no significant differences in HSI among fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{25} diets, among fish fed $Cu_{1.0}$, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets or among those fed CuS_{25}, CuS_{50} and CuS_{195} diets.

Proximate Composition (copper sulfate)

There were significant differences in the proximate composition of beluga sturgeon among treatment groups (Table 30). Whole body lipid contents were negatively correlated with dietary copper sulfate concentrations while ash and moisture contents were positively correlated with dietary copper sulfate concentrations. Whole-body moisture content of fish fed CuS_{195} was significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets. There were no significant differences in whole-body moisture content among fish fed $CuS_{7.0}$, CuS_{13}, CuS_{25} and CuS_{50} diets, among fish fed $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{25} diets or among those fed $Cu_{1.0}$, $CuS_{4.0}$ and CuS_{10} diets. Whole body protein of fish fed CuS_{195} was significantly lower than those of fish fed other diets ($P < 0.05$). Protein content of whole body of fish fed CuS_{50} diet also was significantly lower than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{25} diets. There were no significant differences in whole body protein among fish fed $CuS_{7.0}$, CuS_{10} and CuS_{13} diets, among fish fed $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10} and CuS_{25} diets or among those fed $Cu_{1.0}$, $CuS_{4.0}$, CuS_{10} and CuS_{25} diets.

Whole-body lipid content of fish fed CuS_{195} diet was significantly lower than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets. Lipid content of whole body of fish fed CuS_{50} diet was significantly lower than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{25} diets. However, there were no significant differences in whole body lipid content among fish fed $CuS_{4.0}$, $CuS_{7.0}$ and CuS_{10} diets, among fish fed $CuS_{7.0}$, CuS_{10} and CuS_{13} diets or among those fed CuS_{10}, CuS_{13} and CuS_{25} diets.

Whole body ash content of fish fed CuS_{195} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets. Ash content of whole body of fish fed CuS_{50} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{25} diets. Also, there were no significant differences in whole body ash content among fish fed $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{25} diets or among those fed $CuS_{4.0}$, $CuS_{7.0}$ and CuS_{10} diets.

Muscle moisture contents of fish fed CuS_{195} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets. There were no significant differences in muscle moisture content among fish fed $Cu_{1.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets or among those fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{25} diets. Muscle protein content of fish fed CuS_{13} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{50} and CuS_{195} diets. While, protein content of muscle tissue of fish fed CuS_{195} diet was significantly lower than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets. There were no significant differences in muscle protein content among fish fed $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10} and CuS_{25} diets or among those fed $Cu_{1.0}$, $CuS_{4.0}$ and $CuS_{7.0}$ diets.

Muscle lipid content of fish fed CuS_{195} diet was significantly lower than those of fish fed the other diets. Muscle lipid content of fish fed CuS_{50} diet was also significantly lower than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{25} diets. There were no significant differences in lipid content of muscle among fish fed $Cu_{1.0}$, $CuS_{7.0}$ and CuS_{10} diets or among those fed $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{25} diets.

Enzyme Activity

Hepatic thiobarbituric acid reactive substances (TBARS), copper-zinc superoxide dismutase (Cu-Zn SOD) activity and glutathione peroxidase (GPx) are shown in Table 31. The activities of hepatic and muscle Cu-Zn SOD and GPx of juvenile beluga fed diets containing various levels of copper sulfate supplementation for 12 weeks, increased significantly with the increase of dietary Cu and reached a peak, then showed a decreasing trend ($P < 0.05$). Hepatic Cu-Zn SOD content of fish fed CuS_{10} and CuS_{13} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$ and CuS_{195} diets. There were no significant differences in this parameter among fish fed $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets, among fish fed $CuS_{4.0}$, $CuS_{7.0}$, CuS_{25} and CuS_{50} diets or among those fed $Cu_{1.0}$, $CuS_{4.0}$ and CuS_{195} diets ($P > 0.05$). Broken-line analysis of Cu-Zn SOD suggested that the optimum dietary Cu level was 11.86 mg Cu/kg diet (Figure 11).

Hepatic GPx activity of fish fed CuS_{13} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{25}, CuS_{50} and CuS_{195} diets. Hepatic GPx of fish fed CuS_{195} diet also significantly higher than those of fish fed $Cu_{1.0}$ and $CuS_{4.0}$ diets. Also, hepatic GPx activity of fish fed CuS_{50} diet was significantly lower than those of fish fed CuS_{10}, CuS_{13} and CuS_{25} diets.

There were no significant differences in glutathione peroxidase among fish fed $CuS_{7.0}$, CuS_{10} and CuS_{25} diets, between fish fed $Cu_{1.0}$ and $CuS_{4.0}$ diets or between those fed CuS_{10} and CuS_{13} diets. Hepatic TBARS of juvenile beluga sturgeon fed at CuS_{13} was significantly lower than those of fish fed at ≤7.1 mg Cu/kg and those fed to ≥25.1 mg Cu/kg diet. Hepatic TBARS of fish fed CuS_{195} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets. While, TBARS of fish fed CuS_{13} diet was significantly lower than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{25}, CuS_{50} and CuS_{195} diets. However, there were no significant differences in this parameter between fish fed $CuS_{4.0}$ and CuS_{25} diets, between fish fed $CuS_{7.0}$ and CuS_{10} diets or between those fed CuS_{10} and CuS_{13} diets. Broken-line analysis of TBARS suggested that the optimum dietary Cu level was 11.1 mg Cu/kg diet (Figure 12).

Muscle Cu-Zn SOD increased when dietary Cu increased to about 13.1 mg/kg but then decreased gradually with further increase in dietary Cu. However, this parameter was significantly higher for fish fed CuS_{10} and CuS_{13} diets than for those fed at $Cu_{1.0}$ and $CuS_{4.0}$ diets. There were no significant differences in muscle Cu-Zn SOD among fish fed $Cu_{1.0}$, $CuS_{4.0}$, CuS_{25}, CuS_{50} and CuS_{195} diets, among fish fed $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25}, CuS_{50} and CuS_{195} diets or among those fed $CuS_{4.0}$, $CuS_{7.0}$, CuS_{25}, CuS_{50} and CuS_{195} diets. Muscle GPx of juvenile beluga sturgeon fed CuS_{13} and CuS_{25} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, CuS_{50} and CuS_{195} diets. Also, muscle GPx of fish fed CuS_{195} diet was significantly lower than those of all the fed ones. However, there were no significant differences in this parameter between fish fed at $Cu_{1.0}$ and CuS_{50} diets, among fish fed $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{25} diets, among fish fed $CuS_{4.0}$, $CuS_{7.0}$ and CuS_{10} diets or among those fed $CuS_{4.0}$, $CuS_{7.0}$ and CuS_{50} diets.

Hematological Parameters

Hematological measurements of beluga sturgeon, on the 12th week's exposure at different levels of copper sulfate are shown in Table 32. The activities of hematocrit (PCV), of beluga were found to increase to an optimal at a dietary Cu content of 13.1 mg Cu/kg diet and then declined gradually thereafter. Hematocrit content of fish fed CuS_{13} was significantly higher than those of fish fed $Cu_{1.0}$, CuS_{25}, CuS_{50} and CuS_{195} diets ($P < 0.05$). There were no significant differences in PCV among fish fed $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10} and CuS_{13} diets, among fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$ and CuS_{10} diets or among those fed $Cu_{1.0}$, CuS_{25} and CuS_{50} diets. The activities of hemoglobin (Hb) presented a similar pattern as seen for hematocrit of beluga sturgeon.

Hemoglobin content of fish fed CuS_{10} was significantly higher than those of fish fed CuS_{50} and CuS_{195} diets. There were no significant differences in Hb content among fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{25} diets, among fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{13}, CuS_{25} and CuS_{195} diets or among those fed $Cu_{1.0}$, CuS_{13}, CuS_{25}, CuS_{50} and CuS_{195} diets.

Glucose content of fish fed CuS_{13} diet was significantly lower than those of fish fed $Cu_{1.0}$, $CuS_{7.0}$ and CuS_{25} diets. There were no significant differences in glucose content among fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{25}, CuS_{50} and CuS_{195} diets or among those fed $CuS_{4.0}$, CuS_{10}, CuS_{13}, CuS_{50} and CuS_{195} diets. There were no significant differences in total protein content of serum in all treatments.

Lysozyme activity increased with dietary Cu up to the supplementation level of 13.1 mg/kg diet and then decreased at higer levels. However, lysozyme activities of fish fed CuS_{13} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{25}, CuS_{50} and CuS_{195} diets. Lysozyme activities of fish fed $Cu_{1.0}$, $CuS_{4.0}$ and CuS_{195} diets was also significantly lower than those of fish fed $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets. There were no significant differences in lysozyme activities between fish fed CuS_{10} and CuS_{13} diets or between those fed CuS_{10} and CuS_{25}.

Cholesterol content of fish fed CuS_{195} and CuS_{25} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10} and CuS_{13} diets. There were no significant differences in cholesterol content among fish fed $Cu_{1.0}$, $CuS_{4.0}$ and $CuS_{7.0}$ diets, among fish fed $CuS_{7.0}$, CuS_{10} and CuS_{13} diets or among those fed CuS_{25}, CuS_{50} and CuS_{195} diets ($P > 0.05$).

Peroxidase content of serum of fish fed CuS_{195} diet was significantly higher than those of fish fed $Cu_{1.0}$ and $CuS_{4.0}$ diets. There were no significant differences in peroxidase content among fish fed $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets or among those fed $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25}, CuS_{50} and CuS_{195} diets.

Copper exposure increased the activities of the enzymes glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) serum concentrations with increasing dose. Serum GOT contents of fish fed CuS_{195} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$ and CuS_{10} diets. There were no significant differences in GOT concentrations of serum among fish fed CuS_{13}, CuS_{25}, CuS_{50} and CuS_{195} diets, among fish fed $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{50} diets or among those fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$ and CuS_{10} diets.

Serum GPT concentrations of fish fed CuS_{195} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10} and CuS_{13} diets. There were no significant differences GPT contents among fish fed CuS_{25}, CuS_{50} and CuS_{195} diets, among fish fed $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets or among those fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10} and CuS_{13} diets. Serum calcium concentration was unaffected in the exposure groups compared to the control diet. Serum magnesium concentrations of fish fed CuS_{195} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{50} diets. There were no significant differences in serum magnesium concentrations among fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$ and CuS_{10} diets, among fish fed CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets and among those fed $CuS_{7.0}$, CuS_{10} and CuS_{50} diets.

Copper Concentration (copper sulfate)

Copper concentration in gill, liver, kidney, intestine, muscle, and whole-body of fish fed the experimental diets for 12 weeks was generally dose-dependent, increasing with increase in dietary copper concentration (Table 33). Copper accumulated mostly in the liver, followed by the intestine, gill, muscle and kidney in that order. Liver of beluga sturgeon is a more important storage tissue than other tissues, and the order of Cu accumulation in tissues was liver > intestine > gill > muscle> kidney. During the first 84 days, Cu concentration increased sharply reaching 1.9 to 2.4-fold higher (47.86-53.65 mg Cu/kg dry weight) than in the control group. The lower value (26.49 mg/kg) were found in beluga fed diet with 3.5 mg Cu/kg, the intermediate value (35-38 mg/kg) in beluga fed the diets with 13.1 and 25.1 mg Cu/kg, and the highest liver Cu concentrations (53.65 mg/kg) in beluga fed the diet with the highest dietary Cu (194.6 mg Cu/kg diet), second highest (47.86 mg Cu/kg diet) in fish from 50 mg Cu/kg. The differences in liver Cu concentrations among the four groups were statistically significant.

Intestine copper contents of fish of fish fed CuS_{50} and CuS_{195} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{25} diets ($P < 0.05$). There were no significant differences in intestine Cu content among fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10} and CuS_{13} diets or among those fed CuS_{10}, CuS_{13} and CuS_{25} diets. Gill copper contents of fish of fish fed CuS_{50} and CuS_{195} were significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{25} diets. Also, gill copper concentrations of fish fed CuS_{25} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10} and CuS_{13} diets.

However, there were no significant differences in gill content between fish fed $Cu_{1.0}$ and $CuS_{4.0}$ diets or among those fed CuS_{10} and CuS_{13} diets. Muscle copper content of fish fed CuS_{195} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets. Copper content of muscle tissue of fish fed CuS_{50} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10} and CuS_{13} diets. There were no significant differences in muscle copper content among fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$ and CuS_{10} diets, among fish fed $CuS_{7.0}$, CuS_{10} and CuS_{13} diets or among those fed CuS_{25} and CuS_{50} diets.

Kidney copper contents of fish fed CuS_{195} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{25} diets. There were no significant differences in copper content of kidney among fish fed $Cu_{1.0}$, $CuS_{4.0}$ and $CuS_{7.0}$ diets, among fish fed $CuS_{7.0}$, CuS_{10} and CuS_{13} diets or among those fed CuS_{50} and CuS_{195} diets. Also, there were no significant differences in Cu content of kidney among fish fed CuS_{10}, CuS_{13} and CuS_{25} diets.

Copper plasma contents of fish fed CuS_{195} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets. Copper contents of plasma of fish fed CuS_{50} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$ and CuS_{10} diets. There were no significant differences in plasma Cu among fish fed CuS_{13}, CuS_{25} and CuS_{50} diets, among fish fed CuS_{10}, CuS_{13} and CuS_{25} diets or among those fed $CuS_{7.0}$, CuS_{10} and CuS_{13} diets.

Whole body copper content of fish fed CuS_{195} was significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets. Copper content of whole body of fish fed CuS_{50} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10} and CuS_{13} diets. Also, whole body copper concentration of fish fed CuS_{25} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuS_{4.0}$, $CuS_{7.0}$ and CuS_{10} diets. There were no significant differences in whole body copper content among fish fed $Cu_{1.0}$, $CuS_{4.0}$ and $CuS_{7.0}$ diets, between fish fed $CuS_{7.0}$ and CuS_{10} diets or between those fed CuS_{10} and CuS_{13} diets.

A non significant increase in total copper concentrations was observed in the water from tanks in which fish were fed copper sulfate supplemented diets compared to the control diet. Waterborne Cu level was less than 0.001 ppm at the beginning and increased to 0.013 ppm at the end of the feeding trial.

Discussion

This study is the first report on the safe and toxic dietary copper levels in beluga sturgeon. Copper uptake directly from the water was observed as fish given diets with lower Cu supplementation (i.e. ≤9.7 mg Cu/kg diet) were able to accumulate considerably more Cu than they were fed. Therefore, Cu leakage was a negligible reason for growth performance and body composition in the present trial. In this study, growth reduction has been seen in beluga sturgeon fed either Cu-deficient or excess Cu diet, in agreement with other aquatic animals (Berntssen et al. 1999; Shaw & Handy 2006; Lin et al. 2008; Wu & Huang 2008; Tan et al. 2011). The reduction in growth in beluga sturgeon fed excess Cu diets is most likely explained by reduced feed intake. Loss of appetite as the cause of reduced growth has also been reported in rainbow trout (Lanno et al. 1985) and Nile tilapia, *Oreochromis niloticus* (Shaw & Handy 2006) when exposed to excessive dietary copper.

Gatlin and Wilson (1986) reported that catfish fed a diet with 16 and 32 mg Cu/kg had significantly suppressed growth and feed efficiency values. It is also similar to rainbow trout fry fed on natural diets of invertebrates contaminated with metals (381 mg Cu/kg diet) which showed a 37% depression of growth over 91 days (Woodward et al. 1994). In a further study by Woodward et al. (1995) the energy content of the natural diets were more closely matched, but they observed a 50% reduction in body weight relative to controls over 88 d. Several authors have noted reductions in growth rate during dietary Cu exposure in fish (Baker et al. 1998; Clearwater et al. 2002), but others have not (Lanno et al. 1985; Handy et al. 1999; Campbell et al. 2002) and Clearwater et al. (2002) argues that toxic effects on growth are best rationalised on an exposure dose basis. Moreover, Lundebye et al. (1999) suggested that decreased growth in Atlantic salmon due to increased metabolic costs of intestinal cellular changes and Cu excretion. The physiological changes permitting metal detoxification and homeostasis cost energy and reduced growth caused by exposure to Cu has been attributed to metabolic costs associated with metal detoxification (Marr et al. 1996). However, the oxidation state of the food itself can alter oxidative damage to the tissues (Baker et al. 1998). In the case of beluga, the decreased growth rate is probably due to an increased expenditure of energy for sustaining normal metabolism, leaving less energy available for growth.

Based on the ANOVA results of growth performance and broken-line analysis of WG, the optimum dietary inorganic copper level, in juvenile beluga sturgeon, weighing 2.98 ± 0.64 g (mean ± SD) could be higher than 10.3 but less than 13.1 mg Cu/kg diet. This is a bit higher than values reported in several other species: i.e. Atlantic salmon, *Salmo salar*, parr at 5-10 mg Cu/kg dry diet (Gatlin and Wilson 1986; Lorentzen et al. 1998; Ogino and Yang 1980). Rapidly growing Atlantic salmon fry have a higher Cu requirement of 35 mg Cu/kg dry diet (Berntssen et al. 1999) and 4-6 mg Cu/kg diet for grouper (Lin et al. 2008) fed inorganic Cu. Fish size, basal diet composition, rearing system, water temperature, salinity, and water Cu concentration were identical in the different studies.

In the present study, feed efficiency (FE) was less responsive parameter to elevated dietary Cu exposure than growth response. The poorest FE was observed for fish fed the highest dietary Cu content in beluga sturgeon. Poor absorption or assimilation of the major nutrients is unlikely given that FE, condition factor, and intestinal morphology remained similar throughout the experiment. Similarly, high levels of Cu depressed growth and impaired feed conversion in channel catfish and juvenile yellow catfish (Murai et al. 1981; Tan et al. 2011).

Beluga sturgeon exposed to dietary concentrations ranging between 3.5 and 194.6 mg Cu/kg diet as copper sulfate for 12 weeks exhibited a mean survival rate of 99.63%. Survival may not be good indicators of dietary Cu toxicity in beluga over a relatively short period of time and with moderate concentrations of Cu in diets. Results of this study suggest that the beluga sturgeon is relatively less sensitive to Cu toxicity than other fish species. Exposure to diets 0.1-20 mg Cu/kg diet for 8 weeks did not affect the survival of grouper, *Epinephelus malabaricus,* (Lin et al. 2008); and 16 weeks exposure to 41.8-158 mg Cu/kg diet did not cause any mortality in the juvenile soft-shelled turtles, *Pelodiscus sinensis* (Wu & Huang 2008). Also, exposure to diets with 35-1750 mg Cu/kg diet for 3 months did not affect the survival of Atlantic salmon, *Salmo salar* L. fry (Berntssen et al. 1999). Sturgeon growth exhibited greater sensitivity to Cu toxicity in comparison to survival, but to a much lesser degree in comparison to other fish species exposed to dietary copper.

In this study, fish fed the CuS_{195} diet possessed significantly lower condition factor (CF). In contrast, dietary copper did not affect of CF of the Atlantic salmon (Berntssen et al. 1999) and juvenile yellow catfish (Tan et al. 2011). Reduced CF may be a result of reduced feed intake, energy intake or increased metabolic expenditure. This decline in energy intake is reflected in lower values for hepatosomatic index and CF (fatness) of the Cu-fed fish ($P < 0.05$) compared to controls; i.e. energy intake was less than energy utilisation for the Cu-treated fish.

For comparison, it has been reported that white sturgeon, *A. transmontanus*, exposed to dietary concentrations ranging between 0.4 and 191.1 mg Se/kg for 8 wks exhibited a significant differences in the CF among treatment groups, with the lowest values in sturgeon fed 191.1 mg Se/kg diet (Tashjian et al. 2006). However, feed rejection was not observed and this is in agreement with Lanno et al. (1985) and Brentssen et al. (1999). Lanno et al. (1985) noticed a "pronounced food refusal" which also coincided with the greatest reductions in growth rate. Woodward et al. (1995) report more than a 50% reduction in the feeding activity of trout associated with a 50% reduction in growth. Alternatively, in the dab, *Limundu limundu*, fed 200 mg/kg dry wt diet, body weight was maintained by increasing food (energy) intake (Overnell and McIntosh 1988).

Shrinkage of livers indicated by reduced HSI from fish fed CuS_{50} and CuS_{195} diets, clearly demonstrated an adverse effect of Cu at these concentrations, similar in turtle, *Pelodiscus sinensis* (Wu & Huang 2008) and juvenile yellow catfish (Tan et al. 2011). Duration of the trials could also be a discrepancy factor, as the beluga sturgeon in the present study were fed diets for 12 weeks, which is about 25 days more than those of the studies on shrimp (Lee and Shiau 2002) and fish (Shaw and Handy 2006). It is possible that beluga livers shrink after long-term exposure to high dietary Cu. In fact, in the trial, reduced VSI and HSI may be a result of either reduced feed intake or increased metabolic expenditure for detoxification and maintenance of homeostasis among these treatments (Tan et al. 2011).

Whole-body protein increased with dietary Cu exposure up to the optimum range of 13.1 mg Cu/kg diet and decreased beyond these levels. Whole-body lipid content was negatively correlated while whole-body ash was positively correlated with dietary Cu concentration. Tissue deposition of lipid and protein is dependent on feed intake, metabolic use and intestinal absorption, and these factors can all be influenced by elevated dietary Cu concentrations (Berntssen et al. 1999). The decrease in these two energy sources corresponds with the reduced growth in fish fed high levels of dietary Cu in this study. Berntssen et al. (1999) observed a negative correlation between dietary Cu concentrations and energy stores in Atlantic salmon fed practical diets. DeBoeck et al. (1997) also reported decreased liver and muscle protein, lipid and glycogen content in common carp exposed to waterborne Cu.

Handy et al. (1999) suggested that, compared to fish consuming 11 mg Cu/kg dry diet, lipid stores decreased markedly in rainbow trout after 1 month of exposure to 500 mg Cu/kg diet. Crude lipid and protein in muscle were significantly lower in fish fed dietary Cu ≥50 mg/kg compared to the rest of the treatments ($P < 0.05$). This is in agreement with the previous studies in terrestrial animals. Cheng et al. (2008) reported that dietary copper supplementation, regardless of source and level, significantly reduced 12th rib backfat and kidney fat in lambs. The decrease in backfat depth by dietary copper supplementation was also observed in goat kids (Solaiman et al. 2006) and finishing steers (Engle et al. $2000_{a\&b}$).

Copper plays both pro-and anti-oxidation roles in biological system. The antioxidant enzymes such as Cu-Zn SOD and CAT have been extensively used as biomarkers of oxidative stress (Kopecka-Pilarczyk and Correia 2009). Depressed hepatic Cu-Zn SOD activity in beluga was observed when the dietary Cu was insufficient (≤3.5 mg Cu/kg diet) or in excess >50 mg Cu/kg diet, also depressed muscle Cu-Zn SOD activity in fish was observed when the dietary Cu was insufficient (≤3.5 mg Cu/kg diet), it might be attributed to the increase in production of reactive oxygen species, possibly due to lower rate of fish metabolism. Indeed, some authors found a decrease in the activity of metabolism-related enzymes at low or high Cu concentration (Lin et al. 2008; Wang et al. 2009), which would later return to the original state as the fish acclimatised to the Cu. However, there is little information available on the enzame assay in beluga sturgeon, more studies are required to clarify the role of copper in the enzyme system of sturgeon.

Gatlin and Wilson (1986) also reported that the activity of liver Cu-Zn SOD was significantly reduced in channel catfish fed diets containing 0-2.0 mg Cu/kg as compared to those fed 4 mg Cu/kg diet. Free Cu has been reported to induce the oxidation and destruction of SOD in vitro (Cecconi et al. 2002). Increased free Cu concentration is associated with depressed ceruloplasmin activity in human (Harris 1993). It is likely that low plasma ceruloplasmin activity in beluga may explain the decreased Cu-Zn SOD activity in fish fed high Cu diet, i.e., 194.6 mg Cu/kg diet. Lin et al. (2008) reported that low plasma ceruloplasmin activity in juvenile grouper, *Epinephelus malabaricus*, may explain the decreased Cu-Zn SOD activity in fish fed high Cu concentration in the diet. It has been reported that both a deficiency and excess in dietary Cu reduced Cu-Zn SOD activities and destroyed SOD, respectively in fish (Lin et al. 2008; Wu and Huang 2008). This may be the reason why turtles fed the basal and high Cu diets exhibited higher thiobarbituric acid reaction substances (TBARS) values.

Higher TBARS values observed in low and high dietary Cu fed fish than in those fed adequate Cu indicated that either deficient or excess dietary Cu induced oxidative stress and damage to the tissue caused by high Cu ingestion. These effects can be interpreted as indicators of lipid oxidation. Similar results have been reported for grey mullet (Baker et al. 1998), Atlantic salmon (Berntssen et al. 2000) and juvenile grouper (Lin et al. 2008). The Cu-induced lipid peroxidation in the tissues can be explained by in vivo oxidative damage due to tissue Cu accumulation and/or the intake of oxidative products from rancid feed caused by the supplementation of Cu salts (Baker et al. 1998). Small elevation in free transitional metals can initiate lipid peroxidation in dietary oils (Berntssen et al. 2000). It is therefore not surprising that the diet supplemented with the highest Cu-concentration had increased levels of lipid oxidation products. The higher oxidative state of the food (as seen from increased TBARS levels) could cause increased assimilation of dietary lipid hydroperoxides and hydroalkoxides which in turn may initiate in vivo tissue lipid peroxidation and cause depletion of antioxidants such as α-tocopherol (Baker and Davies 1996).

Glutathione peroxidase (GPx), on the other hand, scavenges H_2O_2, which is a precursor of ROS in addition to its protective role against lipid peroxidation of cell membrane. Lipid peroxidation, a complex, self-propagating and highly destructive process, increases the rigidity (decreases the fluidity) of cellular membranes. In the case of oxidative stress, the MDA level was regarded as a well-suited indicator for the extent of lipid peroxidation (Shi et al. 2004).

The change in hepatic and muscle GPx level was more dramatic ($P < 0.05$) than changes in enzyme values because oxidative stress originating from heavy metals directly affects the GPx level. As an important indicator for oxidative damage, the GPx level decreased in liver and muscle of fish exposed to insufficient (≤3.5 mg Cu/kg diet) and high Cu ingestion (≥50 mg Cu/kg diet). This enzyme catalyzes reactions necessary for the conversion of hydrogen peroxide and fatty acid hydroperoxides into water and fatty acid alcohol by using reduced glutathione, thereby protecting cell membranes against oxidative damage (Abdol-Tawwab et al. 2007).
GPx protects cell membrane against oxidative damage (Rotruck et al. 1973). GPx activity corresponds to either the dietary Cu supplementation level or tissue Cu concentration in fish except at the excess level. Similar increasing trend of hepatic GPx activity to that of hepatic Cu content in fish may reasonably suggest that Cu was used for GPx synthesis in response to the oxidative stress caused by high dietary Cu ingestion (Lin and Shiau 2007).

Hematological parameters are determined as an index of their health status as well (Perottoni et al. 2004). Numerous studies have shown that factors such as age, sex, environmental conditions and diet can influence fish blood values. In the present study all factors, except sex, were the same. Hemoglobin and hematocrit content all increased with dietary Cu up to the supplementation level of 9.7 to 13.1 mg/kg diet respectively, and dropped. It could be suggested that the optimum dietary Cu level could improve hematological characteristics of beluga sturgeon. Also, this could be due to adequate ceruloplamin activity in serum in both cases. Copper deficiency lowers the production of ceruloplasmin which results in suboptimal hemoglobin formation. Excess Cu, on the other hand, may inhibit ceruloplamin activity (Wu and Huang 2008). Similar results were seen in a study in which carp, after a 30-day exposure to waterborne heavy metals including Cu, had reduced RBC, Hb, and PCV values (Dhanapakiam and Ramsasamy 2001). However, waterborne exposure to Cu resulted in significant change to hematological parameters in tilapia (Nussey et al. 1995) and rainbow trout (Dethloff et al. 2001). Fish exposed to copper display a tendency toward decreased antioxidant enzyme activities RBC, HGB, and HCT levels. The toxic effects of Cu on the inhibition of erythropoiesis in the hematopoietic organism and the inhibition of antioxidant enzyme activity have prevented this reaching a statistically significant level (Ates et al. 2008). Decreases in erythrocyte, hemoglobin, and hematocrit values can be an indicator of anemia with the subsequent result of inhibition of erythropoiesis in the hemopoietic organism.

Our observation of significantly higher copper concentrations in the plasma than those in tissues except liver, is consistent with the values reported for the abalone, *Haliotis rufescens* (Viant et al. 2002) and juvenile abalone, *H. discus hannai* Ino (Wang et al. 2009). This is probably due to copper being associated with the oxygen-carrying protein, haemocyanin, which exists in the haemolymph, and that more vacuolated hepatopancreas has higher Cu concentration (Wang et al. 2009). This was further confirmed by the significantly positive correlations between dietary copper concentration and the serum protein level. The freshwater adapted flounder, *Platichtys flesus,* was found to have continuously increased plasma Cu concentrations after 37 days of exposure to 15 pg of Cu per liter and tilapia had elevated plasma Cu concentrations after 6 days of exposure to 50-200 µg of Cu per liter (Pelgrom et al. 1995). Glucose concentration in beluga was similar to levels reported in many sturgeon species (Cataldi et al. 1998; Barton et al. 2000; Belanger et al. 2001; Kieffer et al. 2001; Cech & Crocker 2002). Because these physiological variables are commonly used to describe the stress response in fishes (Barton et al. 2000), and the levels noted for sturgeons are typically lower than those of several teleosts, these results suggest that beluga is not highly stressed while being fed with diet containing high Cu concentration.

Vijayan et al. (1997) reported that catecholamine's rise immediately after application of a stress, and this transient increase results in rapid glycogen breakdown and consequently elevated plasma glucose concentration. A chronic rise in the plasma glucose concentration indicates stimulation of both protein catabolism and gluconeogenesis (Kim et al. 2004). Therefore, dietary Cu exposure probably caused abnormal glycogenesis in beluga sturgeon fish, which resulted in an increase of the plasma glucose concentration and decrease of total protein. The blood serum Ca and Mg in freshwater *H. huso* sturgeon is within the range values observed in freshwater sturgeon of various species. It has been shown that the blood plasma Ca and Mg concentrations are maintained within definite ranges (Kazemi et al. 2003). Following severe exercise, osmotic and ionic redistribution in fish blood may occur (Kieffer et al. 2001). Contrary to this typical response of teleosts, beluga sturgeon magnesium concentrations changed substantially following forced activity. The changes in post-activity plasma ion concentration were either small and transient or not statistically significant (calcium concentration) compared to teleosts (McDonald & Milligan 1997), but are consistent with the post-stress changes for many sturgeon species (Krayushkina 1998; Kieffer et al. 2001).

The specific immune response parameters measured in the present study, i.e. respiratory burst activity, lysozyme activity, is commonly used for evaluating the effect of nutrients on the immunity of fish (Montero et al. 1999; Lin and Shiau 2003 & 2005; Puangkaew et al. 2004). The present results also demonstrated that the lysozyme activity was enhanced with the increment of the dietary Cu levels. The decreases in lysozyme activity in fish fed Cu_1, CuS_4, CuS_7, CuS_{50} and CuS_{195} diets following chasing suggest that forced activity initiated a primary stress response, and also suggesting that immunity in beluga sturgeon was impaired due to oxidative stress induced by high Cu ingestion. These results may be attributed to the hepatocellular damage and kidney failure as a result of toxic effect of Cu (Nesckovic et al. 1996; Abdel-Tawwab et al. 2007). However, little is known about the effects of dietary Cu on lysozyme activities and plasma Cu concentration in a sturgeon fish. Serum lysozyme, deriving from macrophages, could break down the cell wall of pathogenic bacteria and is related to the ability of bacteriolysis (Berglijot 2006). Siwicki (1987) discovered that the concentration and activity of lysozyme increased largely in immunized cyprinids. Evaluation of non-specific immune responses can provide a first-tier evaluation of potential immunotoxicity in aquatic organisms. Of the wide range of immune assays available, phagocytic capacity and lysozyme response enable evaluation of immunotoxicity at the cellular and humoral level (Khoshbavar-Rostami et al. 2004).

Serum Glutamic oxaloacetic transaminase (GOT) and Glutamic pyruvic transaminase (GPT) are important diagnostic tools in medicine and are used to detect the toxic effects by various pollutants (Kim and Kang 2004). Our results show that dietary Cu (Table 32) significantly increased the activity of GOT and GPT in all expousure groups as compared to the control values. These results are in accordance with the results of previous investigators on freshwater fishes (Zikić et al. 1997 & 2001). Kim and Kang (2004) showed that Cu exposure increased GOT and GPT serum concentrations with increasing time and dose in juvenile rockfish, *Sebastes schlegeli*. Under the influence of different heavy metals or in the state of stress, damage of the liver, kidneys, heart and other tissues and organs may occur with concomitant liberation of transaminases into the circulation. Vaglio and Landriscina (1999) suggested that liver is rich in GOT and GPT, and damage to it could result in liberation of large quantities of these enzymes into the blood.

If the water is polluted with different metals, such as zinc, copper and cadmium (Zikić et al. 1997), the activity of plasma transaminases in freshwater fishes are significantly altered. Therefore, increases in GOT and GPT activities in serum of beluga, is assumed to be a result of liver damage by copper.

In the present study, copper accumulated in the tissues and whole body in a dose-dependent manner. The accumulation of heavy metals in the tissues of aquatic organisms may cause various physiological defects and mortality (Karakoc 1999). The lower sensitivity of beluga sturgeon to Cu toxicity in comparison to other fish species was not due to an inability to bio-accumulate Cu, because tissue Cu levels increased significantly during the growth trial in most treatment groups. Furthermore, beluga sturgeon tissue Cu levels were also above tissue Cu levels toxic to other species (Wang et al. 2009). Moreover, the positive Cu accumulation rate observed in tissues suggests that Cu levels in these tissues had not reached equilibrium concentrations after the 12-week exposure. Copper accumulation in all tissues of fish increased with increasing concentrations of the metal and with increasing exposure period (Cogun and Kargin 2004). In this study, the highest Cu accumulation occurred in the liver, followed by the intestine, gill, muscle and kidney, respectively. Studies carried out in various fish species have shown that liver is the main sites of accumulation and storage (Cogun and Kargin 2004). Kamunde et al. (2002) found that the Cu concentration in the liver of rainbow trout was 33-fold higher for fish fed 282 mg Cu/kg diet than for the control group, and he suggested that the role of the liver is central in fish Cu metabolism. This is probably due to copper being associated with the oxygen-carrying protein, haemocyanin, which exists in the haemolymph, and that more vacuolated hepatopancreas has higher Cu concentration. In addition, rainbow trout (Lanno et al. 1985), Atlantic salmon (Lorentzen et al. 1998) and channel catfish (Gatlin and Wilson 1986) all accumulated high concentrations of Cu in the liver. This was further confirmed by the significantly positive correlations between dietary copper concentration and the serum protein level (Wang et al. 2009). The intestine had the second highest Cu accumulation in this study. Handy (1992) reported that 53% of the Cu body burden was restricted to the intestine in rainbow trout exposed to 200 mg Cu/kg diet. The strong intestinal Cu accumulation suggests that dietary Cu uptake is regulated by retention of excess Cu in the intestinal tissue.

In juvenile beluga sturgeon, Cu exposure resulted in increased Cu accumulation in liver as well as intestine, because the liver in fish tends to concentrate metals, and play major role in detoxification and excretion of metals through the induction of metal-binding proteins, such as metallothioneins (MTs) is closely related to heavy metal exposure and metal taken up from the environment can be detoxificated by binding on those proteins (Roesijadi and Robinson 1994).

Therefore, it can be concluded that liver and intestine of juvenile beluga sturgeon are more important storage tissues than other tissues and Cu accumulation clearly reflected the level of dietary exposure. In general, when fish are fed elevated Cu concentrations for approximately a week or more, Cu will accumulate in highest concentrations in the intestinal tissues, liver and gall bladder (bile) and in lower concentrations in the gill, muscle and kidney (Baker et al. 1998; Clearwater et al. 2000; Kamunde et al. 2001; Tan et al. 2011).

The gill of teleost fish plays an important role on ion regulation, gas exchange, acid-base balance and nitrogenous waste excretion, which means it, has a key role at the interface of fish with its environment. Because branchial epithelium of teleost gills is a tissue where both active and positive exchange occurs between animals and its environment it is also likely to be a site action of heavy metal (Ay et al. 1999). So, it is assumed that the gills are major site of Cu uptake due to the large surface area and direct contact with aquatic environment. However, Cu accumulation in the gill of beluga sturgeon was lower than that of liver and intestine. This result was also observed in rainbow trout (Miller et al. 1993; McGeer et al. 2000), tilapia (Pelgrom et al. 1995) and sea bream (Wong et al. 1999). The small increase in gill Cu content than liver and intestine, therefore probably reflects systemic Cu in the gill tissue and distribution of some dietary Cu to the gill from the gut. This phenomenon has been previously observed with high dietary Cu doses in rainbow trout (Handy 1996; Kamunde et al. 2001); grey mullet (Baker et al. 1998) and Nile tilapia (Shaw and Handy 2006).

Generally, kidney plays a major role like liver in detoxification and excretion of toxicants with induction of metal-binding proteins such as MTs (Roesijadi and Robinson 1994). So, tissue likes the kidney which is a major producer of MTs shows high accumulation of Cu in fish (McGeeer et al. 2000). However, Cu content in the kidney of beluga sturgeon was about an order on magnitude lower than those of liver, intestine, gill and muscle.

Freshwater teleost incorporate dissolved metal through the gill and accumulate it mainly in the kidney, whereas marine teleost take in metal by drinking seawater and accumulate it mainly in the liver. Surprisingly, the kidneys of fish fed copper sulfate showed a relatively high new accumulation of copper. These assumptions are, evidently not the case for the new Cu accumulation in the kidney, although it seemed to be so for the liver.

It means that only a fraction of the Cu found in plasma was available to the kidney and that this fraction comprised a relatively large part of the newly accumulated Cu found in the plasma. Regardless of the overestimation of newly accumulated Cu in the kidney, it is evident that the newly accumulated Cu entering the kidney replaced some of the Cu already present in the kidney, since there is no increase in total cu concentration. This means that there could, in fact, be substantial renal Cu excretion (Grosell et al. 1996). Copper concentrations in muscle also increased with dietary Cu content. The muscle is the edible part of beluga sturgeon; hence, care should be taken to maintain the residual mineral content within safe levels for human consumption. Compared to Cu content in liver, intestine, gill, muscle and kidney Cu content was relatively low in beluga sturgeon. The same results were observed the other exposed Cu research (Wong et al. 1999) which Cu accumulation was lower in the muscle than in the liver and intestine tissues. This results was agrees with the view that accumulation in muscle becomes important only when the maximum storage capacity of the liver reached (Kim et al. 2004). However, muscle constitutes more than 30% of the body mass of the fish, the muscle can accumulate a large fraction of the body burden even though Cu concentrations in muscle usually are relatively low (Tan et al. 2011). Copper accumulation in muscle was homeostatically regulated (Kamunde et al. 2001) and related to exposure periods.

Plasma Cu levels exhibited a strong linear correlation with diet at concentrations up to <25.1 mg Cu/kg diet suggesting that Cu levels can serve as bio-indicators of dietary Cu exposure. However, no linear correlation was observed in plasma Cu levels when exposed to >25.1 mg Cu/kg diet. The cause of the non-linearity may be partly due to the rapid depuration of Cu from the tissues after a possible decrease in Cu uptake during the last 6 weeks of the feeding experiment due to decrease in feeding and/or activity as described previously.

In this study, whole-body Cu contents increased with dietary Cu levels, suggesting that beluga could accumulate excess Cu in tissues. Whole body Cu levels were observed to be as high as 6.12 mg Cu/kg DW in this study after the 12-week exposure to dietary copper sulfate, well above whole-body levels correlated with mortality in the juvenile yellow catfish (Tan et al. 2011). So far, there is no information on the tissues copper accumulation from diets in sturgeon.

It is clear that Cu toxicity is a concern in animal nutrition because the low requirement of Cu and the physiological range for Cu tends to be narrows (Lovell 1989; Wu and Huang 2008). beluga growth performance were significantly affected by the dietary Cu, however, its tissue levels of Cu and lipid varied with dietary Cu concentrations, and beluga immune responses were depressed by dietary deficiency and excess copper.

In summary, copper plays a role in promoting growth, enhancing feed utilization efficiency, increasing protein and lipid deposition and intestinal enzyme activities in beluga sturgeon. In the present study, growth performance, hematology and immune responses were depressed by deficiency or excess dietary Cu concentration. Broken-line analysis of WG, Cu-Zn SOD and TBARS suggested that the optimum dietary Cu level for juvenile beluga sturgeon is about 10.3, 11.86 and 11.1 mg Cu/kg, respectively. The ratio of Cu tolerance over requirement may be as low as 25 fold (25 vs. 2.5 mg/kg) for juvenile beluga sturgeon, when copper sulfate ($CuSO_4.5H_2O$) is used as the dietary source of inorganic copper.

Comparison of effects of dietary copper sources and levels on growth, enzyme activity and tissue copper concentration of juvenile beluga sturgeon, *Huso huso*

Abasract

A 2× 8 factorial design was used to compare the effects of dietary organic (Mintrex®Cu) and inorganic copper (copper sulfate) and levels in juvenile beluga sturgeon, *Huso huso*. Three replicate groups of fish averaging 2.46 ± 0.09g (mean ± SD) were fed one of the 15 experimental diets ($Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25}, CuM_{49}, CuM_{194}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25}, CuS_{50} and CuS_{195} diets) that were prepared containing two different dietary copper sources (organic and inorganic Cu) at each of eight different levels of Cu 1, 4, 7, 10, 13, 25, 49 and 194 mg Cu/kg diet. At the end of 12 weeks of the experimental period, there were significant dietary copper sources, levels and their interaction effects on weight gain (WG), feed efficiency (FE), protein efficiency ratio (PER), condition factor (CF), copper-zinc superoxide dismutase activity (Cu-Zn SOD), glutathione peroxidase (GPx) and thiobarbituric acid-reactive substances (TBARS), hematological values (Glutamic oxaloacetic transaminase and Glutamic pyruvic transaminase) and Cu content of tuissues (liver and muscle) and whole boby. There were also significant dietary Cu levels effects and interactive effects of Cu sources and levels on lysozyme activities. Growth performance, nutrient utilization, antioxidant status and hematological values of fish fed organic Cu were relatively greater than inorganic Cu ($P < 0.05$). Copper concentrations in fish tissues and whole-body increased with dietary copper level. There was a trend that fish fed diets with inorganic Cu had higher liver but lower whole-body and muscle copper concentrations than those of fish fed from organinc Cu. WG and specific growth rate (SGR) of fish fed CuM_{10} and CuM_{13} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, CuM_{25}, CuM_{49}, CuM_{194}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{25}, CuS_{50} and CuS_{195} diets. Hepatic Cu-Zn SOD activities of fish fed CuM_{10} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{49}, CuM_{195}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{25}, CuS_{50} and CuS_{195} diets. While, TBARS levels of fish fed CuM_{10} diet was significantly lower than those of fish fed other diets, except for those of fish fed CuS_{13} diet. Broken-line regression analysis of WG suggested that the optimum dietary Cu levels in juvenile beluga could be 9.71 mg/kg and 10.3 mg/kg diet in the form of organic Cu and inorganic Cu, respectively. This study showed that organic Cu is more bioavalible to juvenile beluga sturgeon than inorganic Cu and could use as a new copper source.

Introduction

The trace elements zinc (Zn), iron (Fe), manganese (Mn) and copper (Cu) serve important functions in living cells and are essential element and are required by fish for several biochemical processes (Lall 2002). Fish may absorb elements such as copper from the surrounding water, but the diet is the main source of essential elements (Lall 2002). Copper is a cofactor for antioxidant enzyme systems (e.g. superoxide dismutase, SOD) and enzymes in the electron transport chain (Lin and Shiau 2007). Copper is often supplemented in fish feeds that exceed its requirement level. For example, copper concentrations in commercial fish feed can reach maximum levels of to 34 mg/Cu kg diet (Maage 1994). These concentrations exceed the dietary requirements of copper, and may be on the borderline of toxic concentrations. High levels of dietary Cu in fish cause a toxic syndrome which includes growth depression, increased mortality (Shiau and Ning 2003), oxidative stress (Berntssen et al. 2000) and reduced immune response (Lundebye et al. 1999; Berntssen et al. 1999). Knowledge of bioavailability of supplemental copper sources is critical in selection of a copper source in feed production (Spears et al. 2004; Luo et al. 2005; Shao et al. 2010). The most common form of Cu used in feeds for growth promotion is the sulfate salt (CuSO4). Copper in the form of copper sulfate has been shown to improve growth rate and feed efficiency in broilers (Choi and Paik 1989; Baker et al. 1991) and in pigs (Edmonds et al. 1985). Besides its use as a dietary copper supplement, copper sulfate is used in freshwater aquaculture to control external parasites, bacterial diseases and also as a fungicide. However, use of inorganic salts can result in poor bioavailability of the mineral, mainly due to the numerous nutrient and ingredient antagonisms that impair absorption (Underwood and Suttle 1999).

Chelated forms of various elements have also been found to be effective for some aquatic animals (Apines-Amar et al. 2004; Shao et al. 2010). Improved availability of Cu from organic Cu complexes compared with the commonly used Cu salts has recently been suggested. Chelates are the organic form of Cu and are usually considered for use in animal diet as alternatives to inorganic Cu source. Chelated minerals are widely used in the livestock and poultry industries, yet research concerning these compounds with respect to aquatic species such as fish has been very limited (Apines-Amar et al. 2004; Shao et al. 2010).

More bioavailability of Cu is probably due to better absorption, which enhances its efficiency (Downs et al. 2000; Yu et al. 2000; Guo et al. 2001). Beluga sturgeon is an important fish cultured in Iran (Mohseni et al. 2006) and a series of studies have been conducted to determine the optimal levels of each ingredient from larvae to yearlings based on their growth performance. However, it is crucial for the aqua feed industry to optimize the use of feed ingredient (protein, lipid, carbohydrate, vitamin and mineral) and to improve the feed utilization in the beluga diets. This would allow less dependence on fish meal, reduce the cost of the feed and also reduce the environmental impact through waste output from beluga sturgeon culture. The relationship between optimal Cu levels and tolerance threshold toxicity in this species, but, has not been studied. Thus, this study was designed to evaluate the application of different Cu sources, including dietary organic (Mintrex®Cu) and inorganic (copper sulfate) forms, as feed additives in diets for beluga sturgeon, which is one of the most valuable freshwater fish species cultured in Iran. Furthermore, the biochemical analysis and Cu concentration in beluga tissues were also investigated.

Materials and Methods

Diet Formulation and Preparation

Proximate composition of the basal diet used in this feeding trial is shown in Table 23. Seven experimental diets contained 3.6 ($CuM_{4.0}$), 6.8 ($CuM_{7.0}$), 9.5 (CuM_{10}), 12.7 (CuM_{13}), 24.9 (CuM_{25}), 49.3 (CuM_{49}) and 193.5 (CuM_{194}) mg Cu/kg diet in the form of Mintrex®Cu, a chelate of copper and 2-hydroxy-4-methylthiobutanoic acid, an organic form of Cu, and seven other diets contained 3.5 ($CuS_{4.0}$), 7.1 ($CuS_{7.0}$), 9.7 (CuS_{10}), 13.1 (CuS_{13}), 25.1 (CuS_{25}), 50 (CuS_{50}) and 194.6 (CuS_{195}) mg Cu/kg diet in the form of copper sulfate ($CuSO_4.5H_2O$), were fed to fish of initial body weight 2.46 ± 0.09 g and length 7.84 ± 0.67 cm (mean ± SD) in triplicate groups in a flow-through system for 12 weeks. The control diet without supplementation of Cu contained 1.1 mg Cu/kg diet ($Cu_{1.0}$). The premixture containing the Cu source was added to the diet to replace the equal amount of cellulose in the diet.

Diet formulation and preparation, feeding trial, sample collection and analysis, enzyme assay and copper analysis were conducted in the same manner as in chapter 3, experiment III "The optimum dietary organic copper level in juvenile beluga sturgeon, *Huso huso*".

Statistical analysis

After confirming the normality and homogeneity of variance, two-way ANOVA test was performed to test whether there was any interaction between dietary copper source and dose. When we found the interaction, all data were analyzed by one-way ANOVA to test for the effects of the dietary treatments. When a significant treatment effect was observed, a Tukey's test for multiple comparisons was performed. Treatment effects were considered at $P < 0.05$ level of significance. All statistical analyses were carried out by SAS version 9.0 software (SAS Institute, Cary, NC, USA).

Results

Growth and Survival

Weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER), condition factor (CF) and survival of juvenile Beluga sturgeon, *H. huso*, fed diets containing different levels of organic Cu (Mintrex®Cu) and inorganic Cu (copper sulfate) are shown in Table 34 and 35. There were dietary significant copper source and levels effects on FW, WG, SGR, FE, PER and CF ($P < 0.05$). Furthermore, there were significant their interaction effects on FW, WG, FE, PER and CF content. Also, there were significant copper levels effects on %HSI ($P < 0.05$). However, there were neither significant copper sources nor interaction effects on %HSI for fish fed all diets. Survival did not show any significant differences among fish fed all the experimental diets. Growth performance obviously higher in fish fed diets supplemented with organic copper (Mintrex®Cu), compared to fish fed with equal levels of copper from inorganic ($CuSO_4.5H_2O$), and decreasing trend of growth performance were observed with the increasing level of dietary copper sources. Final weight, WG, SGR, PER and CF peaked at the dietary Cu supplementation level of 10-13 mg/kg diet and dropped.

At the end of 12 weeks of feeding trial, weight gain (WG) of fish fed CuM_{10} and CuM_{13} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, CuM_{25}, CuM_{49}, CuM_{194}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{25}, CuS_{50} and CuS_{195} diets. There were no significant differences in WG among fish fed $CuM_{7.0}$, CuM_{10}, CuM_{13} and CuS_{13} diets, among fish fed $CuM_{7.0}$, CuM_{25}, $CuS_{7.0}$, CuS_{10} and CuS_{13} diets, among fish fed $Cu_{1.0}$, $CuM_{4.0}$, CuM_{49}, CuM_{194}, $CuS_{4.0}$, CuS_{25} and CuS_{50} diets or among those fed $Cu_{1.0}$, CuM_{49}, CuM_{194}, $CuS_{4.0}$, CuS_{25}, CuS_{50} and CuS_{194} diets. Specific growth rate (SGR) of fish fed CuM_{10} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, CuM_{25}, CuM_{49} CuM_{194}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{25}, CuS_{50} and CuS_{195} diets. There were no significant differences in SGR among fish fed $CuM_{7.0}$, CuM_{10}, CuM_{13} and CuS_{13} diets, among fish fed $CuM_{7.0}$, CuM_{25}, $CuS_{7.0}$, CuS_{10} and CuS_{13} diets, among fish fed $CuM_{4.0}$, CuM_{25} CuM_{49}, $CuS_{7.0}$ and CuS_{10} diets.

Feed efficiency (FE) of juvenile beluga sturgeon fed CuS_{195} diet was significantly lower than those of fish fed the other diets, except fish fed CuM_{194} diet. Feed efficiency of fish fed $CuM_{7.0}$ diet was significantly higher than those of fish fed $Cu_{1.0}$, CuM_{25}, CuM_{49}, CuM_{194}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25}, CuS_{50} and CuS_{195} diets.

There were no significant differences in FE among fish fed $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10} and CuM_{13} diets, among fish fed $CuM_{4.0}$, CuM_{10}, CuM_{13} and CuM_{25} diets, among fish fed CuM_{25}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets or between those fed $Cu_{1.0}$ and CuM_{194} diets. Protein efficiency ratio (PER) of fish fed CuS_{195} diet was significantly lower than those of fish fed the other diets, except fish fed CuM_{194} diet. While, PER of fish fed $CuM_{4.0}$ and $CuM_{7.0}$ diets were significantly higher than those of fish fed $Cu_{1.0}$, CuM_{49}, CuM_{194}, $CuS_{4.0}$, CuS_{10}, CuS_{13}, CuS_{25}, CuS_{50} and CuS_{195} diets. Sturgeon survival did not differ significantly among treatment groups after the 12-week dietary Cu exposure, with a mean survival rate of 99.8±1.36% across all groups. The lowest condition factors (CF) and hepatosomatic indices (HSI) were observed in fish fed CuS_{195} diet. Hepatosomatic indices of fish fed $CuM_{4.0}$ and $CuM_{7.0}$ were significantly higher than those of fish fed $Cu_{1.0}$, CuM_{49}, CuM_{194}, CuS_{25}, CuS_{50} and CuS_{195} diets. There were no significant differences in CF among fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10} and CuS_{13} diets, among fish fed $Cu_{1.0}$, CuM_{10}, CuM_{13}, CuM_{25}, CuM_{49}, $CuS_{4.0}$, CuS_{10} and CuS_{13} diets or among those fed CuM_{194}, CuS_{25}, CuS_{50} and CuS_{195} diets. Condition factors of fish fed CuM_{10} was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{25}, CuM_{49}, CuM_{194}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{25}, CuS_{50} and CuS_{195} diets. However, there were no significant differences in CF among fish fed CuM_{10}, CuM_{13} and CuS_{13} diets, among fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{13}, CuM_{25}, $CuS_{7.0}$, CuS_{10} and CuS_{25} diets, or among those fed $Cu_{1.0}$, $CuM_{4.0}$, CuM_{49}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10} and CuS_{50} diets.

Proximate Composition

Table 36 and 37 shows the proximate composition of whole body and muscle of juvenile beluga sturgeon, *Huso huso*, fed the experimental diets. At the end of the experimental period, there were significant copper sources effects ($P < 0.05$) on whole body moisture, protein, lipid, as well as muscle protein and lipid content. Whole body and muscle protein and lipid in fish fed diets supplemented with organic copper (Mintrex®Cu) tended to be relatively higher than fish fed diets with inorganic copper (copper sulfate), while whole body moisture of fish fed inorganic copper significantly higher than fish fed organic copper ($P < 0.05$). Furthermore, there were significant copper levels effects on whole body moisture, protein, lipid, ash, muscle moisture, protein and lipid content. There were also significant their interaction effects on whole body moisture, muscle moisture and protein content ($P < 0.05$).

Dietary copper concentrations were negatively correlated with whole-body protein, lipid as well as muscle protein and lipid content, but positively correlated with ash content. Whole-body moisture content of fish fed CuS_{195} diet was significantly higher than those of fish fed the other diets. Whole-body moisture content of fish fed CuM_{194} was also significantly higher than those of fish fed the other diets, except for that of fish fed CuS_{195} which was significantly lower. There were no significant differences in whole-body moisture content among fish fed $CuS_{7.0}$, CuS_{13}, CuS_{25} and CuS_{50} diets, or among those fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25}, CuM_{49} and $CuS_{4.0}$ diets. Whole-body crude protein content of fish fed $CuM_{7.0}$, CuM_{13} and CuS_{13} diets were significantly higher than that of fish fed $Cu_{1.0}$, CuM_{25}, CuM_{50}, CuM_{194}, $CuS_{4.0}$, CuS_{25}, CuS_{50} and CuS_{195} diets ($P < 0.05$). Whole-body crude protein content of fish fed CuS_{195} was also significantly lower than those of fish fed the other diets. There were no significant differences in whole-body crude protein among fish fed $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, $CuS_{7.0}$, CuS_{10} and CuS_{13} diets, among fish fed $Cu_{1.0}$, $CuM_{4.0}$, CuM_{10}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10} and CuS_{25} diets, among those fed CuM_{25}, CuM_{49} and CuS_{50} diets.

Whole-body crude lipid content of fish fed CuS_{195} and CuM_{194} were significantly lower than those of fish fed the other diets. Also, whole-body crude lipid of fish fed CuM_{49} and CuS_{50} diets were significantly lower than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{25} diets. There were no significant differences in whole-body crude lipid among fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25}, $CuS_{4.0}$ and $CuS_{7.0}$ diets, among fish fed $CuM_{4.0}$, $CuM_{7.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25}, $CuS_{4.0}$, $CuS_{7.0}$ and CuS_{10} diets, among those fed $CuM_{7.0}$, CuM_{13}, CuM_{25}, $CuS_{7.0}$ CuS_{10} and CuS_{13} diets.

Whole-body ash content of fish fed CuS_{195}, CuS_{50}, CuM_{194} and CuM_{49} diets were significantly higher than those of fish fed the other diets. There were no significant differences in whole-body ash among fish fed $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25}, $CuS_{7.0}$, CuS_{10}, CuS_{13}, and CuS_{25} diets or among those fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, $CuS_{4.0}$, $CuS_{7.0}$ and CuS_{10} diets.

Muscle moisture content of fish fed CuS_{195} diet was significantly higher than those of fish fed the other diets except fish fed CuM_{49} and CuM_{194} diets. There were no significant differences in muscle moisture content among fish fed $CuM_{7.0}$, CuM_{25}, CuM_{49}, CuM_{194}, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets or among those fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25}, and CuS_{50} diets.

Muscle crude protein increased with dietary Cu up to the supplementation level of 13 mg/kg diet and then decreased in both Cu sources. Muscle protein content of fish fed CuM_{10} diet was significantly lower than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{13}, CuM_{25}, CuM_{49}, CuM_{194}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{25}, CuS_{50} and CuS_{195} diets. Muscle crude protein content of fish fed CuS_{195} diet was also significantly lower than those of fish fed the other diets. Also, muscle crude protein content of fish fed CuS_{50} and CuM_{194} diets were significantly lower than those of fish fed the other diets, except for that of fish fed CuS_{195} which was significantly higher. There were no significant differences in muscle protein among fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$ and $CuS_{7.0}$ diets.

Muscle lipid content of fish fed CuM_{10}, CuM_{13}, CuM_{25}, CuS_{13} and CuS_{25} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, CuM_{49}, CuM_{194}, $CuS_{4.0}$, CuS_{50} and CuM_{194} diets. Muscle crude lipid content of fish fed CuS_{195} diet was also significantly lower than those of fish fed the other diets. Also, muscle crude lipid content of fish fed CuM_{194} and CuS_{50} diets were significantly lower $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25}, CuM_{49}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{25} diets. There were no significant differences in muscle lipid among fish fed $Cu_{1.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25}, $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{25} diets, among fish fed $Cu_{1.0}$, $CuM_{7.0}$, CuS_{70} and CuS_{10} diets.

Enzyme Activity

Hepatic and muscle thiobarbituric acid reactive substances (TBARS), copper-zinc superoxide dismutase (Cu-Zn SOD) and glutathione peroxidase (GPx) activities are shown in Table 38 and 39. At the end of the first experiment, all parameters were significantly affected by the copper sources and levels. Also, there were copper sources and levels interaction effects on liver Cu-Zn SOD, GPx and TBARS. Furthermore, the highest value ($P < 0.05$) of Cu-Zn SOD and GPx activities in beluga sturgeons liver and muscle were observed in fish fed organic copper (Mintrex®Cu) source. Moreover, fish fed inorganic copper showed the higher value TBARS levels in the liver tissue ($P < 0.05$) than fish fed organic copper. Supplementation with copper improved antioxidant status of fish and the effects of organic copper were relatively greater, also seems to be more effective than inorganic copper in the juvenile beluga sturgeon. The results showed that fish fed with a diet supplemented with 10-13 mg/kg copper from either organic or inorganic copper had a significantly ($P < 0.05$) better liver and muscle TBARS, Cu-Zn SOD and GPx activities than those fed with the diets of ≥ 7 mg/kg diet or ≤ 49 mg Cu/kg diet.

Hepatic Cu-Zn SOD activity of fish fed CuM_{10} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{49}, CuM_{195}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{25}, CuS_{50} and CuS_{195} diets ($P < 0.05$). Hepatic Cu-Zn SOD activities of fish fed CuS_{195} diet was significantly lower than those of fish fed the other diets, except fish fed control diet. However, there were no significant differences in hepatic Cu-Zn SOD activities among fish fed CuM_{10}, CuM_{13}, CuS_{10} and CuS_{13} diets, among fish fed $CuM_{7.0}$, CuM_{13}, CuS_{10}, CuS_{13} and CuS_{25} diets, among fish fed $CuM_{7.0}$, CuM_{13}, CuM_{25}, CuM_{49}, $CuS_{7.0}$, CuS_{10}, CuS_{25} and CuS_{50} diets or among those fed $CuM_{4.0}$, CuM_{194} and CuS_{50} diets.

Hepatic GPx activity of fish fed CuM_{10} diet was significantly higher than those of fish fed the other diets, except fish fed CuM_{13} and CuS_{10} diets. Glutathione peroxidase activity of liver tissue of fish fed $Cu_{1.0}$, $CuM_{4.0}$ and $CuS_{4.0}$ were significantly lower than those of fish fed the other diets. There were no significant differences in GPx activities among fish fed CuM_{10}, CuM_{13} and CuS_{13} diets, among fish fed CuM_{13}, CuM_{25}, $CuS_{7.0}$, CuS_{10}, CuM_{13} and CuS_{25} diets, or among those fed CuM_{194}, CuS_{50} and CuS_{195} diets.

Hepatic TBARS of fish fed CuM_{194} and CuS_{195} diets were significantly higher than those of fish fed the other diets. This parameter was significantly higher for fish fed $Cu_{1.0}$, CuM_{49} and CuS_{50} diets than for those fed $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25}, $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{25} diets. There were no significant differences in hepatic TBARS activity among fish fed CuM_{49}, $CuS_{4.0}$ and CuS_{50} diets or among those fed $CuM_{4.0}$, CuM_{25}, $CuS_{4.0}$ and CuS_{25} diets.

Muscle Cu-Zn SOD activities of fish fed CuM_{10} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, CuM_{194}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{25}, CuS_{50} and CuS_{195} diets. Also, muscle Cu-Zn SOD activity of fish fed $Cu_{1.0}$ diet was significantly lower than those of fish fed other diets, except for that of fish fed $CuS_{4.0}$ diet. However, there were no significant differences in muscle Cu-Zn SOD activity among fish fed $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25}, CuM_{49}, CuS_{10} and CuS_{13} diets, or among those fed $CuM_{4.0}$, $CuM_{7.0}$, CuM_{25}, CuM_{49}, CuM_{194}, $CuS_{7.0}$, CuS_{25}, CuS_{50} and CuS_{195} diets. Muscle GPx activity of fish fed CuM_{10} was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, CuM_{194}, $CuS_{4.0}$, $CuS_{4.0}$, CuS_{50} and CuS_{195} diets. Also, muscle GPx activity of fish fed CuS_{195} was significantly lower than those of fish fed the other diets, except for that of fish fed $Cu_{1.0}$ and CuM_{194} diets.

There were no significant differences in muscle GPx activities among fish fed $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25}, CuM_{49}, $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{25} diets, among fish fed $CuM_{4.0}$, $CuM_{7.0}$, CuM_{13}, CuM_{25}, CuM_{49}, $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{25} diets, among fish fed $CuM_{4.0}$, $CuM_{7.0}$, CuM_{49}, $CuS_{4.0}$, $CuS_{7.0}$ and CuS_{50} diets or among those fed $Cu_{1.0}$, CuM_{194} and CuS_{50} diets.

Hematological Parameters

Hematological and biochemical characteristics of fish fed the experimental diets are shown in Table 40, 41, 42 and 43. At the end of the experimental period, there were significant copper sources effects ($P < 0.05$) on hemoglobin, glucose, cholesterol, peroxidase, enzymes glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and calcium content. Also, there were significant differences copper levels effects on hematocrit, hemoglobin, glucose, cholesterol, peroxidase, lysozyme activity, GOT, GPT, plasma copper, calcium and magnesium content. However, there were neither significant copper sources and levels effects nor their interaction effects on total serum protein. Furthermore, there were significant their interaction effects on lysozyme activity and GOT for fish fed all diets. Hematocrit (packed cell volume, PCV), hemoglobin and lysozyme activity increased to peak values obtained at the optimum dietary Cu (10-13 mg Cu/kg diet) level and dropped beyond this supplementation level in both copper forms, while glucose content decreased to drop values obtained at the optimum dietary Cu level and peaked beyond this supplementation level in both copper forms. Copper exposure increased cholesterol, peroxidase, GOT, GPT serum and plasma copper concentrations with increasing levels.

Hematocrit counts of fish fed CuM_{10} diet was significantly higher than those of fish fed $Cu_{1.0}$, CuM_{49}, CuM_{194}, CuS_{25}, CuS_{50} and CuS_{195} diets ($P < 0.05$). Also, PCV content of fish fed CuS_{195} was significantly lower than those of fish fed ≤25 mg Cu/kg diets in both Cu sources. There were no significant differences in PCV among fish fed $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10} and CuS_{13} diets, among fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{13}, CuM_{25}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10} and CuS_{13} diets or among those fed CuM_{49}, CuM_{194}, CuS_{50} and CuS_{195} diets.

Hemoglobin of fish fed CuM_{13} diet was significantly higher than those of fish fed CuM_{194}, CuS_{25}, CuS_{50} and CuS_{195} diets. There were no significant differences in Hb among fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25}, CuM_{49}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10} and CuS_{13} diets, among fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{25}, CuM_{49}, CuM_{194}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10} and CuS_{13} diets or among those fed $Cu_{1.0}$, CuM_{25}, CuM_{49}, CuM_{194}, CuS_{13}, CuS_{25}, CuS_{50} and CuS_{195} diets.

Glucose content of fish fed CuM_{10} and CuM_{13} diets were significantly lower than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{25} and CuS_{194} diets. There were no significant differences in glucose content among fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{49}, CuM_{194}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{25}, CuS_{50} and CuS_{195} diets or among those fed $CuM_{4.0}$, $CuM_{7.0}$, CuM_{25}, CuM_{49}, CuM_{194}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{50} and CuS_{195} diets. The total serum protein content was not affected by copper exposure. Cholesterol content of fish fed $Cu_{1.0}$, $CuM_{4.0}$ and $CuS_{4.0}$ diets were significantly lower than those of fish fed CuM_{10}, CuM_{13}, CuM_{25}, CuM_{49}, CuM_{194}, CuS_{13}, CuS_{25}, CuS_{50} and CuS_{195} diets. There were no significant differences in cholesterol content between fish fed CuM_{25}, CuM_{49}, CuS_{25}, CuS_{50} and CuS_{195} diets, or among those fed $CuM_{7.0}$, CuM_{10}, CuM_{13}, $CuS_{7.0}$, CuS_{10} and CuS_{13} diets. Peroxidase content of serum of fish fed CuM_{194} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{195} diets. However, there were no significant differences in peroxidase content among fish fed CuM_{13}, CuM_{25}, CuM_{49}, CuS_{50} and CuS_{195} diets, among fish fed $CuM_{7.0}$, CuM_{10}, CuM_{13}, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25}, CuS_{50} and CuS_{195} diets or among those fed $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{25} diets.

Lysozyme activity increased with dietary Cu up to the supplementation levels of 9.5 mg Mintrex®Cu/kg and 13.1 mg Cu sulfate/kg diet, respectively and then decreased. However, lysozyme activity of fish fed CuM_{10} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{13}, CuM_{25}, CuM_{49}, CuM_{194}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25}, CuS_{50} and CuS_{195} diets. However, there were no significant differences in lysozyme activity among fish fed $CuM_{7.0}$, CuS_{10} and CuS_{13} diets, among fish fed $CuM_{7.0}$, CuS_{10} and CuS_{25} diets, or among those fed $Cu_{1.0}$, $CuM_{4.0}$, CuM_{194}, $CuS_{4.0}$ and CuS_{195} diets.

After 12 weeks of exposure, GOT concentrations of fish fed CuS_{195} was significantly higher than those of fish fed other diets except for that of fish fed CuS_{25} diet. There was no significant differences GOT serum concentration between fish fed CuM_{194} and CuS_{25} diets, among fish fed CuM_{49}, CuM_{194}, CuS_{13} and CuS_{50} diets, among fish fed $CuM_{7.0}$, CuM_{10} and CuM_{13} diets or among those fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$ and CuM_{10} diets.

Glutamic pyruvic transaminase (GPT) in the serum of fish fed CuS_{195} was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{50} diets. There were no significant differences GPT serum content among fish fed CuM_{49}, CuM_{194}, $CuS_{7.0}$, CuS_{25} and CuS_{50} diets, among fish fed CuM_{25}, CuM_{49}, CuS_{13}, CuS_{25} and CuS_{50} diets or among those fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10} and CuS_{13} diets. There were no significant differences in serum calcium concentration of fish in all treatments except for serum calcium concentration of fish fed CuS_{195} diet which was significantly lower than those of fish fed CuM_{10} and CuM_{13} diets.

Serum magnesium concentrations of fish fed CuM_{194} and CuS_{195} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, $CuS_{4.0}$, $CuS_{7.0}$ and CuS_{10} diets. There were no significant differences magnesium serum concentration among fish fed CuM_{13}, CuM_{25}, CuM_{49}, Cum_{194}, CuS_{13}, CuS_{25}, CuS_{50} and CuS_{195} diets, among fish fed $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25}, CuM_{49}, CuS_{10}, CuS_{13}, CuS_{25} and Cu_{50} diets or among those fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{25}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13} and CuS_{50} diets.

Copper Concentration

Table 44 and 45 shows the whole-body and tissue Cu concentrations of fish fed the experimental diets. There were significant copper sources effects on whole body, liver, gill and muscle copper concentration. There were also significant copper levels effects on whole body, and liver, intestine, kidney, gill and muscle Cu content. Furthermore, there were significant their interaction effects on whole body, liver and muscle copper concentration. However, there were no significant their interaction effects on intestine, kidney and gill Cu content for fish fed all diets.

Copper content in the liver of fish fed by from either dietary organic or inorganic copper sources showed a statistically significant rise, also, the results showed that hepatic Cu accumulations rate was higher in fish fed diets with inorganic Cu than in fish fed diets with organic copper. But, there was a trend that fish fed diets with organic copper had relatively higher muscle copper concentrations than those fed diets with equal levels of copper from inorganic copper. Copper accumulated in the whole body, liver, intestine, gill, muscle and kidney tissues in a dose-dependent manner. Copper accumulated most in liver, followed by intestine, gills, muscle and then kidney. Copper concentration in liver of fish fed CuS_{195} diet was significantly higher than values for the other treatments ($P < 0.05$).

Copper content of liver tissue of fish fed CuS_{50} diet was also significantly higher than those of fish fed the other diets, except for that of fish fed CuS_{195} diet which was significantly lower.

Also, liver capper concentration of fish fed CuM_{194} diet was significantly higher than those of fish fed the other diets, except for that of fish fed CuS_{50} and CuS_{195} diets which were significantly lower. Liver copper concentration of fish fed CuM_{49} diet was also significantly higher than those of fish fed the other diets, except for that of fish fed CuS_{50}, CuS_{195} and CuM_{194} diets which was significantly lower. For those other treatments, this parameter was significantly higher at each dietary Cu supplementation level than they were for the preceding one. There were no significant differences in liver Cu content among fish fed $Cu_{1.0}$, $CuM_{4.0}$ and $CuS_{4.0}$ diets or among those fed $CuM_{7.0}$, CuM_{10}, $CuS_{7.0}$ and CuS_{10} diets.

Intestine copper contents of fish fed CuM_{49}, CuM_{194}, CuS_{50} and CuS_{195} diets were significantly higher than those of fish fed ≤25 mg Cu/kg diets, independent on copper sources. Also, Cu content of intestine tissue of fish fed CuM_{25} and CuS_{25} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, $CuS_{4.0}$, $CuS_{7.0}$ and CuS_{10} diets. There were no significant differences in intestine Cu content among fish fed $CuM_{7.0}$, CuM_{10}, $CuS_{7.0}$, CuS_{10}, and CuS_{13} diets, among fish fed $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10} and CuS_{13} diets or among those fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, $CuS_{4.0}$, $CuS_{7.0}$ and CuS_{10} diets.

Gill Cu contents of fish fed CuM_{194} diet was significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets. There were no significant differences in gill Cu content among fish fed CuM_{49}, CuS_{50} and CuS_{195} diets, or among those fed $Cu_{1.0}$, $CuM_{4.0}$ and $CuS_{4.0}$ diets.

Muscle copper concentrations of fish fed CuM_{194} diet was significantly higher than values for the other diets. Copper content of muscle tissue of fish fed CuS_{194} diet was also significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, CuM_{25}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{13}, CuS_{25} and CuS_{50} diets. There were no significant differences in Cu content of muscle among fish fed CuM_{25}, CuM_{49} and CuS_{50} diets, among fish fed CuM_{25}, CuS_{25} and CuS_{50} diets, among fish fed CuM_{13}, CuS_{25} and CuS_{50} diets or among those fed $CuM_{4.0}$, $CuM_{7.0}$, $CuS_{4.0}$, $CuS_{7.0}$ and CuS_{10} diets.

Kidney copper concentrations of fish fed CuM_{194} and CuS_{195} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10} and CuS_{13} diets. There were no significant differences Cu content of kidney among fish fed CuM_{13}, CuM_{25}, CuM_{49}, CuM_{194}, CuS_{25}, CuS_{50} and CuS_{195} diets, among fish fed CuM_{13}, CuM_{25}, CuM_{49}, CuS_{13}, CuS_{25} and CuS_{50} diets or among those fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, $CuS_{4.0}$, $CuS_{7.0}$ and CuS_{10} diets.

Copper plasma contents of fish fed CuM_{194} and CuS_{195} diets were significantly higher than those of fish fed other diets. Also, plasma copper concentration of fish fed CuM_{49} diets were significantly higher than those of fish fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{10}, CuM_{13}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10} and CuS_{13} diets. However, there were no significant differences in plasma copper content among fish fed CuM_{25}, CuS_{13}, CuS_{25} and CuS_{50} diets, among fish fed CuM_{13}, CuM_{25}, CuS_{13} and CuS_{25} diets, or among those fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$ and $CuS_{4.0}$ diets.

Whole-body Cu concentrations of fish fed CuM_{194} diet were significantly higher than those of fish fed the other diets. Also, whole-body Cu concentrations of fish fed CuS_{195} and CuM_{49} diets were significantly higher than those of fish fed the other diets, except for that of fish fed CuM_{194} which was significantly higher. Whole body Cu concentration of fish fed CuS_{50} diet was also significantly higher than those of fish fed ≤13 mg Cu/kg diets of both Cu sources. However, there were no significant differences in muscle Cu concentrations among fish fed CuM_{13}, CuM_{25}, CuS_{10} and CuS_{25} diets or among those fed $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, $CuS_{4.0}$ and $CuS_{7.0}$ diets.

Discussion

Although Cu concentrations in tanks of fish fed the copper-supplemented diets were higher than that in the tanks of fish fed the control diet, the differences were not statistically significant. It is therefore unlikely that the leaching of Cu from feed and/or faeces to water had an effect on growth or whole-body Cu contents in the present study. In the present study, optimum dietary Cu level determined with Mintrex®Cu for beluga were 7-10 mg Cu/kg diet. This value is considerably lower than the value (10-13 mg Cu/kg diet) obtained for the same species fed copper sulfate. It should be noted that, growth performance obviously higher in fish fed diets supplemented with Mintrex®Cu, compared to fish fed the with equal levels of copper from inorganic. This close agreement suggests that beluga have higher ability to retain and absorbtion copper from chelated sources. Lin et al. (2010) reported that grouper, *E. malabaricus*, can better utilize organic Cu (Cu peptide) than inorganic Cu.

Several factors can impede intestinal absorption of inorganic nutrients. Phytic acid, which complexes many inorganic nutrients (Spinelli et al. 1983), apparently is responsible to some degree for the lower net retention of the minerals in the practical diet. Interaction with other minerals or nutrients can decrease intestinal absorption of inorganic nutrients (Paripatananont and Lovell 1997). This study indicates that chelated trace elements are appreciably more absorbable and bioavailability than inorganic sources, and suggests that lower amounts of the chelated forms may be used as supplements in commercial feeds. Chelated Cu might be absorbed and transported intact to target tissues and be more available in metabolic processes than inorganic copper. Paripatananont and Love11 (1995) found that bioavailability of chelated zinc for growth of channel catfish was 352% of that of zinc sulfate and subsequently found that digestibility of the organic zinc was only 140% of that of the inorganic zinc (Paripatananont and Lovell 1997). Apparently, other factors besides net absorption are responsible for the higher bioavailability of organic compared to inorganic trace elements. Kucukbay et al. (2009) reported that selenomethionine supplementation seems to be more effective than sodium selenite on performance of rainbow trout reared under crowding conditions. In this study, fish fed 10 mg Mintrex®Cu/kg diet had highest WG (1777) compares favorably with those in similar sized fish but fed inorganic Cu a nutritionally adequate diet for 12 wks, in which 1623 WG was observed. Similar results were found in grouper, *E. malabaricus* (Lin et al. 2010).

Selenomethionine is a predominant chemical form of organic selenium in feedstuffs due to their excellent bioavailability and has been reported to have higher bioavailability than inorganic Se (sodium selenite) for Atlantic salmon (Bell and Cowey 1989; Lorentzen et al. 1994) and channel catfish (Wang and Lovell 1997). Copper functions biochemically as a component of several Cu dependent enzymes and as a cofactor for numerous other enzymes (Das et al. 2010). It is possible that high dietary Cu concentration enhance growth of beluga sturgeon by stimulating activities of the enzymes involved in nutrient utilization. Lim and Paik (2006) found dietary supplementation of 100 ppm Cu-methionine chelate increased the performance of layer compared to the control diet. Beluga survival is not very sensitive to dietary copper levels, fed a diet containing between 1.1 and 195 mg Cu/kg for 12 wks, and exhibited a mean survival rate of 99.8 ± 1.36 % across all groups. For comparison, it has been reported that white sturgeon, *A. transmontanus*, exposed to dietary concentrations ranging between 0.4 and 191.1 mg Se/kg diets for 8 wks exhibited a mean survival rate of survival rate of 99.0 ± 0.34 % (Tashjian et al. 2006).

Both the type of diet and the source of mineral had a significant effect on proximate composition of all minerals. In the present experiment, whole body protein and lipid were decreased with elevated dietary copper, independent on copper source, and there was a trend that fish fed diets with Mintrex®Cu had relatively higher whole body protein and lipid than those fed diets with equal levels of copper from copper sulfate. DeBoeck et al. (1997) reported decreased liver and muscle protein, lipid and glycogen content in the common carp exposed to waterborne Cu despite normal feed consumption. Increased metabolic expenditure for detoxification and maintenance of homeostasis was assumed to cause this change in energy stores. Since elevated dietary Cu concentrations are known to cause an onset of adaptive responses in Atlantic salmon (Berntssen et al. 1999), depletion of energy stores due to increased metabolic rate may also occur simultaneously. In organisms, oxidative metabolism generates the continuous production of superoxide radicals and hydrogen peroxide. When reactive oxygen species (ROS) overwhelms the cellular defences, such as in stress conditions, oxidative stress occurs (Kelly et al. 1998). Under oxidative stress induced by ROS, the lipid peroxidation process can lead to cell membrane damage (Sweetman et al. 2010). Reactive oxygen species could be effectively scavenged by the antioxidant defence systems including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Atencio et al. 2009).

In this study, fish consuming diets supplemented with 9.5 mg Cu/kg from Mintrex®Cu, or 13.1 mg Cu/kg from copper sulfate had significantly higher Cu-Zn SOD activities than those fed low and high Cu concentrations. It was further found that either deficient or excess dietary copper induced oxidative stress for lower SOD and GPx activities observed in fish fed low-Cu and high-Cu diets. Therefore, the protective function of Cu serving as the antioxidation should be within a certain range of concentrations. However, in the present study, beluga sturgeon fed copper from the Mintrex®Cu had better growth and higher GPx and Cu-Zn SOD activities than those fed Cu from the copper sulfate. Reasons for this variation in bioavailability among measurement criteria are not readily apparent. This indicates that the positive effect of chelation on bioavailability of Cu goes beyond absorption. Wang and Lovell (1997) observed that growth and hepatic GSH-Px activity in channel catfish fed selenium from sodium selenite (Na_2SeO_3) significantly lower than fish fed selenomethionine (Se-M). Gatlin and Wilson (1986) reported that liver Cu-Zn SOD in channel catfish was reduced when dietary copper level reached 8 mg/kg, but recovered when copper level increased to 10 mg/kg, and similar results were found in grouper (Lin et al. 2008).

In our study, thiobarbituric acid reactive substances (TBARS) value were obviously lower in fish fed diets supplemented with CuM_{10} than fish fed the $Cu_{1.0}$, $CuM_{4.0}$, $CuM_{7.0}$, CuM_{25}, CuM_{49}, CuM_{194}, $CuS_{4.0}$, $CuS_{7.0}$, CuS_{10}, CuS_{25}, CuS_{50} and CuS_{195} diets ($P < 0.05$). The hepatic TBARS value showed an inverse trend with the performance of hepatic Cu-Zn SOD activity (Table 39). The high hepatic TBARS values in fish fed the Cu-deficient diet might be due to low activity of Cu-Zn SOD. It is suggested, then, that excess Cu stored in the liver caused high oxidative stress and destroyed the enzyme activity. Although there was no apparently toxicity to the beluga fed high levels of dietary Cu, hepatic lipid peroxidation was found to be enhanced by elevating dietary Cu. This could be due to the increasing level of Cu stored in liver tissue when dietary Cu was increased. This is further supported by the fact that liver TBARS was positively correlated to liver Cu content in fish fed high Cu concentration. It has also been well documented that transition metals such as copper and iron are potent catalysts for lipid peroxidation in biological systems (Kanner et al. 1987) and are capable of initiating or promoting lipid peroxidation through either non-enzymatic or enzymatic pathways (McDonald & Hultin 1987). Thus, it is surprising that TBARS increase with tissue Cu content.

Táati et al. (2011) also observed that hemoglobin and hematocrit values in beluga sturgeon ranged, respectively, from 4 g/dl and 23% to 6 g/dl and 26%. The beluga in the present study fed the high Cu-supplemented diet of both Cu forms, had lower-than-normal hematocrit (21 %), but only fish fed high Cu-supplemented had lower-than-normal hemoglobin (3.6 g/dl) levels. Also, in current study, fish fed inorganic Cu had higher Glutamic oxaloacetic transaminase (GOT) and Glutamic pyruvic transaminase (GPT) than fish fed organic Cu ($P < 0.05$). There was a statistically significant increase in GOT and GPT levels in Cu supplementation up to 25 mg/kg form Mintrex®Cu, while the level in 13 mg/kg form copper sulfate when compared with the control diet. Increased serum GPT activity usually is suggestive of either hepatocyte death or sublethal hepatocyte injury, but necrosis or sublethal damage to muscle cells must be considered as well (Gharaei et al. 2011). Most of orally administered copper was distributed in various tissues and organs, especially to vessel organs such as liver via blood, shortly after intake (NRC 2000). Serum enzymes can be result in increased serum enzyme activity by two ways, either due to leakage from damage or due to induction that increases the amount of enzyme and hence the amount that is also likely to leak. It also important to mention that the induction may occur as a result of the organism's mobilizing energy sources, including amino acids, to manage stress and to repair damage caused by metals (Masola et al. 2008). The influence of supplemental Cu-salt with different dose to beluga sturgeon could not produce any significant alteration in plasma glucose and proteins and enzyme concentrations.

There were significant copper levels and also significant copper sources and levels interaction effects on lysozyme activity ($P < 0.05$). Lysozyme activity of fish fed 10 mg Cu/kg diet was significantly higher than those of fish fed other levels. Highest lysozyme activity results were obtained with juvenile beluga sturgeon fed the diet containing CuM_{10} diet. Lysozyme activity is commonly used as a non-specific immune response indicator in fish (El-Mowafi et al. 1997; Lin & Shiau 2003, 2007; Puangkaew et al. 2004). The result showed the similar pattern as for antioxidant indicators. It clearly provided the evidence that adequate Cu is an immunostimulant for beluga sturgeon. But neither lysozyme activity, complement haemolytic activity, total protein in serum nor blood haemoglobin of Atlantic salmon was affected by dietary Mg (El-Mowafi et al. 1997), which had also no effect on the resistance of juvenile channel catfish to Edwarsiella ictaluri challenge (Lim & Klesius 2003). Maybe different species led to the differences.

Although previous data and the present study support that magnesium (Mg), has a strong relation with the immune system (both non-specific and specific immunity) (Tam et al. 2003), further research is necessary to investigate the role of Cu in different biological processes related directly or indirectly with the immune system in beluga. Furthermore, in the rainbow trout, *Oncorhynchus mykiss*, lysozyme activity rose while phagocytosis decreased after 5-d exposure to distinct concentrations of mercury (Hg), cadmium (Cd), and zinc (Zn) (Sánchez-Dardón et al. 1996). Lysozyme activity shows different responses to chemical contamination in different species, perhaps reflecting distinct physiological and biochemical mechanisms which control the normal body burden and toxicity of xenobiotics.

Higher dietary copper significantly increased copper deposition and accumulation in beluga tissues. It was obvious that the copper contents in beluga sturgeon tissue were markedly changed with the addition of dietary Cu and higher content of muscle and gill Cu was found in Mintrex®Cu, compared to fish fed the copper sulfate. The results indicated that probably, Mintrex®Cu and copper sulfate had different metabolic pathways (Lin et al. 2010), although both inorganic and organic forms cross the intestinal barrier. One goal of aquaculture is to produce fish fillets for human consumption. Hence, it is of interest whether the nutritional value of fish fillets can be manipulated through the diet. No significant differences were found in muscle Cu concentrations between fish fed ≤50 mg Cu/kg diet in both Cu sources. However, the result is lower Cu content of fish fed organic Cu than the values obtained for inorganic Cu (Table 44).

Liver copper concentration is regarded as the most sensitive in evaluating copper status (Apines-Amar et al. 2003). In the present experiment, liver copper concentrations were increased with elevated dietary copper, independent on copper source, and there was a trend that fish fed diets with higher 25 mg $CuSO_4$/kg had relatively higher liver copper concentrations than those fed diets with equal levels of copper from Mintrex®Cu. This phenomenon was also reported in pig (Coffey et al. 1994), cow (Du et al. 1996), ewe (Eckert et al. 1999) and juvenile grouper, *Epinephelus malabaricus* (Lin et al. 2010). This lower accumulation rate of Cu in organic Cu-fed fish also provides additional evidence for why organic Cu had a higher toxicity threshold for beluga than inorganic Cu in this study. High levels of Cu accumulation in the liver might cause toxic oxidative stress for fish (Lin and Shiau 2007; Lin et al. 2010).

Addition of Cu increases plasma Cu concentration (Luo and Dove 1996; Mondal et al. 2007) indicating that dietary Cu levels were reflected in plasma Cu concentration which is consistent with the present findings. However, considering that normal plasma copper concentration in fish ranges from 8 to 15 mg/l Cu, and there was a significant asymptotic effect of dietary copper supplementation level upon plasma copper concentration, it is suggested that beluga sturgeon fed diets supplemented with less than 7 mg Cu/kg of both Cu scoures, were Cu-deficient, as observed in the present study. Supplemental Cu in the organic form including Cu-lysine, Cu-methionoine and Cu- proteinate was absorbed equal to or greater than that from cupric sulphate (Kincaid et al. 1986; Baker et al. 1991) increase plasma Cu concentration, which corroborates the present findings.

Bioavailability indicates the absorption and utilization of a nutrient. The bioavailability values based on tissue copper concentrations was higher in fish fed Mintrex®Cu than fish fed copper sulfate, which was similar in response to the growth performance stated above. In addition, adding chelated trace minerals to the diet may minimize inhibitory interaction with other minerals and dietary factors (e.g., phytate and fiber) compared with inorganic sources (Peter and Mahan 2008; Lin et al. 2010). Another potential benefit of stable chelated organic minerals for aquatic species is reduced solubility in water (Guo et al. 2001). Although there may be numerous advantages to using organic Cu sources in aquaculture, organic Cu studies in fish nutrition thus far have only been done on one fish species. Apines-Amar et al. (2003) and Apines-Amar et al. (2004) indicated that trace elements chelated with amino acids, which include copper, zinc, and manganese (Mn), seem to be more available for mineral deposition than those from inorganic compounds in rainbow trout. This is because organic minerals are generally considered less sensitive to the inhibitory action of other compounds due to the different absorption pathway (Ashmead 1992). Wieght gain peaked at the dietary Mintrex®Cu supplementation level of 9.5 mg/kg diet and dropped at higher levels. But WG peaked at the dietary inorganic Cu ($CuSO_4.5H_2O$) supplementation level of 13.1 mg/kg diet and dropped. These differential levels required for growth reduction suggest that beluga sturgeon have a lower toxicity threshold for inorganic Cu than organic Cu. Similarly, Acda et al. (2002) reported for pig and Lin et al. (2010) for juvenile grouper, *Epinephelus malabaricus*.

One reason for the higher bioavailability of organic Cu to beluga sturgeon is enhanced absorption. Also, WG was highest in fish fed the diet with CuM_{10} and CuM_{13} diets, followed by fish fed the diet with CuM_7 and CuS_{13} diets (Table 34-35). The higher cost of most mineral chelates relative to inorganic sources has generally limited their use in aquaculture to date. If only considering cost, a half-supplementation level of organic Cu, which, according to this study, should produce growth equivalent to a whole supplementation of inorganic Cu, in fish diets may not be a good candidate as a dietary source. Considering the economic aspect, supplementing diets with 10 mg organic Cu/kg diet enables the fish farmer to use 5% less feed when compared with fish fed the supplemented diets with inorganic Cu, which is equivalent to savings of about 100 g feed per kg body weight gain. This aspect is of practical importance for the fish farmer both from an environmental and an economic viewpoint. This is in accordance with Becker et al. (1999) and Mohseni et al (2008), who addressed the cost-effectiveness of feeding an $_L$-carnitine supplemented diet to hybrid tilapia and beluga sturgeon. The optimization of feed consumption and improved growth can be obtained simultaneously because of better feed conversion ratio. Thus, less feed is required to attain the same final weight. Considering the reduction in feed costs, and despite the high price of organic Cu, the farmer would still benefit from a substantial reduction in the production costs. Nevertheless, Cu solubility and its excretion from cultured animals due to high ingestion continue to draw environmental concern and hence lend importance to organic mineral research.

In conclusion, this study of fish utilization of organic Cu was the first of its kind, revealing through growth performance and physiological responses, that beluga can better utilize organic Cu (Mintrex®Cu) than inorganic Cu (copper sulfate $CuSO_4.5H_2O$). Use of organic copper will have advantages environmentally because less copper will go into the aquacultural system. Optimal copper requirements for juvenile beluga sturgeon were ranging from 9.71 to 10.3 mg Cu/kg, independent of copper sources (organic and inorganic Cu, respectively). Although the cost of using organic minerals is high even relative to the lower adequate dietary Cu requirement for organic-form Cu found in this study, the lower environmental impact due to higher utilization rate and lower solubility support the need for further research.

Chapter 4: General discussion and summary

I. Discussion

Four feeding trials were conducted to evaluate the optimum dietary organic (Mintrex®Cu, a chelated dietary copper source) and inorganic (copper sulfate, $CuSO_4.5H_2O$) copper levels on growth performance, nutrient utilization, whole-body proximate composition, immune responses, hematological parameters and tissue copper concentration in juvenile olive flounder, *Paralichthys olivaceus* (initial body weight 6.88 ± 0.05 g), and beluga sturgeon, *Huso huso* (initial body weight 2.46 ± 0.09 g). The result of the present study clearly demonstrates that juvenile olive flounder and beluga sturgeon have Cu requirements that cannot be met by Cu in the rearing water, thus, dietary supplementation is necessary.

Optimum copper supplementation in juvenile olive flounder and beluga sturgeon diets both in organic and inorganic forms improved growth performance, antioxidant status and hematological values of both species but the effects of organic Cu were relatively greater than those of inorganic Cu. The study has been showed 30% of fish fed by inorganic Cu and 70% of fish fed organic Cu, could be used for selective breeding and caviar extraction according to growth performance and remaining 70% and 30% will be discarded. Further studies are needed to declare the better growth performance with the use of copper sources.

Dietary copper source and concentration significantly influenced body composition of juvenile olive flounder and beluga sturgeon. Whole body protein and lipid content negatively correlated with dietary copper concentrations, while ash content was positively correlated with dietary Cu contents. Tissue deposition of lipid, protein and ash is dependent on feed intake, metabolic use and intestinal absorption, and these factors can all be influenced by elevated dietary Cu concentrations.

Furthermore, both a deficiency and excess in dietary Cu reduced growth performance and superoxide dismutase (Cu-Zn SOD) activities. This may be the reason why juvenile olive flounder and beluga sturgeon fed the basal and high Cu diets exhibited higher thiobarbituric acid reactive substances (TBARS) values. Moreover, free Cu has been reported to induce the oxidation and destruction of SOD in vitro.

It is suggested, then, that excess Cu stored in the liver caused high oxidative stress and destroyed the enzyme activity. This further clarifies why the lower Cu accumulation rate in organic Cu may be favorable for fish fitness.

Generally, Blood parameters of fish are suitable biomarkers for evaluating the potential risk of chemicals. However, the present findings indicate that in the juvenile olive flounder and beluga sturgeon, sub-chronic dietary exposure to Cu has significant effect on blood parameters and serum chemistry. Generally, metal exposure can result in gill damage, which in turn can affect blood parameters. Serum GOT and GPT are important diagnostic tools in medicine and are used to detect the toxic effects by various pollutants. Our results show that dietary Cu exposure increased GOT and GPT serum concentrations with increasing time and dose. Therefore, increases in GOT and GPT activities in serum of juvenile beluga sturgeon, is assumed to be a result of liver damage by Cu. In the present study, lysozyme activity increased with dietary Cu up to the optimum supplementation levels and then decreased at higer levels. Also, in this study, lysozyme activity was negatively correlated with TBARS values, suggesting that immunity in beluga was impaired due to oxidative stress induced by high Cu ingestion.

Liver of juvenile olive flounder and beluga sturgeon is a more important storage tissue than other tissues and Cu accumulation clearly reflected the level of dietary exposure. Our study also suggested that hepatic Cu accumulation rate was higher in fish fed diets with inorganic Cu than in fish fed diets with organic Cu. High levels of Cu accumulation in the liver might cause toxic oxidative stress for fish. This lower accumulation rate of Cu in organic Cu-fed fish also provides additional evidence for why organic Cu had a higher toxicity threshold for juvenile olive flounder and beluga sturgeon than inorganic Cu in this study.

The present experiment indicated that, fish utilization of organic Cu was the first of its kind, revealing through growth performance, physiological response and Cu retention data that juvenile olive flounder and beluga sturgeon can better utilize organic Cu than inorganic Cu. Although the cost of using organic minerals is high even relative to the lower adequate dietary Cu requirement for organic-form Cu found in this study, the lower environmental impact due to higher utilization rate and lower solubility support the need for further research.

II. Summary

I - The optimum dietary organic copper level in juvenile olive flounder:

The optimum dietary Cu level could be higher than 8.44 mg Cu/kg diet but less than 10 mg Cu/kg diet in juvenile olive flounder, *P. olivaceus,* when Mintrex®Cu is used as the dietary source of organic copper, by growth performance, red blood cell, copper-zinc superoxide dismutase (Cu-Zn SOD), thiobarbituric acid-reactive substances (TBARS) and Broken Line analysis. Whole-body and muscle crude lipid decreased while whole-body crude ash increased with dietary Cu content. Copper concentrations in tissue and whole-body from fish increased with dietary copper level.

II- The optimum dietary inorganic copper level in juvenile olive flounder:

The optimum dietary Cu level could be higher than 9.05 mg Cu/kg diet but less than 10 mg Cu/kg diet in juvenile olive flounder, *P. olivaceus,* when copper sulfate is used as the dietary source of inorganic copper, by growth performance, red blood sell, copper-zinc superoxide dismutase (Cu-Zn SOD), thiobarbituric acid-reactive substances (TBARS) and Broken Line analysis. Whole-body lipid content of fish decreased while whole-body ash increased with dietary copper levels. Whole-body and tissue copper concentrations increased with dietary copper levels.

Comparison of effects of dietary copper sources and levels on growth, enzyme activity and tissue copper concentration in juvenile olive flounder:

This research had demonstrated that different Cu source supplemented in basal diet could improve the growth performance; hence, dietary copper is essential for juvenile olive flounder. The optimum dietary level for organic Cu could be > 8.44 but ≤ 10 mg Cu/kg diet, and for inorganic Cu level could be > 9.05 but ≤10 mg Cu/kg diet. This study indicates that Cu allowance in diets of olive flounder can be reduced when organic Cu replaces inorganic copper. Organic Cu supproted better performance, increases nutrient utilization, improves physiological responses and greater effectiveness than inorganic Cu.

III - The optimum dietary organic copper level in juvenile beluga sturgeon:

The optimum dietary Cu level could be higher than 9.71 mg Cu/kg diet but less than 13 mg Cu/kg diet in juvenile beluga sturgeon, *Huso huso,* when Mintrex®Cu is used as the dietary source of organic copper, by growth performance, copper-zinc superoxide dismutase (Cu-Zn SOD), lysozyme activity, thiobarbituric acid-reactive substances (TBARS) and Broken Line analysis. Crude protein in muscle and whole-body as well as lysozyme activity peaked at the dietary Cu supplementation level of 13.1 mg/kg diet and dropped. Glutamic oxaloacetic transaminase (GOT), Glutamic pyruvic transaminase (GPT) and Cu concentration in tissues increased with dietary copper.

IV- The optimum dietary inorganic copper level in juvenile beluga sturgeon:

The optimum dietary Cu level could be higher than 10.3 mg Cu/kg diet but less than 13 mg Cu/kg diet in juvenile beluga sturgeon, *Huso huso,* when copper sulfate is used as the dietary source of inorganic copper, by growth performance, red blood sell, copper-zinc superoxide dismutase (Cu-Zn SOD), thiobarbituric acid-reactive substances (TBARS) and Broken Line analysis. Whole-body lipid content of fish decreased while whole-body ash increased with dietary copper levels. Whole-body and tissue copper concentrations increased with dietary copper levels.

Comparison of effects of dietary copper sources and levels on growth, enzyme activity and tissue copper concentration in juvenile beluga sturgeon:

This research had demonstrated that different Cu source supplemented in basal diet could improve the growth performance; hence, dietary copper is essential for juvenile beluga sturgeon. The optimum dietary level for organic Cu could be > 9.71 but ≤ 13 mg Cu/kg diet, and for inorganic Cu level could be > 10.3 but ≤ 13 mg Cu/kg diet. Weight gain, SGR and feed efficiency in fish fed diets supplemented with Mintrex®Cu tended to be relatively higher than fish fed diets with copper sulfate. While, fish fed with copper sulfate had relatively higher liver Cu concentrations than those fed diets with equal levels of copper from Mintrex®Cu. Organic Cu supproted better performance, increased nutrient utilization, improved physiological responses and greater effectiveness than inorganic Cu in juvenile beluga sturgeon.

Table 1. Meat quality of some sturgeon species

Species	Meat (%)	Water (%)	Protein (%)	Oil (%)	Energy (kcal/100 g)
Huso huso	63	73.8	16.6	6.7	136
A. *Guldenstadti*	64	67.0	18.8	12.5	188
A. *Nudiventris*	62	69.7	18.7	10.2	-
A. *stellatus*	63	70.7	19.0	8.6	153

Table 2. Composition of the basal diet

Ingredients	% Dry matter (DM)
Casein §	42
Gelatin§	7
Flounder muscle †	10
Wheat meal‡	4
Dextrin§	8
Corn starch§	10
Fish oil (EPA + DHA enriched) ¶	9
Vitamin premix††	3
Mineral premixture (Cu-free) ‡‡	2
Alhpa cellulose §	5
Premix of Cu (by using organic or inorganic copper)	

† Lyophilized Japanese flounder muscle.
‡Young Nam Flour Mills Co., Busan, Korea.
isheries Co-operative Feeds Co. Ltd., Korea.
§ United States Biochemical, Cleveland, OH, USA.
¶ E-Wha oil Co. Ltd., Pusan, Korea.

†† Vitamin mixture (Contains as mg/kg diet): ascorbic acid, 300; dl-calcium pantothenate, 150; choline bitartrate, 3000; inositol, 150; menadione, 6; niacian, 150; pyridoxine·HCl, 15; riboflavin, 30; thiamine mononitrate, 15; dl-(-tocopherol acetate, 201; retinyl acetate, 6; biotin, 1.5;folic acid, 5.4; B12, 0.06; cholecalciferol, 2.4. Vitamin mixture was prepared by our laboratory (Feeds and Foods Nutrition Research Center, Pukyong National University, Busan, Korea) and the individual vitamins were purchased from United States Biochemical.

‡‡ Mineral mixture (Cu free; as g/kg pre-mixture): MnSO4, 1.94; ZnSO4, 4; FeSO4, 6.81; CaCO3, 255.1; MgSO4, 173.9; K2SO4, 164.7;NaCl, 60.52; NaSeO3, 0.01; KI, 0.2; AlCl3, 0.37; CoCl2, 0.08. Mineral mixture was prepared by our laboratory (Feeds and Foods NutritionResearch Center, Pukyong National University, Busan, Korea) and the individual minerals purchased from Junsei Chemical, Tokyo, Japan.

Table 3. Growth performance of juvenile olive flounder fed different levels of organic Cu[1]

Diet	Final weight (g)	WG (%)[2]	SGR (%/day)[3]	FE (%)[4]	PER[5]	Survival (%)
$Cu_{1.0}$	24.9^{c}	262.8^{c}	1.53^{c}	111.3^{a}	1.14^{ab}	87
$CuM_{6.0}$	26.1^{b}	278.9^{b}	1.59^{b}	119.4^{a}	1.15^{ab}	90
CuM_{10}	27.1^{a}	302.2^{a}	1.66^{a}	124.1^{a}	1.25^{ab}	89
CuM_{15}	26.3^{b}	290.9^{ab}	1.62^{ab}	123.4^{a}	1.27^{a}	87
CuM_{19}	24.6^{c}	261.8^{c}	1.53^{c}	108.2^{a}	1.17^{ab}	82
CuM_{42}	23.7^{d}	244.3^{d}	1.47^{d}	108.1^{a}	1.12^{b}	84
CuM_{86}	17.6^{e}	161.0^{e}	1.14^{e}	68.4^{b}	0.74^{c}	82
Pooled SEM[6]	0.95	9.51	0.04	4.52	0.04	2.22

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Weight gain (%): (final wt. - initial wt.) × 100 / initial wt.

[3]Specific growth rate (%/day): 100× (Ln final wt. - Ln initial wt.)/days

[4]Feed efficiency (%): (wet weight gain (g) / dry feed intake (g)) × 100

[5]Protein efficiency ratio: (wet weight gain / protein intake)

[6]Pooled standard error of means: SD/√n

Table 4. Proximate composition (% dry matter) of juvenile olive flounder fed different levels of organic Cu[1]

Diet	Whole body moisture	Whole body protein	Whole body lipid	Whole body ash	Muscle protein	Muscle lipid
$Cu_{1.0}$	78.4	71.4^{ab}	12.0^{a}	12.7^{c}	86.7	3.16^{a}
$CuM_{6.0}$	78.0	72.3^{ab}	11.7^{a}	13.1^{bc}	87.4	3.03^{ab}
CuM_{10}	78.6	72.4^{a}	11.2^{ab}	13.2^{abc}	87.7	2.95^{ab}
CuM_{15}	79.2	71.3^{ab}	11.3^{ab}	13.4^{abc}	87.8	2.91^{b}
CuM_{19}	78.4	71.8^{ab}	10.5^{c}	13.7^{ab}	87.2	2.82^{b}
CuM_{42}	78.3	70.9^{ab}	9.6^{c}	13.8^{ab}	87.0	2.37^{c}
CuM_{86}	78.4	70.6^{b}	9.6^{c}	13.9^{a}	86.8	2.34^{c}
Pooled SEM[2]	0.122	0.187	0.247	0.119	0.143	0.090

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: $SD/\sqrt{n}$

Table 5. Hepatic thiobarbituric acid reactive substances (TBARS), Cu-Zn superoxide dismutase (Cu-Zn SOD) activity and hepatosomatic index (HSI) of juvenile olive flounder fed different levels of organic Cu[1]

Diet	TBARS (nmol MDA/ml tissue)	Cu-Zn SOD Units/g tissue	HSI (%)
$Cu_{1.0}$	10.3^{c}	325.3^{e}	2.55^{a}
$CuM_{6.0}$	8.65^{e}	370.7^{b}	2.51^{ab}
CuM_{10}	8.03^{f}	382.7^{a}	2.49^{ab}
CuM_{15}	8.27^{ef}	376.6^{ab}	2.55^{ab}
CuM_{19}	9.70^{d}	360.0^{c}	2.39^{bc}
CuM_{42}	13.5^{b}	331.1^{de}	2.51^{ab}
CuM_{86}	14.2^{a}	337.6^{d}	2.26^{c}
Pooled SEM[2]	0.54	5.03	4.699

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: SD/√n

Table 6. Hematological characteristics of juvenile olive flounder fed different levels of organic Cu[1]

Diet	RBC[2]	PCV[3]	WBC[4]	Hb[5]	MCV[6]	MCH[7]	MCHC[8]
$Cu_{1.0}$	2.83^{b}	21.7^{bc}	47.7^{d}	11.45^{b}	766.6	40.2^{ab}	52.4
$CuM_{6.0}$	2.84^{b}	22.2^{abc}	49.4^{d}	11.85^{ab}	782.0	41.7^{a}	53.4
CuM_{10}	3.15^{a}	23.6^{a}	51.5^{cd}	12.80^{a}	748.9	40.7^{ab}	54.4
CuM_{15}	3.09^{a}	22.9^{ab}	55.3^{bc}	11.40^{b}	742.5	37.0^{b}	49.8
CuM_{19}	2.90^{b}	22.5^{abc}	58.5^{b}	11.25^{bc}	775.9	38.8^{ab}	50.0
CuM_{42}	2.77^{bc}	21.9^{abc}	59.7^{b}	11.20^{bc}	792.0	40.5^{ab}	51.2
CuM_{86}	2.65^{c}	20.8^{c}	65.7^{a}	10.25^{c}	786.4	38.8^{ab}	49.3
Pooled SEM[9]	0.05	0.25	1.66	0.20	6.54	0.46	0.60

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]RBC ($\times 10^6$ cell μl): Red blood cell count

[3]PCV (%): Hematocrit (Packed cell volume, PCV)

[4]WBC ($\times 10^3$ mm^3): Whiteblood cell

[5]Hb (g/100ml): Hemoglobin

[6]MCV (FI): Mean corpuscular volume

[7]MCH (pg): Mean corpuscular hemoglobin

[8]MCHC (%): Mean corpuscular hemoglobin concentration

[9]Pooled standard error of means SD/√n

Table 7. Copper content (mg/kg) of juvenile olive flounder fed different levels of organic Cu[1]

Diet	Liver	Intestine	Kidney	Gill	Muscle	Whole Body (DM)
$Cu_{1.0}$	19.0^{f}	10.4^{f}	10.0^{f}	5.2^{e}	1.42^{d}	5.3^{d}
$CuM_{6.0}$	19.1^{f}	10.4^{f}	10.6^{fe}	5.3^{e}	1.46^{d}	5.5^{d}
CuM_{10}	21.4^{e}	11.0^{e}	11.0^{e}	5.6^{de}	1.58^{cd}	5.6^{d}
CuM_{15}	27.4^{d}	12.9^{d}	12.6^{d}	5.9^{cd}	1.96^{bc}	7.3^{c}
CuM_{19}	32.8^{c}	17.5^{c}	14.5^{c}	6.2^{bc}	2.19^{b}	8.1^{b}
CuM_{42}	41.0^{b}	30.3^{b}	16.3^{b}	6.5^{b}	2.96^{a}	8.8^{a}
CuM_{86}	42.9^{a}	35.6^{a}	18.4^{a}	7.1^{a}	3.37^{a}	8.8^{a}
Pooled SEM[2]	2.587	2.679	0.819	0.179	0.198	0.404

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: $SD/\sqrt{n}$

Table 8. Growth performance of juvenile olive flounder fed different levels of inorganic Cu[1]

Diet	Final weight (g)	WG (%)[2]	SGR (%/day)[3]	FE[4] (%)	PER[5]	Survival (%)
$Cu_{1.0}$	24.9^{bc}	261.0^{bc}	1.53^{bc}	114.6^{a}	1.12^{ab}	93
$CuS_{6.0}$	25.2^{b}	266.5^{b}	1.55^{b}	115.7^{a}	1.15^{ab}	90
CuS_{10}	26.6^{a}	285.5^{a}	1.61^{a}	114.4^{a}	1.22^{a}	84
CuS_{15}	25.1^{bc}	265.6^{b}	1.54^{b}	105.1^{a}	1.19^{a}	80
CuS_{19}	24.3^{c}	254.8^{c}	1.51^{c}	107.0^{a}	1.14^{ab}	84
CuS_{42}	22.6^{d}	229.8^{d}	1.42^{d}	102.5^{a}	1.04^{b}	89
CuS_{86}	16.9^{e}	148.2^{e}	1.08^{e}	62.8^{b}	0.69^{c}	82
Pooled SEM[6]	0.69	9.85	0.038	4.34	0.04	2.05

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2] Weight gain (%): (final wt. - initial wt.) × 100 / initial wt.

[3]Specific growth rate (%/day): 100× (Ln final wt. - Ln initial wt.)/days

[4]Feed efficiency (%): (wet weight gain/dry feed intake) × 100.

[5]Protein efficiency ratio: (wet weight gain / protein intake)

[6]Pooled standard error of means: SD/√n

Table 9. Proximate composition (% dry matter) of juvenile olive flounder fed different levels of inorganic Cu[1]

Diet	Whole body moisture	Whole body protein	Whole body lipid	Whole body ash	Muscle protein	Muscle lipid
$Cu_{1.0}$	78.6	71.1^{b}	11.8^{a}	12.5^{d}	86.9^{ab}	3.14^{a}
$CuS_{6.0}$	79.5	72.4^{a}	11.3^{a}	13.3^{c}	87.4^{ab}	3.15^{a}
CuS_{10}	78.2	71.9^{ab}	11.0^{ab}	13.4^{bc}	87.6^{a}	3.05^{ab}
CuS_{15}	78.7	71.7^{ab}	10.1^{bc}	13.5^{bc}	87.7^{a}	2.89^{bc}
CuS_{19}	79.1	71.8^{ab}	9.6^{c}	13.9^{abc}	86.7^{ab}	2.78^{cd}
CuS_{42}	80.1	71.0^{b}	9.6^{c}	14.0^{ab}	86.5^{b}	2.43^{e}
CuS_{86}	79.2	70.9^{b}	9.3^{c}	14.4^{a}	86.8^{ab}	2.60^{de}
Pooled SEM[2]	0.23	0.15	0.26	0.15	0.13	0.07

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: $SD/\sqrt{n}$

Table 10. Hepatic thiobarbituric acid reactive substances (TBARS) value, Cu-Zn superoxide dismut ase
(Cu-Zn SOD) activity and hepatosomatic index (HSI) of juvenile olive flounder fed different levels of inorganic Cu[1]

Diet	TBARS (nmol MDA/ml tissue)	Cu-Zn SOD Units/g tissue	HSI (%)
$Cu_{1.0}$	10.3^{e}	324.2^{e}	2.54^{a}
$CuS_{6.0}$	9.7^{cd}	360.8^{bc}	2.48^{abc}
CuS_{10}	8.3^{e}	375.5^{a}	2.51^{ab}
CuS_{15}	9.3^{d}	367.0^{b}	2.47^{abc}
CuS_{19}	9.9^{c}	357.8^{c}	2.40^{bc}
CuS_{42}	13.7^{b}	329.1^{de}	2.36^{c}
CuS_{86}	14.4^{a}	335.2^{d}	2.21^{d}
Pooled SEM[2]	0.48	4.19	0.03

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: $SD/\sqrt{n}$

Table 11. Hematological characteristics of juvenile olive flounder fed different levels of inorganic Cu[1]

Diet	RBC[2]	PCV[3]	WBC[4]	Hb[5]	MCV[6]	MCH[7]	MCHC[8]
$Cu_{1.0}$	2.83^{a}	21.2^{bcd}	47.7^{f}	12.3^{a}	747^{b}	43.3^{a}	57.9^{a}
$CuS_{6.0}$	2.85^{a}	23.0^{a}	50.5^{ef}	11.5^{a}	807^{a}	40.3^{ab}	49.9^{b}
CuS_{10}	2.78^{ab}	22.4^{a}	53.3^{de}	11.0^{bc}	807^{a}	39.5^{b}	48.9^{b}
CuS_{15}	2.70^{bc}	22.2^{ab}	56.3^{cd}	10.8^{bc}	822^{a}	40.0^{ab}	48.7^{b}
CuS_{19}	2.66^{cd}	22.1^{abc}	58.8^{c}	10.6^{c}	831^{a}	39.7^{b}	47.7^{b}
CuS_{42}	2.60^{de}	21.0^{cd}	62.8^{b}	10.4^{c}	808^{a}	40.0^{ab}	49.5^{b}
CuS_{86}	2.57^{e}	20.3^{d}	67.0^{a}	10.1^{c}	788^{ab}	39.3^{b}	49.9^{b}
Pooled SEM[2]	0.03	0.24	1.76	0.13	6.53	0.19	0.42

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]RBC ($\times 10^6$ cell µl): Red blood cell count

[3]PCV (%): Hematocrit (Packed cell volume, PCV)

[4]WBC ($\times 10^3$ mm^3): Whiteblood cell

[5]Hb (g/100ml): Hemoglobin

[6]MCV (Fl): Mean corpuscular volume

[7]MCH (pg): Mean corpuscular hemoglobin

[8]MCHC (%): Mean corpuscular hemoglobin concentration

[9]Pooled standard error of means SD/√n

Table 12. Copper content (mg/kg) of juvenile olive flounder fed different levels of inorganic Cu[1]

Diet	Liver	Intestine	Kidney	Gill	Muscle	Whole Body (DM)
$Cu_{1.0}$	19.1^{f}	10.4^{e}	10.1^{f}	5.3^{d}	1.4^{d}	5.3^{f}
$CuS_{6.0}$	19.2^{f}	10.6^{e}	10.8^{ef}	5.5^{cd}	1.7^{d}	5.7^{f}
CuS_{10}	21.7^{e}	11.5^{e}	11.6^{e}	5.9^{c}	1.8^{cd}	6.3^{e}
CuS_{15}	27.9^{d}	13.3^{d}	13.0^{d}	6.7^{b}	2.3^{bc}	7.7^{d}
CuS_{19}	34.3^{c}	20.7^{c}	15.6^{c}	6.8^{b}	2.8^{b}	8.4^{c}
CuS_{42}	41.7^{b}	35.7^{b}	17.1^{b}	7.2^{ab}	3.4^{a}	9.6^{b}
CuS_{86}	46.5^{a}	45.2^{a}	18.6^{a}	7.7^{a}	3.9^{a}	11.3^{a}
Pooled SEM[2]	2.85	3.59	0.85	0.24	0.24	0.56

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: SD/√n

Table 13. Effect of dietary copper sources and levels on growth performance and feed utilization of fingerling olive flounder[1]

Dietary factors	FW[2]	WG[3]	SGR[4]	FE[5]	PER[6]	Survival
Copper source (mg/kg)						
Organic Cu	24.2[a]	256[a]	1.50[a]	104	1.12[a]	86.0
Inorganic Cu	23.6[b]	243[b]	1.46[b]	103	1.10[b]	85.7
Pooled SEM[8]	0.49	7.06	0.03	3.23	0.03	1.53
Copper levels (mg/kg)						
1	24.9[c]	262[c]	1.53[c]	113.0[a]	1.130[cd]	90.0
6	25.6[b]	273[b]	1.57[b]	117.5[a]	1.153[bcd]	90.0
10	26.8[a]	294[a]	1.63[a]	119.3[a]	1.232[a]	86.7
15	25.7[b]	278[b]	1.58[b]	114.3[a]	1.230[ab]	83.3
19	24.5[c]	258[c]	1.52[c]	90.9[ab]	1.157[abc]	83.3
42	23.1[d]	237[d]	1.45[d]	105.3[a]	1.077[d]	86.7
86	17.2[e]	155[e]	1.11[e]	65.6[b]	0.72[e]	82.2
Pooled SEM[8]	0.49	7.06	0.03	3.23	0.03	1.53
ANOVA						
Copper sources	<0.0001	<0.0001	<0.0001	0.088	0.0028	0.92
Copper levels	<0.0001	<0.0001	<0.0001	0.0004	<0.0001	0.83
Source × levels	0.0016	0.001	0.0006	0.4002	0.5611	0.94

[1]Values in same column with different superscript are significantly different at $P < 0.05$.

[2]Final Weight (g)

[3]Weight gain (WG, %) = (final weight-initial weight) ×100/initial weight

[4]Specific growth rate (SGR, % BWday^{-1}) = (loge final wt. - loge initial wt.)/ days.

[5]Feed efficiency (FE, %) = wet weight gain (g) × 100/dry feed intake (g)

[6]Protein efficiency ratio (PER) = Wet weight gain/protein intake

[7]Hepatosomatic index (HSI, %) = 100× (Liver weight/fish weight)

[8]Pooled standard error of means: SD/√n

Table 14. Growth performance of juvenile olive flounder fed with various levels of supplemental copper from organic or inorganic sources[1]

Diet	Final weight (g)	WG (%)[2]	SGR (%/day)[3]	FE (%)[4]	PER[5]	Survival (%)
$Cu_{1.0}$	24.9^{cd}	261.9^{c}	1.53^{c}	112.9^{a}	1.13^{bcd}	90
$CuM_{6.0}$	26.1^{b}	278.9^{b}	1.59^{b}	119.4^{a}	1.15^{abcd}	90
CuM_{10}	27.1^{a}	302.2^{a}	1.66^{a}	124.1^{a}	1.25^{ab}	89
CuM_{15}	26.3^{b}	290.9^{ab}	1.62^{ab}	123.4^{a}	1.27^{a}	87
CuM_{19}	24.6^{cd}	261.8^{c}	1.53^{c}	108.2^{a}	1.17^{abc}	82
CuM_{42}	23.7^{e}	244.3^{d}	1.47^{d}	108.1^{a}	1.12^{cd}	84
CuM_{86}	17.6^{g}	161.0^{f}	1.14^{f}	68.5^{b}	0.74^{e}	82
$CuS_{6.0}$	25.2^{c}	266.5^{c}	1.55^{c}	115.7^{a}	1.15^{abcd}	90
CuS_{10}	26.6^{ab}	285.5^{b}	1.61^{b}	114.4^{a}	1.22^{abc}	84
CuS_{15}	25.1^{c}	265.6^{c}	1.54^{c}	105.1^{a}	1.19^{abc}	80
CuS_{19}	24.3^{de}	254.8^{cd}	1.51^{cd}	107.0^{a}	1.14^{bcd}	84
CuS_{42}	22.6^{f}	229.8^{e}	1.42^{e}	102.5^{a}	1.04^{d}	89
CuS_{86}	16.9^{g}	148.2^{g}	1.08^{g}	62.8^{b}	0.69^{e}	82
Pooled SEM[6]	0.49	7.01	0.03	3.22	0.03	1.53

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Weight gain (%): (final wt. - initial wt.) × 100 / initial wt.

[3]Specific growth rate (%/day): (100× (Ln final wt. - Ln initial wt.)/days

[4]Feed efficiency (%): (wet weight gain (g) / dry feed intake (g)) × 100

[5]Protein efficiency ratio: (wet weight gain / protein intake)

[6]Pooled standard error of means: SD/√n

Table 15. Effect of dietary copper sources and levels on proximate composition (% dry matter) of juvenile olive flounder[1]

Dietary factors	Whole body moisture	Whole body protein	Whole body lipid	Whole body ash	Muscle protein	Muscle lipid
Copper sources (mg/kg)						
Organic Cu	78.5^{b}	71.5^{a}	10.8^{a}	13.4^{b}	87.2	2.79^{b}
Inorganic Cu	79.1^{a}	71.6^{a}	10.4^{b}	13.6^{a}	87.1	2.86^{a}
Pooled SEM[8]	0.14	0.12	0.18	0.1	0.1	0.06
Copper levels (mg/kg)						
1	78.5	71.2^{bc}	11.9^{a}	12.6^{d}	86.8^{ab}	3.15^{a}
6	78.8	72.3^{a}	11.5^{ab}	13.2^{c}	87.4^{ab}	3.09^{ab}
10	78.4	72.1^{a}	11.1^{bc}	13.3^{c}	87.7^{ab}	3.00^{bc}
15	78.9	71.5^{abc}	10.7^{c}	13.5^{bc}	87.7^{a}	2.89^{cd}
19	78.8	71.8^{ab}	10.0^{d}	13.8^{ab}	87.0^{ab}	2.79^{d}
42	79.2	71.0^{bc}	9.64^{de}	13.9^{ab}	86.8^{b}	2.40^{e}
86	78.8	70.8^{c}	9.43^{e}	14.2^{a}	86.8^{ab}	2.47^{e}
Pooled SEM[8]	0.14	0.12	0.18	0.1	0.1	0.06
ANOVA						
Copper source	0.03	0.79	0.0001	0.013	0.39	0.0061
Copper levels	0.69	0.0001	0.0001	0.0001	0.009	<0.0001
Source × levels	0.2	0.69	0.0053	0.391	0.84	0.0235

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: $SD/\sqrt{n}$

Table 16. Proximate composition (% dry matter) of juvenile olive flounder fed with various levels of supplemental copper from organic or inorganic sources[1]

Diet	Whole body moisture	Whole body protein	Whole body lipid	Whole body ash	Muscle protein	Muscle lipid
$Cu_{1.0}$	78.5	71.2^{abc}	12.0^{a}	12.7^{e}	86.8	3.15^{a}
$CuM_{6.0}$	78.0	72.3^{a}	11.7^{a}	13.1^{de}	87.4	3.03^{ab}
CuM_{10}	78.6	72.4^{a}	11.2^{ab}	13.2^{cde}	87.7	2.95^{abc}
CuM_{15}	79.2	71.3^{abc}	11.3^{ab}	13.4^{bcd}	87.8	2.91^{bc}
CuM_{19}	78.4	71.8^{abc}	10.5^{cd}	13.7^{bcd}	87.2	2.82^{c}
CuM_{42}	78.3	70.9^{bc}	9.6^{ef}	13.8^{abc}	87.0	2.37^{ef}
CuM_{86}	78.4	70.6^{c}	9.6^{ef}	13.9^{ab}	86.8	2.34^{f}
$CuS_{6.0}$	79.5	72.4^{a}	11.4^{ab}	13.4^{bcd}	87.4	3.15^{a}
CuS_{10}	78.2	71.9^{abc}	11.0^{bc}	13.4^{bcd}	87.6	3.05^{ab}
CuS_{15}	78.7	71.7^{abc}	10.1^{de}	13.5^{bcd}	87.7	2.89^{bc}
CuS_{19}	79.1	71.8^{abc}	9.6^{ef}	13.9^{ab}	86.7	2.78^{cd}
CuS_{42}	80.1	71.1^{abc}	9.6^{ef}	14.0^{ab}	86.5	2.43^{ef}
CuS_{86}	79.2	70.9^{bc}	9.3^{f}	14.4^{a}	86.8	2.60^{de}
Pooled SEM[2]	0.14	0.12	0.18	0.10	0.10	0.06

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: $SD/\sqrt{n}$

Table 17. Effect of dietary copper source and levels on hepatic thiobarbituric acid reactive substances (TBARS), Cu-Zn superoxide dismutase (Cu-Zn SOD) activity and hepatosomatic index (%HSI) of juvenile olive flounder[1]

Dietary factors	TBARS (nmol MDA/ml tissue)	Cu-Zn SOD Units/g tissue	HSI (%)
Copper sources (mg/kg)			
Organic Cu	10.5^{b}	354^{a}	2.47^{a}
Inorganic Cu	10.9^{a}	349^{b}	2.42^{b}
Pooled SEM[2]	0.36	3.24	0.02
Copper levels (mg/kg)			
1	10.3^{c}	325^{g}	2.55^{a}
6	9.18^{e}	366^{c}	2.49^{a}
10	8.18^{g}	379^{a}	2.53^{a}
15	8.77^{f}	372^{b}	2.49^{a}
19	9.82^{d}	359^{d}	2.45^{ab}
42	13.62^{b}	330^{f}	2.38^{b}
86	14.3^{a}	333^{e}	2.34^{c}
Pooled SEM[2]	0.36	3.24	0.02
ANOVA			
Copper source	<0.0001	<0.0001	0.021
Copper levels	<0.0001	<0.0001	<0.0001
Source × levels	0.0003	0.009	0.908

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different (P < 0.05).

[2]Pooled standard error of means: SD/√n

Table 18. Hepatic thiobarbituric acid reactive substances (TBARS) value, Cu-Zn superoxide dismutase (Cu-Zn SOD) activity and hepatosomatic index (%HSI) of juvenile olive flounder fed with various levels of supplemental copper from organic or inorganic sources[1]

Diet	TBARS nmol MDA/ml tissue	Cu-Zn SOD Units/g tissue	HSI (%)
$Cu_{1.0}$	10.3^{d}	325^{h}	2.55^{ab}
$CuM_{6.0}$	8.65^{f}	371^{bc}	2.51^{abc}
CuM_{10}	8.03^{g}	383^{a}	2.49^{abc}
CuM_{15}	8.27^{fg}	377^{ab}	2.55^{ab}
CuM_{19}	9.70^{de}	360^{e}	2.39^{bcd}
CuM_{42}	13.5^{c}	331^{fgh}	2.51^{abc}
CuM_{86}	14.2^{ab}	338^{f}	2.26^{de}
$CuS_{6.0}$	9.73^{de}	361^{de}	2.48^{abc}
CuS_{10}	8.33^{fg}	376^{b}	2.51^{abc}
CuS_{15}	9.27^{e}	367^{cd}	2.47^{abc}
CuS_{19}	9.93^{d}	358^{e}	2.40^{bcd}
CuS_{42}	13.7^{bc}	329^{gh}	2.36^{cde}
CuS_{86}	14.43^{a}	335^{fg}	2.21^{e}
Pooled SEM[2]	0.36	3.23	0.02

[1]Values are means from groups (n = 3) of fish where the means in each column with a different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means SD/√n

Table 19. Effect of dietary copper source and levels on hematological characteristics of juvenile olive flounder[1]

Dietary factors	RBC[2]	PCV[3]	Hb[4]	WBC[5]	MCV[6]	MCH[7]	MCHC[8]
Copper sources (mg/kg)							
Organic Cu	2.89^{a}	22.2^{a}	11.4^{a}	55.2^{b}	771^{b}	39.7	51.5^{a}
Inorganic Cu	2.71^{b}	21.7^{b}	10.9^{b}	56.5^{a}	801^{a}	40.3	50.4^{b}
Pooled SEM[9]	0.03	0.18	0.15	1.22	5.73	0.30	0.54
Copper levels (mg/kg)							
1	2.83^{bc}	21.4^{bc}	11.8^{a}	47.4^{f}	757^{b}	41.7^{a}	55.2^{a}
6	2.84^{bc}	22.6^{a}	11.65^{ab}	49.9^{e}	794^{ab}	41.0^{ab}	51.7^{b}
10	2.96^{a}	23.0^{a}	11.9^{a}	51.7^{e}	778^{ab}	40.1^{abc}	51.6^{b}
15	2.89^{ab}	22.6^{a}	11.1^{bc}	50.8^{d}	782^{ab}	38.8^{c}	49.2^{b}
19	2.78^{c}	22.3^{ab}	10.9^{c}	58.6^{c}	803^{a}	39.2^{bc}	48.9^{b}
42	2.68^{d}	21.5^{bc}	10.8^{c}	61.2^{b}	780^{ab}	40.3^{abc}	50.4^{b}
86	2.61^{d}	20.5^{c}	10.2^{d}	66.3^{a}	787^{ab}	39.0^{bc}	49.6^{b}
Pooled SEM[9]	0.03	0.18	0.15	1.22	5.73	0.30	0.54
ANOVA							
Copper source	<0.0001	0.0049	<0.0001	0.0011	0.0006	0.11	0.046
Copper levels	<0.0001	<0.0001	<0.0001	<0.0001	0.05	0.0034	0.0002
Source × levels	<0.0001	0.0841	<0.0001	0.068	0.024	0.018	0.0002

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]RBC ($\times 10^6$ cell μl): Red blood cell count

[3]PCV (%): Hematocrit (Packed cell volume, PCV)

[4]Hb (g/100ml): Hemoglobin

[5]WBC ($\times 10^3$ mm^3): Whiteblood cell

[6]MCV (FI): Mean corpuscular volume

[7]MCH (pg): Mean corpuscular hemoglobin

[8]MCHC (%): Mean corpuscular hemoglobin concentration

[9]Pooled standard error of means SD/√n

Table 20. Hematological values of juvenile olive flounder fed with various levels of supplemental copper from organic or inorganic sources[1]

Diet	RBC[2]	PCV[3]	Hb[4]	WBC[5]	MCV[6]	MCH[7]	MCHC[8]
$Cu_{1.0}$	2.83^{bc}	21.4^{cdef}	11.8^{ab}	47.4^{h}	757^{bc}	41.7^{a}	55.2^{a}
$CuM_{6.0}$	2.84^{bc}	22.2^{abcde}	11.9^{ab}	49.4^{h}	782^{abc}	41.7^{a}	53.4^{ab}
CuM_{10}	3.15^{a}	23.6^{a}	12.8^{a}	50.0^{gh}	749^{c}	40.7^{ab}	54.4^{ab}
CuM_{15}	3.09^{a}	22.9^{abc}	11.4^{bc}	55.3^{ef}	743^{c}	37.0^{b}	49.8^{ab}
CuM_{19}	2.90^{b}	22.5^{abcd}	11.3^{bcd}	58.5^{de}	776^{abc}	38.8^{ab}	50.0^{ab}
CuM_{42}	2.77^{cd}	21.9^{bcde}	11.2^{bcd}	59.7^{cd}	792^{abc}	40.5^{ab}	51.2^{ab}
CuM_{86}	2.65^{ef}	20.8^{ef}	10.3^{cd}	65.7^{ab}	786^{abc}	38.8^{ab}	49.3^{ab}
$CuS_{6.0}$	2.85^{bc}	23.0^{ab}	11.5^{bc}	50.5^{gh}	807^{abc}	40.3^{ab}	49.9^{ab}
CuS_{10}	2.78^{cd}	22.4^{abcd}	11.0^{bcd}	53.3^{g}	807^{abc}	39.5^{ab}	48.9^{ab}
CuS_{15}	2.70^{de}	22.2^{abcde}	10.8^{bcd}	56.3^{def}	822^{ab}	40.0^{ab}	48.7^{ab}
CuS_{19}	2.66^{def}	22.1^{abcde}	10.6^{cd}	58.8^{de}	831^{a}	39.7^{ab}	47.7^{b}
CuS_{42}	2.60^{ef}	21.0^{def}	10.4^{cd}	62.8^{bc}	808^{abc}	40.0^{ab}	49.5^{ab}
CuS_{86}	2.57^{f}	20.25^{f}	10.1^{d}	67.00^{a}	788^{abc}	39.3^{ab}	49.9^{ab}
Pooled SEM[9]	0.03	0.18	0.15	1.22	5.73	0.30	0.54

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$)

[2]RBC ($\times 10^6$ cell μl): Red blood cell count

[3]PCV (%): Hematocrit (Packed cell volume, PCV)

[4]Hb (g/100ml): Hemoglobin

[5]WBC ($\times 10^3$ mm^3): Whiteblood cell

[6]MCV (FI): Mean corpuscular volume

[7]MCH (pg): Mean corpuscular hemoglobin

[8]MCHC (%): Mean corpuscular hemoglobin concentration

[9]Pooled standard error of means: $SD/\sqrt{n}$

Table 21. Effect of dietary copper sources and levels on copper content (mg/kg) of juvenile olive flounder[1]

Dietary factors	Liver	Intestine	Kidney	Gill	Muscle	Whole Body (DM)
Copper sources (mg/kg)						
Organic Cu	29.1^{b}	18.3^{b}	13.5^{b}	5.97^{b}	2.13^{b}	7.06^{b}
Inorganic Cu	30.1^{a}	21.1^{a}	13.8^{a}	6.42^{a}	2.47^{a}	7.76^{a}
Pooled SEM[2]	1.89	2.21	0.58	0.15	0.16	0.35
Copper levels (mg/kg)						
1	19.1^{f}	10.4^{e}	10.1^{g}	5.23^{e}	1.43^{f}	5.32^{f}
6	19.2^{f}	10.5^{e}	10.7^{f}	5.37^{e}	1.55^{ef}	5.58^{f}
10	21.6^{e}	11.2^{e}	11.28^{e}	5.73^{d}	1.70^{e}	5.97^{e}
15	27.7^{d}	13.1^{d}	12.8^{d}	6.30^{c}	2.13^{d}	7.52^{d}
19	33.5^{c}	19.1^{c}	15.0^{c}	6.50^{bc}	2.50^{c}	8.26^{c}
42	41.3^{b}	33.0^{b}	16.7^{b}	6.81^{b}	3.20^{b}	9.15^{b}
86	44.7^{a}	40.4^{a}	18.5^{a}	7.43^{a}	3.62^{a}	10.1^{a}
Pooled SEM[2]	1.89	2.21	0.58	0.15	0.16	0.35
ANOVA						
Copper source	<0.0001	<0.0001	0.0001	<0.0001	<0.0001	<0.0001
Copper levels	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Source × levels	<0.0001	<0.0001	0.051	0.016	0.043	<0.0001

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different (P < 0.05).

[2]Pooled standard error of means: SD/√n

Table 22. Copper content (mg/kg) in juvenile olive flounder fed with various levels of supplemental copper from organic or inorganic sources[1]

Diet	Liver	Intestine	Kidney	Gill	Muscle	Whole body (DM)
$Cu_{1.0}$	19.06^{h}	10.39^{g}	10.05^{g}	5.23^{g}	1.43^{f}	5.32^{h}
$CuM_{6.0}$	19.08^{h}	10.42^{g}	10.58^{fg}	5.27^{g}	1.46^{f}	5.51^{gh}
CuM_{10}	21.37^{g}	11.03^{efg}	10.98^{f}	5.59^{fg}	1.58^{ef}	5.60^{gh}
CuM_{15}	27.43^{f}	12.86^{ef}	12.62^{e}	5.93^{ef}	1.96^{de}	7.21^{f}
CuM_{19}	32.76^{e}	17.48^{e}	14.48^{d}	6.22^{de}	2.19^{d}	8.10^{de}
CuM_{42}	40.95^{c}	30.30^{c}	16.34^{bc}	6.45^{cd}	2.96^{bc}	8.76^{c}
CuM_{86}	42.91^{b}	35.60^{b}	18.44^{a}	7.14^{b}	3.37^{ab}	8.84^{c}
$CuS_{6.0}$	19.22^{h}	10.55^{fg}	10.82^{fg}	5.46^{gh}	1.65^{ef}	5.65^{gh}
CuS_{10}	21.73^{g}	11.45^{efg}	11.59^{f}	5.87^{ef}	1.80^{def}	6.33^{g}
CuS_{15}	27.87^{f}	13.33^{e}	12.97^{e}	6.67^{bcd}	2.30^{d}	7.73^{ef}
CuS_{19}	34.27^{d}	20.72^{d}	15.57^{c}	6.78^{bc}	2.81^{c}	8.41^{cd}
CuS_{42}	41.70^{bc}	35.73^{b}	17.09^{b}	7.16^{b}	3.40^{ab}	9.55^{b}
CuS_{86}	46.53^{a}	45.15^{a}	18.57^{a}	7.71^{a}	3.87^{a}	11.34^{a}
Pooled SEM[2]	1.89	2.21	0.58	0.15	0.16	0.35

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: SD/√n

Table 23. Composition of the basal diet

Ingredients	% Dry matter (DM)
Casein §	38
Gelatin§	8
Fish meal †	5
Wheat flour	8
Dextrin§	7
Corn starch§	11
Oil (Corn + Fish oil) ¶	12
Vitamin premix††	2
Mineral premixture (Cu-free) ‡‡	1
Alhpa cellulose §	4
Premix of Cu (by using organic or inorganic Cu)	-

† 62% crude protein.

§ United States Biochemical, Cleveland, OH, USA.

¶ Aras oil Co. Ltd., Anzali, Iran (Lipid source is a 1:1 blend of fish oil and corn oil.).

†† Vitamin mixture (Contains as mg/kg diet): DL-alpha tocopherol acetate. 60 IU; DL-cholecalciferol, 3000 IU; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; B12, 0.05 mg; nicotinic acid, 175 mg; folic acid, 5 mg; ascorbic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthoteate, 50 mg; choline chloride, 2000 mg.

‡‡ Mineral mixture (Cu free; as g/kg pre-mixture): calcium carbonate (40% Ca), 2.15 g; agnesium oxide (60% Mg), 1.24 g; ferric citrate, 0.2 g; potassium iodide (75% I), 0.4 mg; zinc sulphate (36% Zn), 0.4 g; manganese sulphate (33% Mn), 0.3 g; dibasic calcium phosphate (20% Ca, 18% P), 5 g; cobalt sulphate, 2 mg; sodium selenite (30% Se), 3 mg; KCl, 0.9 g; NaCl, 0.4 g.

Table 24. Performance of juvenile beluga sturgeon fed different levels of organic Cu[1]

Diet	Final weight (g)	WG (%)[2]	SGR (%/day)[3]	FE (%)[4]	PER[5]	Survival (%)	HSI (%)[6]	CF[7]
$Cu_{1.0}$	130^{e}	1436^{c}	3.94^{c}	126de	1.28^{bc}	100	3.06^{abc}	0.58^{b}
$CuM_{4.0}$	136^{d}	1449^{c}	3.97^{c}	152^{ab}	1.48^{a}	100	3.24^{ab}	0.57^{b}
$CuM_{7.0}$	148^{b}	1657^{ab}	4.15^{ab}	158^{a}	1.49^{a}	100	3.34^{a}	0.65^{b}
CuM_{10}	162^{a}	1777^{a}	4.28^{a}	153^{ab}	1.47^{a}	100	3.01^{abcd}	0.73^{a}
CuM_{13}	164^{a}	1776^{a}	4.25^{a}	148^{ab}	1.38^{ab}	100	2.76^{bcd}	0.65^{b}
CuM_{25}	145^{c}	1577^{b}	4.01^{b}	144^{bc}	1.37^{ab}	100	2.74^{cd}	0.63^{b}
CuM_{49}	130^{e}	1443^{c}	3.97^{c}	133^{cd}	1.30^{bc}	100	2.56^{d}	0.56^{b}
CuM_{194}	124^{f}	1341^{c}	3.87^{c}	117^{e}	1.18^{c}	100	2.04^{e}	0.46^{c}
Pooled SEM[8]	2.95	35.4	0.031	2.90	0.02	-	0.05	0.01

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$)

[2] Weight gain (WG; %): (final wt. - initial wt.)×100/initial wt

[3]Specific growth rate (SGR; %/day): 100× (Ln final wt. - Ln initial wt.)/days

[4]Feed efficiency (FE; %): (wet weight gain/dry feed intake) × 100

[5]Protein efficiency ratio (PER): (wet weight gain/protein intake)

[6]Hepatosomatic index (HSI; %) = Liver weight×100/fish weight

[7]Condition factor (CF; g/cm^3) = [Body weight (g)/fork langth (cm^3)] × 100

[8]Pooled standard error of means: SD/√n

Table 25. Proximate composition (% dry matter) of juvenile beluga sturgeon fed levels of organic Cu[1]

Diet	Whole body moisture	Whole body protein	Whole body lipid	Whole body ash	Muscle moisture	Muscle protein	Muscle lipid
$Cu_{1.0}$	4.78^{b}	64.53^{c}	21.82^{a}	9.93^{c}	4.24^{ab}	63.38^{cd}	20.90^{abc}
$CuM_{4.0}$	4.95^{b}	65.29^{ab}	21.69^{a}	10.46^{bc}	4.26^{ab}	63.76^{cd}	20.38^{bc}
$CuM_{7.0}$	4.80^{b}	65.52^{a}	21.15^{a}	10.74^{bc}	4.44^{ab}	64.36^{ab}	21.47^{ab}
CuM_{10}	4.76^{b}	65.61^{a}	21.21^{a}	10.73^{bc}	4.07^{b}	65.82^{a}	21.65^{ab}
CuM_{13}	5.08^{b}	65.27^{ab}	21.05^{a}	11.02^{bc}	4.05^{b}	65.31^{ab}	21.87^{a}
CuM_{25}	4.93^{b}	64.75^{bc}	21.19^{a}	11.32^{b}	4.38^{ab}	64.49^{ab}	21.99^{a}
CuM_{49}	5.24^{b}	62.79^{d}	18.47^{b}	12.63^{a}	4.72^{a}	62.72^{d}	19.93^{cd}
CuM_{194}	7.82^{a}	61.66^{e}	16.48^{c}	13.19^{a}	4.83^{a}	60.94^{e}	18.80^{d}
Pooled SEM[2]	0.29	0.47	0.47	0.27	0.08	0.54	0.37

[1]Values are means from groups (n = 3) of fish where the means in each column with a different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means SD/√n.

Table 26. Thiobarbituric acid reactive substances (TBARS) value, Cu-Zn superoxide dismutase (Cu-Zn SOD) and glutathione peroxidase (GPx) activity of juvenile beluga sturgeon fed different levels of organic Cu[1]

Diet	Cu-Zn SOD Units/mg liver	GPX/liver	TBARS nmol MDA/ml liver	Cu-Zn SOD Units/mg muscle	GPX/muscle
$Cu_{1.0}$	99.56^{e}	191.5^{d}	6.70^{b}	12.63^{e}	149.8^{b}
$CuM_{4.0}$	111.26^{de}	194.1^{d}	5.20^{d}	13.57^{d}	151.8^{a}
$CuM_{7.0}$	127.71^{abc}	222.4^{bc}	4.10^{e}	15.16^{abc}	152.2^{a}
CuM_{10}	141.04^{a}	234.3^{a}	3.20^{e}	15.71^{a}	153.4^{a}
CuM_{13}	133.33^{ab}	227.9^{ab}	3.95^{e}	15.31^{ab}	152.9^{a}
CuM_{25}	131.45^{abc}	226.5^{b}	5.25^{cd}	14.83^{bc}	153.2^{a}
CuM_{49}	125.63^{bc}	220.7^{bc}	6.15^{bc}	14.64^{c}	152.4^{a}
CuM_{194}	117.55^{cd}	215.44^{c}	9.0^{a}	14.1^{d}	149.6^{b}
Pooled SEM[2]	3.29	5.34	0.44	0.25	0.37

[1]Values are means from groups (n = 3) of fish where the means in each column with a different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means SD/√n.

Table 27. Hematological and biochemical characteristics of juvenile beluga sturgeon fed different levels of organic Cu[1]

Diet	PCV[2]	Hb[3]	Glucose (mg/dL)	Total protein (mg/dL)	Lysozyme Activity (U/mL)	Cholesterol	Peroxidase	GOT[4]	GPT[5]	Calcium (mg d/L)	Magnesium (meq/L)
$Cu_{1.0}$	23.3^{bc}	4.50^{abc}	3.73^{a}	1.58	13.2^{e}	16.3^{e}	2.30^{f}	233^{c}	1.30^{c}	6.85	1.20^{b}
$CuM_{4.0}$	24.0^{ab}	5.11^{abc}	3.43^{ab}	1.71	13.8^{e}	$17. 7^{e}$	2.58^{ef}	235^{c}	1.37^{c}	7.13	1.30^{ab}
$CuM_{7.0}$	25.3^{ab}	5.20^{abc}	3.41^{abc}	1.76	$25. 7^{b}$	19.3^{de}	2.95^{de}	243^{c}	1.50^{c}	7.43	1.34^{ab}
CuM_{10}	26.3^{a}	5.40^{a}	2.66^{d}	1.80	29.3^{a}	21.0^{de}	3.03^{cd}	251^{c}	1.67^{c}	7.53	1.37^{ab}
CuM_{13}	25.8^{ab}	5.30^{ab}	2.72^{cd}	1.60	23.0^{bc}	22.7^{cd}	3.13^{bcd}	260^{bc}	1.83^{c}	7.54	1.52^{ab}
CuM_{25}	24.7^{ab}	4.60^{abc}	3.00^{bcd}	1.53	21.0^{c}	28.0^{b}	3.50^{ab}	293^{ab}	2.33^{bc}	7.00	1.43^{ab}
CuM_{49}	21.2^{c}	4.30^{bc}	3.1^{abcd}	1.55	19.0^{cd}	27.3^{bc}	3.43^{abc}	306^{a}	3.33^{ab}	7.09	1.63^{a}
CuM_{194}	21.2^{c}	4.20^{c}	3.27^{abcd}	1.49	14.9^{de}	34.0^{a}	3.57^{a}	324^{a}	3.67^{a}	6.73	1.71^{a}
Pooled SEM[6]	0.25	0.08	0.08	0.02	1.25	0.79	0.09	6.92	0.19	0.07	0.04

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P< 0.05$).

[2]PCV (%): Hematocrit (Packed cell volume, PCV)

[3]Hb (g/dL): Hemoglobin

[4]GOT (AST; U/L): Glutamic oxaloacetic transaminase (Aspartate transminase)

[5]GPT (ALT; U/L): Glutamic pyruvic transaminase (Alanine transminase)

[6]Pooled standard error of means SD/√n.

Table 28. Copper content (mg/kg) of juvenile beluga sturgeon fed different levels of organic Cu[1]

Diet	Liver	Intestine	Kidney	Gill	Muscle	Plasma copper	Whole body (DM)
$Cu_{1.0}$	25.40^{f}	3.79^{e}	3.05^{c}	1.35^{e}	1.49^{e}	5.33^{e}	3.46^{f}
$CuM_{4.0}$	26.88^{f}	4.02^{e}	3.15^{c}	1.39^{e}	1.57^{e}	6.70^{e}	3.61^{f}
$CuM_{7.0}$	28.97^{e}	4.16^{de}	3.24^{c}	2.14^{d}	1.86^{d}	8.10^{d}	3.88^{ef}
CuM_{10}	30.75^{e}	4.74^{cd}	3.50^{bc}	2.47^{c}	1.95^{d}	10.47^{d}	4.23^{de}
CuM_{13}	36.52^{d}	4.92^{bc}	4.21^{ab}	2.75^{c}	2.50^{c}	12.17^{c}	4.66^{cd}
CuM_{25}	40.27^{c}	5.40^{b}	4.36^{a}	3.34^{b}	2.98^{b}	14.33^{b}	5.06^{c}
CuM_{49}	43.33^{b}	6.46^{a}	4.52^{a}	4.03^{a}	3.16^{b}	15.90^{b}	6.10^{b}
CuM_{194}	45.30^{a}	6.73^{a}	4.80^{a}	4.16^{a}	4.20^{a}	24.40^{a}	6.90^{a}
Pooled SEM[2]	2.09	0.27	0.17	0.24	0.18	1.2	0.22

[1]Values are means from groups (n = 3) of fish where the means in each column with a different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means SD/√n.

Table 29. Performance of juvenile beluga sturgeon fed different levels of inorganic Cu[1]

Diet	Final weight (g)	WG (%)[2]	SGR (%/day)[3]	FE (%)[4]	PER [5]	Survival (%)	HIS (%)[6]	CF[7]
$Cu_{1.0}$	129de	1429^{c}	3.90^{c}	126^{b}	1.28^{a}	100	3.05^{ab}	0.55^{bc}
$CuS_{4.0}$	134^{cd}	1440^{bc}	3.96^{bc}	136^{a}	1.32^{a}	100	3.16^{a}	0.55^{bc}
$CuS_{7.0}$	142^{bc}	1572^{ab}	4.08^{ab}	140^{a}	1.35^{a}	100	3.20^{a}	0.59^{bc}
CuS_{10}	149^{ab}	1623^{a}	4.13^{a}	138^{a}	1.31^{a}	100	2.89^{ab}	0.59^{bc}
CuS_{13}	151^{a}	1674^{a}	4.17^{a}	138^{a}	1.33^{a}	100	3.02^{ab}	0.71^{a}
CuS_{25}	131^{d}	1428^{c}	3.95^{bc}	138^{a}	1.31^{a}	100	2.53^{abc}	0.61^{b}
CuS_{50}	129de	1394^{c}	3.92^{c}	137^{a}	1.31^{a}	100	2.43^{bc}	0.51^{c}
CuS_{195}	122^{e}	1314^{c}	3.84^{c}	110^{c}	1.06^{b}	97	1.91^{c}	0.40^{d}
Pooled SEM[8]	2.06	27.4	0.03	1.37	0.01	0.04	0.07	0.01

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$)

[2] Weight gain (WG; %): (final wt. - initial wt.) × 100/initial wt

[3]Specific growth rate (SGR; %/day): 100× (Ln final wt. - Ln initial wt.)/days

[4]Feed efficiency (FE; %): (wet weight gain/dry feed intake) × 100

[5]Protein efficiency ratio (PER): (wet weight gain / protein intake)

[6]Hepatosomatic index (HIS; %) = Liver weight×100/fish weight

[7]Condition factor (CF; g/cm^3) = [Body weight (g)/fork langth (cm^3)] × 100

[8]Pooled standard error of means: SD/√n

Table 30. Proximate composition (% dry matter) of juvenile beluga sturgeon fed different levels of inorganic Cu[1]

Diet	Whole body moisture	Whole body protein	Whole body lipid	Whole body ash	Muscle moisture	Muscle protein	Muscle lipid
$Cu_{1.0}$	4.83^{d}	64.5^{c}	21.9^{a}	9.95^{e}	4.25^{bc}	63.3^{cd}	20.9^{bc}
$CuS_{4.0}$	5.11^{cd}	64.8^{bc}	21.6^{ab}	10.25^{de}	4.08^{c}	62.2^{bc}	20.3^{c}
$CuS_{7.0}$	5.45^{bc}	65.4^{ab}	21.0^{abc}	10.64^{cde}	4.11^{bc}	63.8^{bc}	21.5^{ab}
CuS_{10}	5.27^{cd}	65.0^{abc}	20.7^{bcd}	10.73^{cd}	4.33^{bc}	64.4^{ab}	21.6^{ab}
CuS_{13}	5.46^{bc}	65.5^{a}	20.3^{cd}	11.05^{c}	4.34^{bc}	65.7^{a}	21.8^{a}
CuS_{25}	5.48^{bc}	64.7^{bc}	19.9^{d}	11.19^{c}	4.44^{bc}	65.2^{ab}	21.9^{a}
CuS_{50}	5.89^{b}	62.7^{d}	17.6^{e}	12.49^{b}	4.51^{b}	60.5^{d}	19.2^{d}
CuS_{195}	8.79^{a}	59.8^{e}	16.2^{f}	13.34^{a}	5.15^{a}	59.3^{e}	17.5^{e}
Pooled SEM[2]	0.30	0.46	0.48	0.28	0.07	0.3	0.28

[1]Values are means from groups (n = 3) of fish where the means in each column with a different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means SD/√n.

Table 31. Hepatic and muscle thiobarbituric acid reactive substances (TBARS), Cu-Zn superoxide dismutase (Cu-Zn SOD) and glutathione peroxidase (GPx) of juvenile beluga fed different levels of inorganic Cu[1]

Diet	Cu-Zn SOD Units/mg liver	GPX/Liver	TBARS nmol MDA/ml liver	Cu-Zn SOD Unit/mg muscle	GPX/muscle
$Cu_{1.0}$	99. 6^{c}	191^{e}	6.68^{b}	12.6^{c}	149.4^{d}
$CuS_{4.0}$	108.7^{bc}	193^{e}	5.65^{cd}	13.4^{bc}	151.3^{bc}
$CuS_{7.0}$	125.0^{ab}	222^{bc}	4.65^{e}	14.4^{ab}	151.9^{abc}
CuS_{10}	129.1^{a}	226^{ab}	4.20^{ef}	15.2^{a}	152.6^{ab}
CuS_{13}	138.5^{a}	231^{a}	3.60^{f}	15.5^{a}	153.0^{a}
CuS_{25}	126.5^{ab}	226^{b}	5.45^{d}	14.3^{abc}	153.0^{a}
CuS_{50}	122.0^{ab}	220^{c}	6.20^{bc}	14.2^{abc}	151.0^{cd}
CuS_{195}	95.9^{c}	214^{d}	9.05^{a}	14.0^{abc}	148.4^{e}
Pooled SEM[2]	3.73	3.68	0.41	0.24	0.40

[1]Values are means from groups (n = 3) of fish where the means in each column with a different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means SD/√n.

Table 32. Hematological and biochemical characteristics of juvenile beluga sturgeon fed different levels of inorganic Cu[1]

Diet	PCV[2]	Hb[3]	Glucose (mg/dL)	Total Protein (mg/dL)	Lysozyme Activity	Cholesterol	Peroxidase	GOT[4]	GPT[5]	Calcium (mg/dL)	Magnesium (mg/dL)
$Cu_{1.0}$	23.5^{bc}	4.50^{abc}	3.67^{a}	1.60	13.1^{d}	16.50^{d}	2.30^{c}	239^{c}	1.30^{d}	6.89	1.21^{d}
$CuS_{4.0}$	24.7^{ab}	4.70^{ab}	3.40^{ab}	1.60	13.6^{d}	16.67^{d}	2.55^{bc}	262^{c}	1.50^{cd}	6.97	1.26^{d}
$CuS_{7.0}$	25.2^{ab}	4.80^{ab}	3.51^{a}	1.80	20.0^{c}	19.00^{cd}	2.84^{ab}	273^{bc}	1.80^{bcd}	7.12	1.31^{cd}
CuS_{10}	25.3^{ab}	4.90^{a}	3.10^{ab}	1.60	26.3^{ab}	20.67^{c}	2.80^{abc}	276^{bc}	2.00^{bcd}	7.11	1.34^{bcd}
CuS_{13}	25.6^{a}	4.40^{abc}	2.80^{b}	1.80	28.0^{a}	22.67^{bc}	2.88^{ab}	318^{ab}	2.30^{bcd}	6.90	1.49^{b}
CuS_{25}	22.3^{c}	3.80^{abc}	3.60^{a}	1.60	24.3^{b}	27.33^{a}	2.93^{ab}	339^{a}	3.20^{ab}	6.54	1.52^{ab}
CuS_{50}	21.3^{cd}	3.60^{c}	3.20^{ab}	1.60	18.0^{c}	26.33^{ab}	3.06^{ab}	310^{ab}	2.80^{abc}	6.56	1.45^{bc}
CuS_{195}	19.5^{d}	3.80^{bc}	3.50^{ab}	1.50	14.7^{d}	29.33^{a}	3.14^{a}	344^{a}	4.00^{a}	6.46	1.68^{a}
Pooled SEM[6]	0.25	0.08	0.08	0.02	1.16	1.00	0.06	9.02	0.2	0.07	0.03

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]PCV (%): Hematocrit (Packed cell volume, PCV)

[3]Hb (g/dL): Hemoglobin

[4]GOT (AST; U/L): Glutamic oxaloacetic transaminase (Aspartate transminase)

[5]GPT (ALT; U/L): Glutamic pyruvic transaminase (Alanine transminase)

[6]Pooled standard error of means SD/√n.

Table 33. Copper content (mg/kg) of juvenile beluga sturgeon fed different levels of inorganic Cu[1]

Diet	Liver	Intestine	Kidney	Gill	Muscle	Plasma copper	Whole body (DM)
$Cu_{1.0}$	25.40^{f}	3.79^{c}	3.06^{e}	1.36^{e}	1.49^{e}	5.45^{e}	3.47^{f}
$CuS_{4.0}$	26.49^{f}	3.93^{c}	3.13^{e}	1.37^{e}	1.58^{e}	6.70^{e}	3.55^{f}
$CuS_{7.0}$	28.94^{e}	4.11^{c}	3.43^{de}	2.07^{d}	1.81^{de}	9.00^{de}	3.84^{ef}
CuS_{10}	30.24^{e}	4.47^{bc}	3.73^{cd}	2.41^{c}	1.93^{de}	11.0^{cd}	4.26^{de}
CuS_{13}	35.56^{d}	4.62^{bc}	3.89^{bcd}	2.59^{c}	2.22^{cd}	12.7^{bcd}	4.51^{cd}
CuS_{25}	38.05^{c}	5.31^{b}	4.30^{bc}	3.02^{b}	2.68^{bc}	14.3^{bc}	4.91^{bc}
CuS_{50}	47.86^{b}	6.36^{a}	4.46^{ab}	3.76^{a}	2.83^{b}	15.7^{b}	5.30^{b}
CuS_{195}	53.65^{a}	6.67^{a}	4.92^{a}	3.99^{a}	3.42^{a}	24.7^{a}	6.12^{a}
Pooled SEM[2]	1.51	0.191	0.117	0.168	0.122	1.22	0.159

[1]Values are means from groups (n = 3) of fish where the means in each column with a different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means SD/√n.

Table 34. Effect of the dietary copper source and levels on growth performance and feed utilization of fingerling beluga sturgeon[1]

Dietary factors	FW[2]	WG[3]	SGR[4]	FE[5]	PER[6]	HSI[7]	CF[8]	Survival
Copper sources (mg/kg)								
Organic Cu	142.2^{a}	1557^{a}	4.07^{a}	141^{a}	1.37^{a}	2.84	0.60^{a}	100
Inorganic Cu	136.0^{b}	1484^{b}	3.99^{b}	133^{b}	1.28^{b}	2.77	0.57^{b}	99.6
Pooled SEM[9]	1.83	21.36	0.02	1.87	0.02	0.06	0.01	0.19
Copper levels (mg/kg)								
1	129.7^{d}	1433^{cd}	3.93^{de}	126^{d}	1.28^{c}	3.05^{a}	0.57^{c}	100
4	134.8^{c}	1445^{cd}	3.97^{cd}	144^{ab}	1.40^{a}	3.20^{a}	0.56^{c}	100
7	144.9^{b}	1615^{b}	4.12^{b}	149^{a}	1.42^{a}	3.27^{a}	0.62^{b}	100
10	155.1^{a}	1700^{a}	4.20^{a}	146^{ab}	1.39^{ab}	2.95^{ab}	0.66^{a}	100
13	157.6^{a}	1725^{a}	4.21^{a}	143^{ab}	1.36^{abc}	2.89^{abc}	0.68^{a}	100
25	138.1^{c}	1503^{c}	4.02^{c}	141^{cd}	1.34^{abc}	2.64^{bc}	0.62^{b}	100
49	129.2^{d}	1418^{d}	3.94^{d}	135^{c}	1.30^{bc}	2.50^{c}	0.53^{c}	100
194	123.1^{e}	1328^{e}	3.85^{e}	113^{e}	1.11^{d}	1.98^{d}	0.43^{d}	98.5
Pooled SEM[9]	1.83	21.36	0.02	1.87	0.02	0.06	0.01	0.19
ANOVA								
Copper source	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.2784	<0.0001	0.92
Copper levels	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.83
Source × levels	<0.0001	0.0286	0.0663	<0.0001	0.0194	0.6772	0.0001	0.94

[1]Values in same column with different superscript are significantly different at $P < 0.05$.

[2]Final Weight (g)

[3]Weight gain (WG, %) = (final weight-initial weight) ×100/initial weight

[4]Specific growth rate (SGR, % BWday^{-1}) = (loge final wt. - loge initial wt.)/ days.

[5]Feed efficiency (FE, %) = wet weight gain (g) × 100/dry feed intake (g)

[6]Protein efficiency ratio (PER) = Wet weight gain/protein intake

[7]Hepatosomatic index (HSI, %) = 100× (Liver weight/fish weight)

[8]Condition factor (CF; g/cm^3) = [Body weight (g)/fork langth (cm^3)] × 100

[9]Pooled standard error of means: SD/√n

Table 35. Growth Performance of juvenile beluga sturgeon fed with various levels of supplemental copper from organic or inorganic sources[1]

Diet	Final weight (g)	WG (%)[2]	SGR (%/day)[3]	FE (%)[4]	PER[5]	Survival (%)	HSI (%)[6]	CF[7]
$Cu_{1.0}$	130^{efg}	1433^{de}	3.93^{fg}	126^{fg}	1.28^{cd}	100	3.05^{abc}	0.57^{cde}
$CuM_{4.0}$	136^{de}	1449^{cd}	3.97^{def}	152^{abc}	1.48^{a}	100	3.24^{a}	0.57^{cde}
$CuM_{7.0}$	148^{bc}	1657^{ab}	4.15^{abc}	158^{a}	1.49^{a}	100	3.34^{a}	0.64^{bc}
CuM_{10}	162^{a}	1777^{a}	4.28^{a}	153^{ab}	1.47^{ab}	100	3.01^{abc}	0.73^{a}
CuM_{13}	164^{a}	1776^{a}	4.25^{ab}	148^{abc}	1.38^{abc}	100	2.76^{abc}	0.65^{abc}
CuM_{25}	145^{bc}	1577^{bc}	4.09^{cd}	144^{bcd}	1.37^{abc}	100	2.74^{abc}	0.63^{cd}
CuM_{49}	130^{efg}	1443^{de}	3.97^{def}	133^{ef}	1.30^{cd}	100	2.56^{bcd}	0.56^{de}
CuM_{194}	124^{gh}	1341^{de}	3.87^{fg}	117^{gh}	1.17^{de}	100	2.04^{de}	0.46^{fg}
$CuS_{4.0}$	134^{ef}	1440^{de}	3.96^{defg}	136^{def}	1.32^{c}	100	3.16^{ab}	0.55^{de}
$CuS_{7.0}$	142^{cd}	1572^{bc}	4.08^{cde}	140^{cde}	1.35^{abc}	100	3.20^{a}	0.59^{cde}
CuS_{10}	149^{b}	1623^{b}	4.12^{bc}	138^{cde}	1.31^{c}	100	2.89^{abc}	0.59^{cde}
CuS_{13}	151^{b}	1674^{ab}	4.17^{abc}	138^{cde}	1.33^{bc}	100	3.02^{abc}	0.72^{ab}
CuS_{25}	131^{ef}	1428^{de}	3.95^{efg}	138^{cde}	1.31^{c}	100	2.53^{cde}	0.61^{cd}
CuS_{50}	128^{fgh}	1394^{de}	3.92^{fg}	137^{cde}	1.30^{cd}	100	2.43^{cde}	0.51^{ef}
CuS_{195}	122^{h}	1314^{e}	3.84^{g}	110^{h}	1.06^{e}	97	1.91^{e}	0.40^{g}
Pooled SEM[8]	1.92	23.30	0.02	1.94	0.02	0.20	0.06	0.01

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$)

[2] Weight gain (WG; %): (final wt. - initial wt.) × 100/initial wt

[3]Specific growth rate (SGR; %/day): 100× (Ln final wt. - Ln initial wt.)/days

[4]Feed efficiency (FE; %): (wet weight gain/dry feed intake) × 100

[5]Protein efficiency ratio (PER): (wet weight gain / protein intake)

[6]Hepatosomatic index (HIS; %) = Liver weight×100/fish weight

[7]Condition factor (CF; g/cm^3) = [Body weight (g)/fork langth (cm^3)] × 100

[8]Pooled standard error of means: SD/√n

Table 36. Effect of the dietary copper sources and levels on proximate composition (% dry matter) of juvenile beluga sturgeon[1]

Dietary factors	Whole body moisture	Whole body protein	Whole body lipid	Whole body ash	Muscle moisture	Muscle protein	Muscle lipid
Copper sources (mg/kg)							
Organic Cu	5.29^{b}	64.3^{a}	20.38^{a}	11.25	4.38	63.84^{a}	20.87^{a}
Inorganic Cu	5.78^{a}	64.05^{b}	19.90^{b}	11.20	4.40	63.05^{b}	20.57^{b}
Pooled SEM[2]	0.20	0.31	0.33	0.19	0.05	0.76	0.23
Copper levels (mg/kg)							
1	4.80^{e}	64.52^{c}	21.84^{a}	9.94^{e}	4.24^{c}	63.36^{d}	20.88^{bc}
4	5.03^{d}	65.03^{b}	21.62^{a}	10.35^{de}	4.17^{c}	62.97^{e}	20.35^{c}
7	5.12^{cd}	64.45^{a}	21.09^{b}	10.69^{cd}	4.28^{c}	64.05^{c}	21.46^{ab}
10	5.01^{d}	65.15^{b}	20.96^{bc}	10.73^{cd}	4.20^{c}	65.08^{b}	21.61^{a}
13	5.27^{c}	65.53^{a}	20.68^{bc}	11.04^{c}	4.20^{c}	65.50^{a}	21.85^{a}
25	5.20^{cd}	64.74^{c}	20.56^{c}	11.25^{c}	4.41^{bc}	64.86^{b}	21.93^{a}
49	5.57^{b}	62.73^{d}	18.03^{d}	12.56^{b}	4.62^{b}	61.60^{f}	19.54^{d}
194	8.30^{a}	60.24^{e}	16.34^{e}	13.26^{a}	4.99^{a}	60.13^{g}	18.15^{e}
Pooled SEM[2]	0.20	0.31	0.33	0.19	0.05	0.76	0.23
ANOVA							
Copper sources	<0.0001	0.0007	<0.0002	0.585	0.6123	<0.0001	0.0094
Copper levels	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Source × levels	0.003	0.0475	0.0583	0.9651	0.0168	<0.0001	0.0555

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: $SD/\sqrt{n}$

Table 37. Proximate composition (% dry matter) of juvenile beluga sturgeon fed with various levels of supplemental copper from organic or inorganic sources[1]

Diet	Whole body moisture	Whole body protein	Whole body lipid	Whole body ash	Muscle moisture	Muscle protein	Muscle lipid
$Cu_{1.0}$	4.80^{ef}	64.52^{c}	21.84^{a}	9.94^{d}	4.24^{cd}	63.36^{d}	20.88^{bc}
$CuM_{4.0}$	4.95^{ef}	65.29^{ab}	21.69^{ab}	10.46^{bcd}	4.26^{cd}	63.76^{d}	20.38^{cd}
$CuM_{7.0}$	4.80^{ef}	65.52^{a}	21.15^{abc}	10.74^{bcd}	4.45^{bcd}	64.36^{c}	21.48^{ab}
CuM_{10}	4.76^{f}	65.28^{ab}	21.21^{ab}	10.73^{bcd}	4.07^{d}	65.82^{a}	21.65^{a}
CuM_{13}	5.08^{def}	65.61^{a}	21.05^{abc}	11.02^{bc}	4.05^{d}	65.31^{ab}	21.87^{a}
CuM_{25}	4.93^{ef}	64.75^{cd}	21.19^{abc}	11.32^{b}	4.39^{bcd}	64.49^{c}	21.30^{a}
CuM_{49}	5.24^{def}	62.80^{d}	18.48^{e}	12.64^{a}	4.73^{abc}	62.72^{e}	19.9^{d}
CuM_{194}	7.82^{b}	60.66^{e}	16.48^{f}	13.19^{a}	4.83^{ab}	60.94^{f}	18.81^{e}
$CuS_{4.0}$	5.11^{def}	64.77^{bc}	21.56^{ab}	10.25^{cd}	4.08^{d}	62.19^{e}	20.32^{cd}
$CuS_{7.0}$	5.45^{cd}	65.38^{ab}	21.03^{abc}	10.64^{bcd}	4.11^{d}	63.75^{d}	21.45^{ab}
CuS_{10}	5.27^{de}	65.03^{abc}	20.71^{bcd}	10.73^{bcd}	4.34^{bcd}	64.35^{c}	21.65^{ab}
CuS_{13}	5.46^{cd}	65.46^{a}	20.31^{cd}	11.05^{bc}	4.34^{bcd}	65.7^{ab}	21.83^{a}
CuS_{25}	5.48^{cd}	64.74^{bc}	19.94^{d}	11.19^{b}	4.44^{bcd}	65.23^{b}	21.87^{a}
CuS_{50}	5.89^{c}	62.66^{d}	17.59^{e}	12.49^{a}	4.51^{bcd}	60.49^{f}	19.16^{e}
CuS_{195}	8.79^{a}	59.83^{f}	16.20^{f}	13.34^{a}	5.15^{a}	59.3^{g}	17.50^{f}
Pooled SEM[2]	0.21	0.33	0.34	0.20	0.06	0.33	0.25

[1]Values are means from groups (n = 3) of fish where the means in each column with a different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means SD/√n.

Table 38. Effect of the dietary copper source and levels on hepatic thiobarbituric acid reactive substances (TBARS) and Cu-Zn superoxide dismutase (Cu-Zn SOD) activity of juvenile beluga sturgeon[1]

Dietary factors	Cu-Zn SOD Units/mg liver	GPX/ liver	TBARS nmol MDA/ml liver	Cu-Zn SOD Units/mg muscle	GPX/ muscle
Copper sources (mg/kg)					
Organic Cu	123.4^{a}	216.6^{a}	5.42^{b}	14.49^{a}	151.9^{a}
Inorganic Cu	118.2^{b}	215.4^{b}	5.68^{a}	14.22^{b}	151.3^{b}
Pooled SEM[2]	2.47	2.61	0.30	0.17	0.28
Copper levels (mg/kg)					
1	99.56^{d}	191.5^{g}	6.69^{b}	12.63^{d}	149.6^{c}
4	110.0^{c}	193.4^{f}	5.43^{d}	13.48^{c}	151.5^{b}
7	126.4^{b}	222.3^{c}	4.38^{e}	14.79^{ab}	152.1^{b}
10	135.1^{a}	230.3^{a}	3.70^{f}	14.48^{a}	153.0^{a}
13	135.9^{a}	229.7^{a}	3.78^{f}	15.41^{a}	153.0^{a}
25	129.0^{ab}	226.1^{b}	5.35^{d}	14.59^{b}	153.1^{a}
49	123.8^{b}	220.3^{d}	6.18^{c}	14.42^{b}	151.7^{b}
194	106.7^{cd}	214.7^{e}	8.98^{a}	14.03^{bc}	149.0^{c}
Pooled SEM[2]	2.47	2.61	0.30	0.17	0.28
ANOVA					
Copper source	0.0003	<0.0001	0.0025	0.0312	0.0010
Copper levels	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Source × levels	0.001	<0.0001	0.0081	0.5214	0.2118

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: SD/√n

Table 39. Copper zinc superoxide dismutase (Cu-Zn SOD), glutathione peroxidase (GPx) activity and thiobarbituric acid reactive substances (TBARS) value of juvenile beluga sturgeon fed with various levels of supplemental copper from organic or inorganic sources[1]

Diet	Cu-Zn SOD Units/mg liver	GPX/ liver	TBARS nmol MDA/ml liver	Cu-Zn SOD Units/mg muscle	GPX/ muscle
$Cu_{1.0}$	99.6^{gh}	191.5^{g}	6.69^{b}	12.6^{h}	149.58^{ef}
$CuM_{4.0}$	111.3^{efg}	194.1^{g}	5.20^{ef}	13.8^{cde}	151.76^{bcd}
$CuM_{7.0}$	127.7^{bcd}	222.4^{cd}	4.10^{gh}	15.2^{abcde}	152.21^{abcd}
CuM_{10}	141.0^{a}	234.3^{a}	3.20^{i}	15.7^{a}	153.41^{a}
CuM_{13}	133.3^{abc}	227.9^{abc}	3.95^{gh}	15.3^{abc}	152.92^{abc}
CuM_{25}	131.4^{abc}	226.5^{bcd}	5.25^{ef}	14.8^{abcde}	153.24^{ab}
CuM_{49}	125.6^{cd}	220.8^{cde}	6.15^{bc}	14.6^{abcdef}	152.39^{abcd}
CuM_{194}	117.6^{def}	215.4^{ef}	8.90^{a}	14.1^{defg}	149.65^{ef}
$CuS_{4.0}$	108.7^{fg}	192.6^{g}	5.65^{cde}	13.4^{gh}	151.34^{cd}
$CuS_{7.0}$	125.0^{cd}	222.0^{bcd}	4.64^{fg}	14.4^{bcdefg}	151.92^{abcd}
CuS_{10}	129.1^{abcd}	226.2^{bcd}	4.20^{gh}	15.2^{abcd}	152.61^{abc}
CuS_{13}	138.5^{ab}	231.5^{ab}	3.60^{hi}	15.5^{ab}	152.99^{ab}
CuS_{25}	126.5^{bcd}	225.6^{bcd}	5.45^{de}	14.3^{bcdefg}	152.95^{ab}
CuS_{50}	122.0^{cde}	219.7^{def}	6.20^{bc}	14.2^{cdefg}	150.95^{de}
CuS_{195}	95.9^{h}	213.9^{f}	9.05^{a}	14.0^{efg}	148.39^{f}
Pooled SEM[2]	2.42	2.52	0.31	0.17	0.27

[1]Values are means from groups (n = 3) of fish where the means in each column with a different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means SD/√n.

Table 40. Effect of the dietary copper source and levels on hematological characteristics of juvenile beluga sturgeon[1]

Dietary factors	Hematocrit (%)	Hemoglobin (g/100ml)	Glucose (mg/dL)	Total protein (mg/dL)	Cholesterol	Peroxidase
Copper sources (mg/kg)						
Organic Cu	24.0^{a}	4.84^{a}	3.16^{b}	1.63	23.29^{a}	3.07^{a}
Inorganic Cu	23.4^{b}	4.31^{b}	3.35^{a}	1.63	22.31^{b}	2.85^{b}
Pooled SEM[2]	0.24	0.08	0.05	0.02	0.78	0.06
Copper levels (mg/kg)						
1	23.4^{c}	4.46^{abc}	3.73^{a}	1.58	16.4^{e}	2.30^{d}
4	24.3^{bc}	4.92^{ab}	3.44^{ab}	1.66	17.17^{e}	2.57^{cd}
7	25.3^{ab}	5.01^{a}	3.45^{ab}	1.76	19.17^{de}	2.89^{bc}
10	25.8^{a}	5.09^{a}	2.89^{cd}	1.72	20.83^{cd}	2.92^{bc}
13	25.8^{a}	4.94^{ab}	2.77^{d}	1.69	22.67^{c}	3.00^{b}
25	23.5^{c}	4.21^{bc}	3.29^{abc}	1.55	27.67^{b}	3.22^{ab}
49	21.3^{d}	3.64^{c}	3.11^{bcd}	1.56	26.83^{b}	3.25^{ab}
194	20.3^{d}	4.01^{c}	3.36^{ab}	1.50	31.67^{a}	3.39^{a}
Pooled SEM[2]	0.24	0.08	0.05	0.02	0.78	0.06
ANOVA						
Copper sources	<0.0546	<0.0001	0.0156	0.9449	0.0351	0.0005
Copper levels	<0.0001	<0.0001	<0.0001	0.247	<0.0001	<0.0001
Source × levels	0.1221	0.5049	0.4042	0.6494	0.1908	0.2344

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[9]Pooled standard error of means SD/√n

Table 41. Effect of the dietary copper source and levels on hematological characteristics of juvenile beluga sturgeon[1]

Dietary factors	Lysozyme Activity (U/mL)	GOT[2]	GPT[3]	Calcium (mg d/L)	Magnesium (meq/L)
Copper sources (mg/kg)					
Organic Cu	20.0	268.3^{b}	2.13^{b}	7.16^{a}	1.43
Inorganic Cu	19.7	295.3^{a}	2.38^{a}	6.82^{b}	1.41
Pooled SEM[4]	0.81	5.41	0.13	0.06	0.03
Copper levels (mg/kg)					
1	13.1^{e}	236.3^{f}	1.32^{e}	6.87^{ab}	1.21^{d}
4	13.7^{e}	248.7^{e}	1.43^{de}	7.05^{ab}	1.28^{d}
7	22.9^{c}	258.0^{de}	1.67^{de}	7.27^{a}	1.33^{cd}
10	27.8^{a}	263.8^{d}	1.83^{de}	7.32^{a}	1.35^{bcd}
13	25.5^{b}	289.2^{c}	2.08^{cd}	7.22^{ab}	1.51^{abc}
25	22.7^{c}	316.2^{b}	2.77^{bc}	6.77^{ab}	1.48^{bc}
49	18.6^{d}	308.3^{b}	3.08^{b}	6.82^{ab}	1.54^{ab}
194	14.9^{e}	334.0^{a}	3.83^{a}	6.59^{b}	1.69^{a}
Pooled SEM[4]	0.81	5.41	0.13	0.06	0.03
ANOVA					
Copper sources	0.5168	<0.0001	0.0268	0.0016	0.3056
Copper levels	<0.0001	<0.0001	<0.0001	0.0059	<0.0001
Source × levels	<0.0001	<0.0001	0.1663	0.7501	0.6645

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]GOT (AST; U/L): Glutamic oxaloacetic transaminase (Aspartate transminase)

[3]GPT (ALT; U/L): Glutamic pyruvic transaminase (Alanine transminase)

[4]Pooled standard error of means SD/√n.

Table 42. Hematological and biochemical characteristics of juvenile beluga sturgeon fed with various levels of supplemental copper from organic or inorganic sources[1]

Dietary factors	Hematocrit (%)	Hemoglobin (g/100ml)	Glucose (mg/dL)	Total protein (mg/dL)	Cholesterol	Peroxidase
$Cu_{1.0}$	23.4^{bcde}	4.46^{abcd}	3.7^{a}	1.6	16.40^{f}	2.30^{g}
$CuM_{4.0}$	24.0^{abcd}	5.11^{ab}	3.4^{abc}	1.7	17.67^{ef}	2.58^{efg}
$CuM_{7.0}$	25.3^{ab}	5.23^{ab}	3.4^{abc}	1.8	19.33^{def}	2.95^{def}
CuM_{10}	26.3^{a}	5.28^{ab}	2.7^{d}	1.8	21.00^{de}	3.03^{cde}
CuM_{13}	25.8^{ab}	5.45^{a}	2.7^{d}	1.6	22.67^{cd}	3.13^{abcd}
CuM_{25}	24.7^{abc}	4.61^{abcd}	3.0^{bcd}	1.5	28.00^{b}	3.50^{ab}
CuM_{49}	21.2^{ef}	4.34^{abcd}	3.1^{abcd}	1.5	27.33^{b}	3.43^{abc}
CuM_{194}	21.2^{ef}	4.23^{bcd}	3.3^{abcd}	1.5	34.00^{a}	3.57^{a}
$CuS_{4.0}$	24.7^{abc}	4.74^{abc}	3.4^{abc}	1.6	16.67^{ef}	2.55^{fg}
$CuS_{7.0}$	25.2^{ab}	4.79^{abc}	3.5^{abc}	1.8	19.00^{def}	2.84^{def}
CuS_{10}	25.3^{ab}	4.91^{abc}	3.1^{abcd}	1.6	20.67^{def}	2.80^{def}
CuS_{13}	25.7^{ab}	4.44^{abcd}	2.8^{cd}	1.8	22.67^{cd}	2.88^{def}
CuS_{25}	22.3^{cde}	3.82^{cd}	3.6^{ab}	1.6	27.33^{b}	2.93^{def}
CuS_{50}	21.3^{def}	3.56^{d}	3.2^{abcd}	1.6	26.33^{bc}	3.06^{bcd}
CuS_{195}	19.5^{f}	3.78^{cd}	3.5^{abc}	1.5	29.33^{b}	3.14^{abcd}
Pooled SEM[2]	0.25	0.08	0.05	0.02	0.79	0.06

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means SD/√n.

Table 43. Hematological and biochemical characteristics of juvenile beluga sturgeon fed with various levels of supplemental copper from organic or inorganic sources[1]

Diet	Lysozyme Activity (U/mL)	GOT[2]	GPT[3]	Calcium (mg d/L)	Magnesium (meq/L)
$Cu_{1.0}$	13.1^{h}	236^{i}	1.32^{e}	6.87^{abc}	1.21^{d}
$CuM_{4.0}$	13.8^{h}	235^{i}	1.37^{e}	7.13^{abc}	1.30^{cd}
$CuM_{7.0}$	25.7^{bc}	243^{hi}	1.50^{e}	7.43^{abc}	1.34^{bcd}
CuM_{10}	29.3^{a}	251^{ghi}	1.67^{e}	7.53^{ab}	1.37^{bcd}
CuM_{13}	23.0^{de}	260^{fgh}	1.83^{de}	7.54^{a}	1.52^{abc}
CuM_{25}	21.0^{ef}	293de	2.33^{cde}	7.00^{abc}	1.43^{abcd}
CuM_{49}	19.0^{fg}	306^{cd}	3.33^{abc}	7.09^{abc}	1.63^{ab}
CuM_{194}	14.9^{h}	324^{bc}	3.67^{ab}	6.73^{abc}	1.71^{a}
$CuS_{4.0}$	13.6^{h}	262^{fg}	1.50^{e}	6.97^{abc}	1.26^{cd}
$CuS_{7.0}$	20.0^{fg}	273^{f}	1.83^{de}	7.12^{abc}	1.31^{cd}
CuS_{10}	26.3^{bc}	276^{ef}	2.00^{de}	7.11^{abc}	1.34^{bcd}
CuS_{13}	28.0^{ab}	318^{c}	2.33^{cde}	6.90^{abc}	1.49^{abcd}
CuS_{25}	24.3^{cd}	339^{ab}	3.20^{abc}	6.54^{bc}	1.52^{abc}
CuS_{50}	18.0^{g}	310^{cd}	2.83^{bcd}	6.56^{abc}	1.45^{abcd}
CuS_{195}	14.7^{h}	344^{a}	4.00^{a}	6.46^{c}	1.68^{a}
Pooled SEM[4]	0.9	5.7	0.14	0.07	0.03

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]GOT (AST; U/L): Glutamic oxaloacetic transaminase (Aspartate transminase);

[2]GPT (ALT; U/L): Glutamic pyruvic transaminase (Alanine transminase);

[4]Pooled standard error of means SD/√n.

Table 44. Effect of the dietary copper source and levels on copper content (mg/kg) of juvenile beluga sturgeon[1]

Dietary factors	Liver	Intestine	Gill	Muscle	Kidney	Plasma Cu	Whole body (DM)
Copper sources (mg/kg)							
Organic Cu	34.68^{b}	5.03	2.70^{a}	2.46^{a}	3.85	12.4	4.74^{a}
Inorganic Cu	35.77^{a}	4.91	2.57^{b}	2.25^{b}	3.86	12.2	4.49^{b}
Pooled SEM[2]	1.53	0.19	0.17	0.14	0.12	0.84	0.18
Copper levels (mg/kg)							
1	25.4^{h}	3.8^{e}	1.36^{g}	1.49^{e}	3.05^{e}	5.40^{f}	3.46^{g}
4	26.7^{g}	4.0^{e}	1.38^{g}	1.57^{e}	3.14^{e}	6.68^{ef}	3.58^{fg}
7	29.0^{f}	4.1^{de}	2.11^{f}	1.83^{d}	3.33^{de}	8.55^{e}	3.86^{f}
10	30.5^{e}	4.6^{cd}	2.44^{e}	1.94^{d}	3.61^{d}	10.73^{d}	4.25^{e}
13	36.0^{d}	4.8^{c}	2.67^{d}	2.36^{c}	4.05^{c}	12.42^{cd}	4.58^{d}
25	39.1^{c}	5.4^{b}	3.18^{c}	2.83^{b}	4.33^{bc}	14.33^{bc}	4.99^{c}
49	45.6^{b}	6.4^{a}	3.89^{b}	3.00^{b}	4.49^{ab}	15.78^{b}	5.70^{b}
194	49.5^{a}	6.7^{a}	4.08^{a}	3.81^{a}	4.86^{a}	14.53^{a}	6.51^{a}
Pooled SEM[2]	1.53	0.19	0.17	0.14	0.12	0.84	0.18
ANOVA							
Copper sources	<0.0001	0.1141	<0.0001	<0.0001	0.8753	0.4074	<0.0001
Copper levels	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Source × levels	<0.0001	0.9479	0.0520	0.0003	0.4827	0.9876	0.0007

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: SD/√n

Table 45. Copper content (mg/kg) of juvenile beluga sturgeon fed with various levels of supplemental copper from organic or inorganic sources[1]

Diet	Liver	Intestine	Kidney	Gill	Muscle	Plasma Cu	Whole body (DM)
$Cu_{1.0}$	25.4^{i}	3.79^{e}	3.05^{f}	1.36^{i}	1.49^{j}	5.4^{h}	3.46^{h}
$CuM_{4.0}$	26.9^{i}	4.02^{de}	3.16^{f}	1.39^{i}	1.57^{ij}	6.7^{gh}	3.61^{h}
$CuM_{7.0}$	29.0^{h}	4.16^{cde}	3.24^{ef}	2.14^{gh}	1.86^{ghij}	8.1^{fgh}	3.89^{gh}
CuM_{10}	30.8^{h}	4.74^{bcd}	3.50^{def}	2.47^{ef}	1.95^{gh}	10.5^{ef}	4.23^{fg}
CuM_{13}	36.5^{fg}	4.92^{bc}	4.21^{abcd}	2.75^{de}	2.50^{ef}	12.2^{de}	4.66^{def}
CuM_{25}	40.3^{e}	5.40^{b}	4.36^{abc}	3.34^{c}	2.98^{cd}	14.3^{bcd}	5.06^{cd}
CuM_{49}	43.3^{d}	6.46^{a}	4.52^{ab}	4.03^{ab}	3.16^{bc}	15.9^{b}	6.10^{b}
CuM_{194}	45.3^{c}	6.73^{a}	4.80^{a}	4.16^{a}	4.20^{a}	24.4^{a}	6.90^{a}
$CuS_{4.0}$	26.5^{i}	3.93^{de}	3.13^{f}	1.37^{i}	1.58^{hij}	6.7^{gh}	3.55^{h}
$CuS_{7.0}$	29.0^{h}	4.11^{cde}	3.43^{ef}	2.07^{h}	1.81^{hij}	9.0^{fg}	3.84^{gh}
CuS_{10}	30.2^{h}	4.47^{cde}	3.74^{cdef}	2.41^{fg}	1.93^{ghi}	11.0^{ef}	4.26^{de}
CuS_{13}	35.6^{g}	4.62^{bcd}	3.89^{bcde}	2.59^{ef}	2.22^{fg}	12.7^{cde}	4.51^{ef}
CuS_{25}	38.1^{f}	5.31^{b}	4.30^{abc}	3.02^{d}	2.68^{de}	14.3^{bcd}	4.91^{cde}
CuS_{50}	47.9^{b}	6.36^{a}	4.46^{ab}	3.76^{b}	2.83^{cde}	15.7^{bc}	5.30^{c}
CuS_{195}	53.7^{a}	6.67^{a}	4.91^{a}	4.00^{ab}	3.42^{b}	24.7^{a}	6.12^{b}
Pooled SEM[2]	1.5	0.19	0.12	0.18	0.14	0.83	0.19

[1]Values are means from groups (n = 3) of fish where the means in each column with a different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means SD/√n.

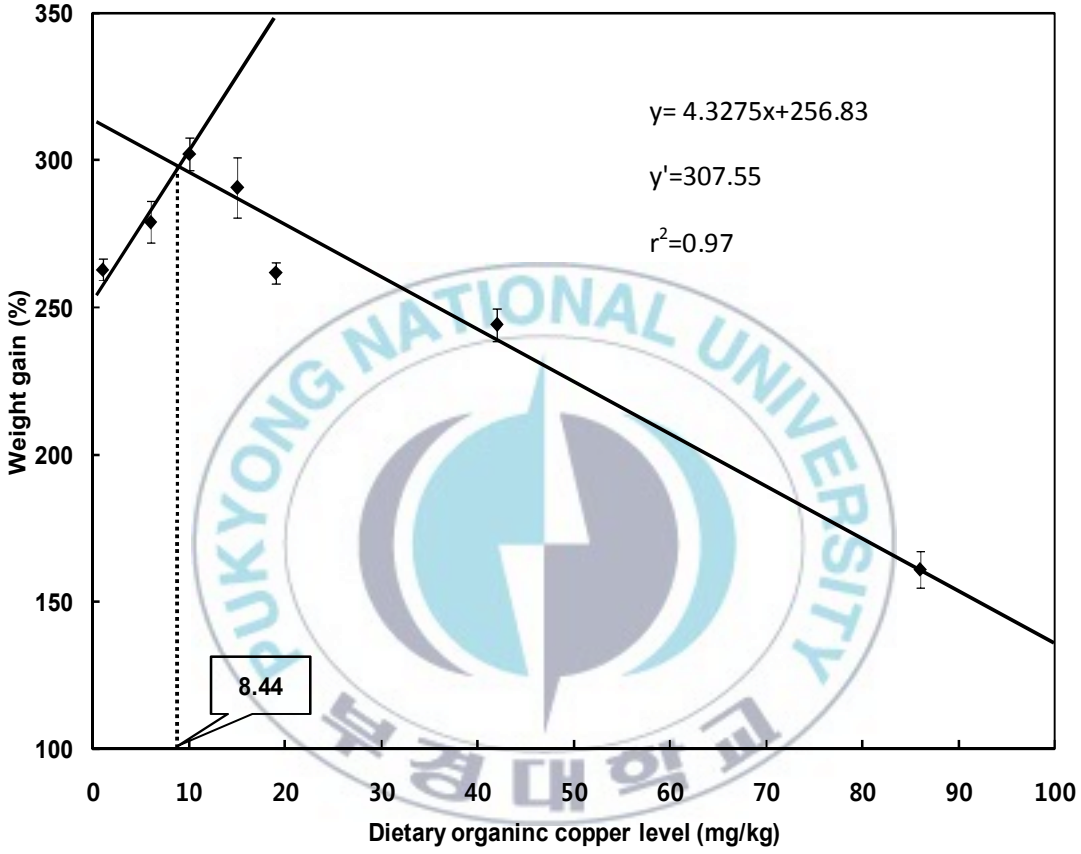

Figure 1. The relationship between dietary copper concentration and weight gain in juvenile olive flounder, *P. olivaceus*, fed different levels of organic copper. Each point represents the mean of three groups of fish. Requirement derived with the broken-line analysis method is 8.44 mg/kg diet.

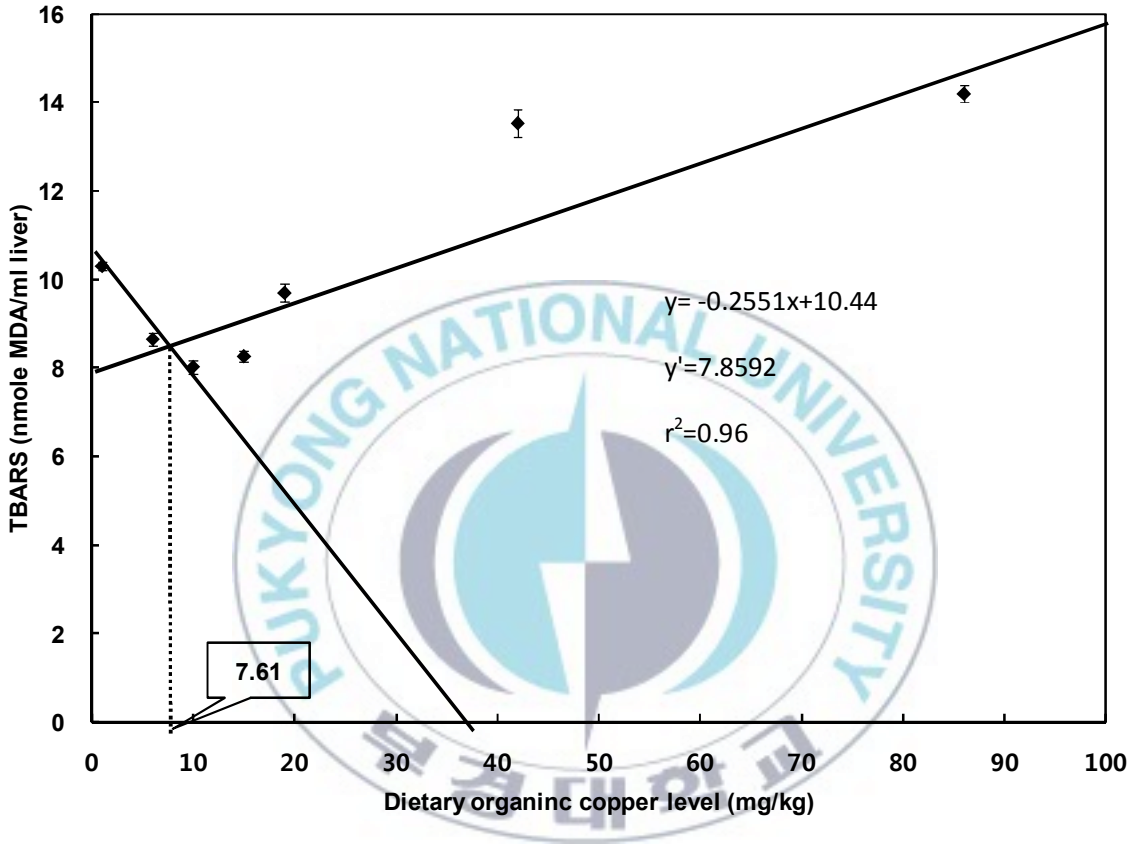

Figure 2. The relationship between dietary copper concentration and TBARS values in juvenile olive flounder, P. olivaceus, fed different levels of organic copper. Each point represents the mean of three groups of fish. Requirement derived with the broken-line analysis method is 7.61 mg/kg diet.

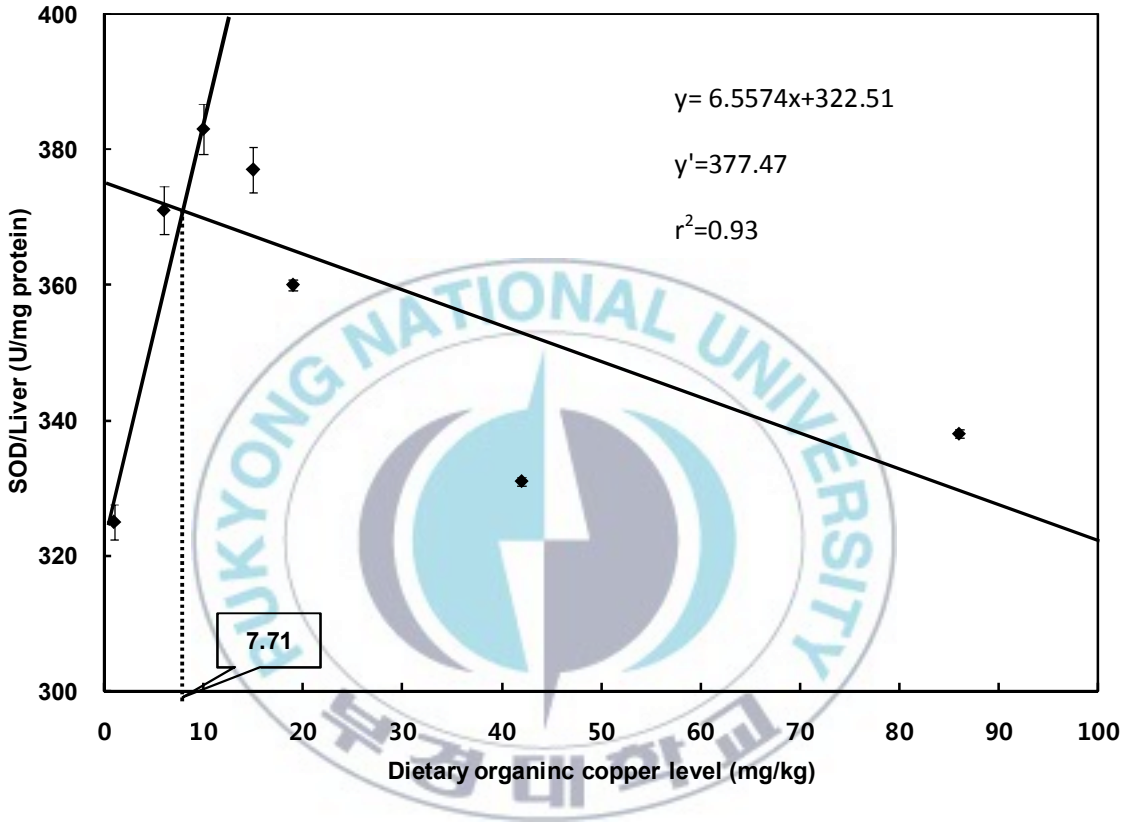

Figure 3. The relationship between dietary copper concentration and Cu-Zn SOD activity in juvenile olive flounder, *P. olivaceus*, fed different levels of organic copper. Each point represents the mean of three groups of fish. Requirement derived with the broken-line analysis method is 7.71 mg/kg diet.

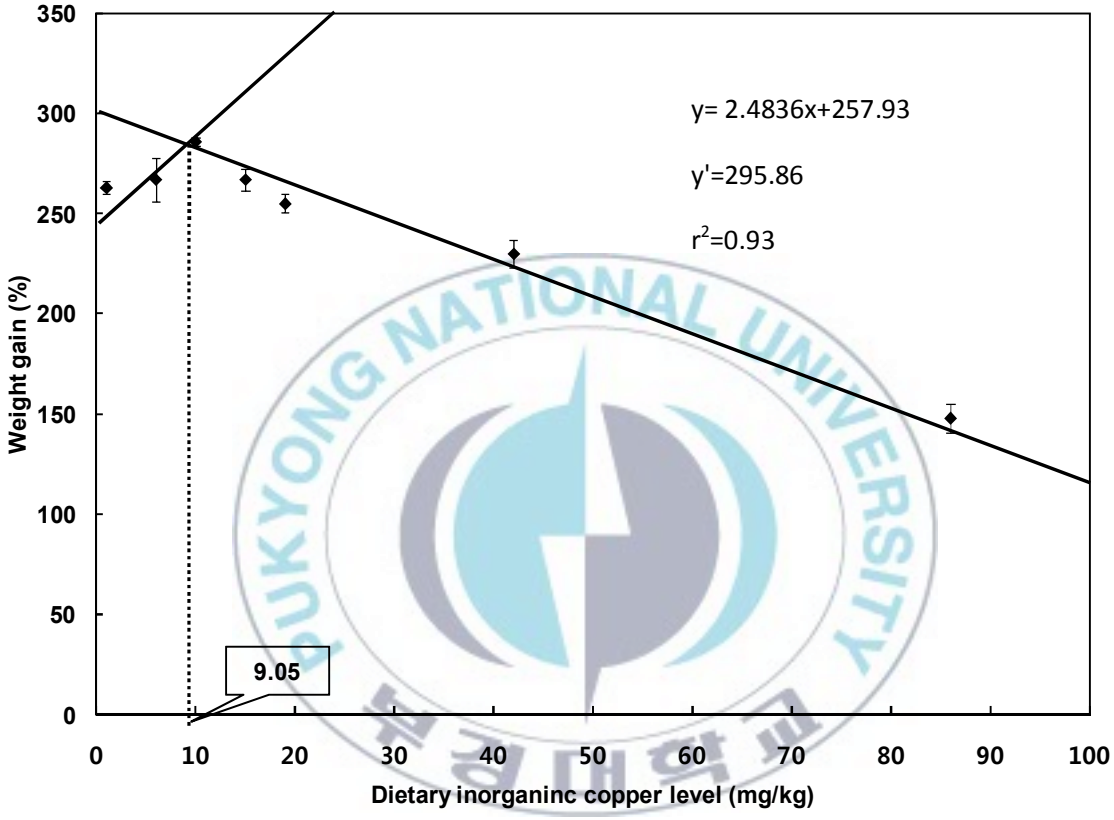

Figure 4. The relationship between dietary copper concentration and weight gain in juvenile olive flounder, *P. olivaceus*, fed different levels of inorganic copper. Each point represents the mean of three groups of fish. Requirement derived with the broken-line analysis method is 9.05 mg/kg diet.

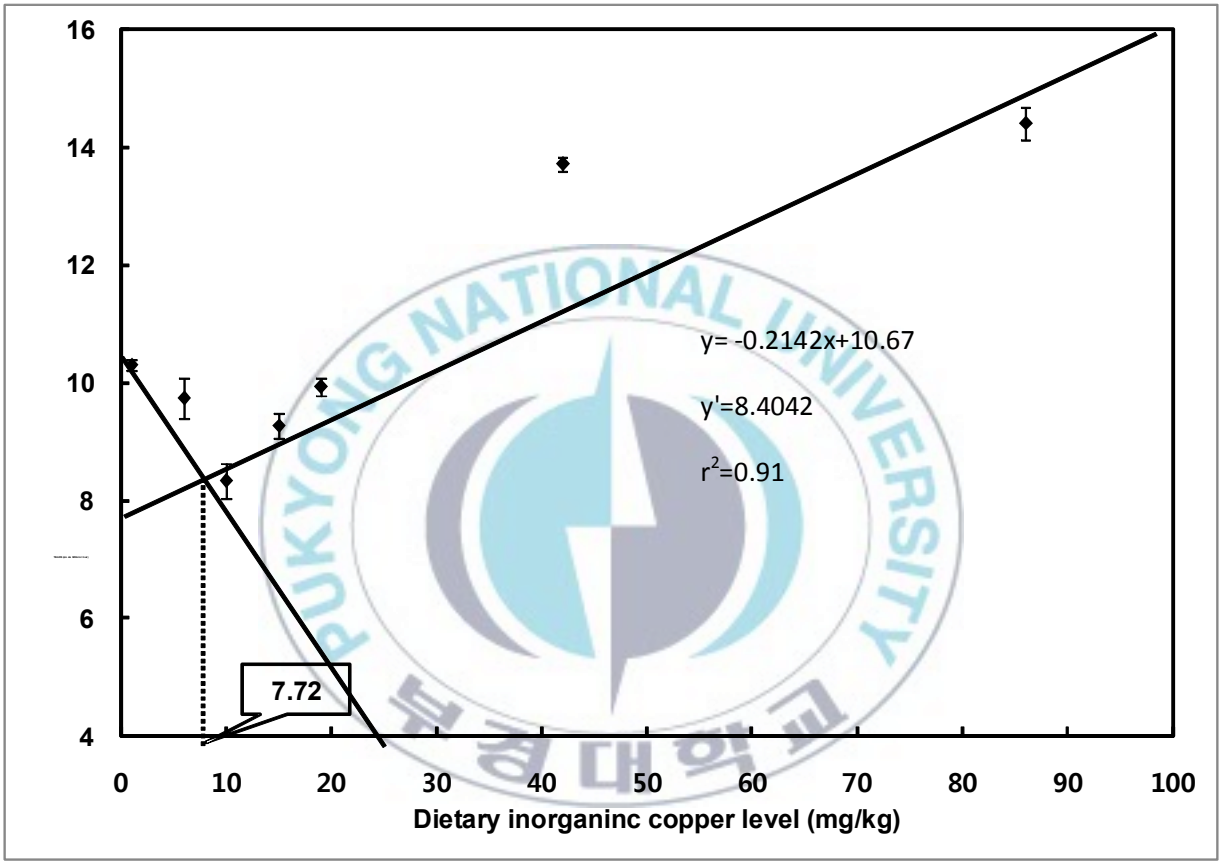

Figure 5. The relationship between dietary copper concentration and TBARS values in juvenile olive flounder, *P. olivaceus*, fed different levels of inorganic copper. Each point represents the mean of three groups of fish. Requirement derived with the broken-line analysis method is 7.72 mg/kg diet.

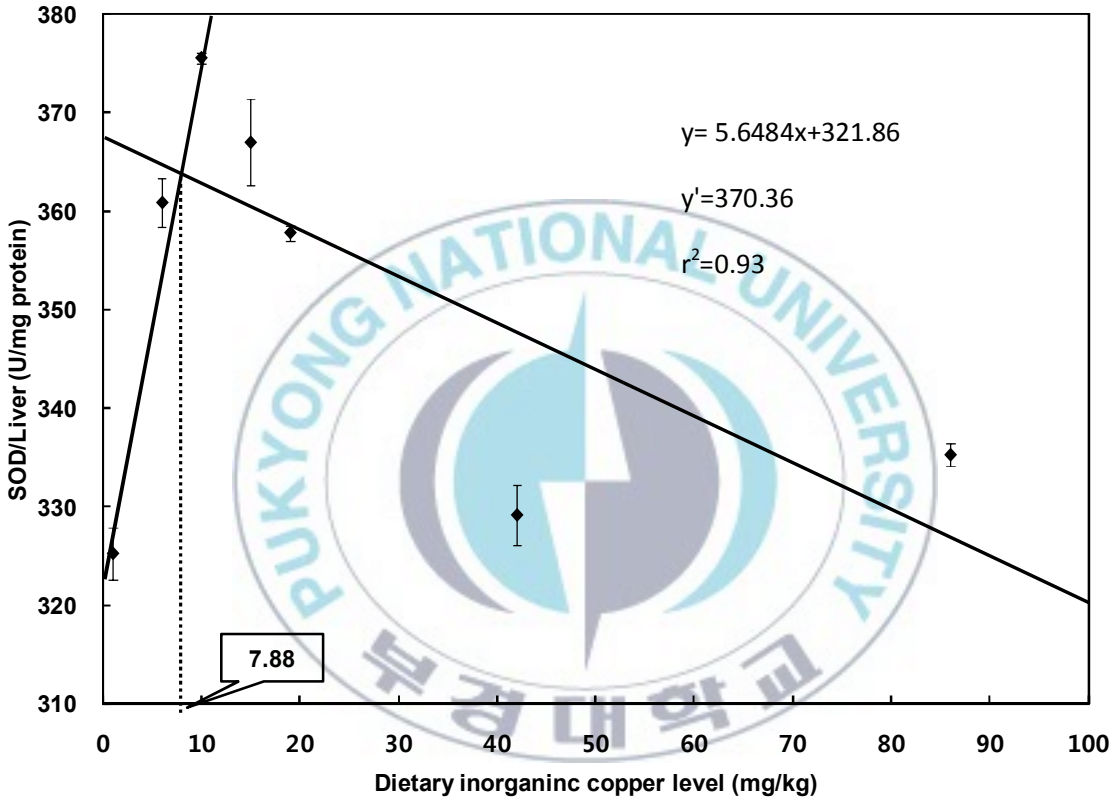

Figure 6. The relationship between dietary copper concentration and Cu-Zn SOD activity in juvenile olive flounder, *P. olivaceus*, fed different levels of inorganic copper. Each point represents the mean of three groups of fish. Requirement derived with the broken-line analysis method is 7.88 mg/kg diet.

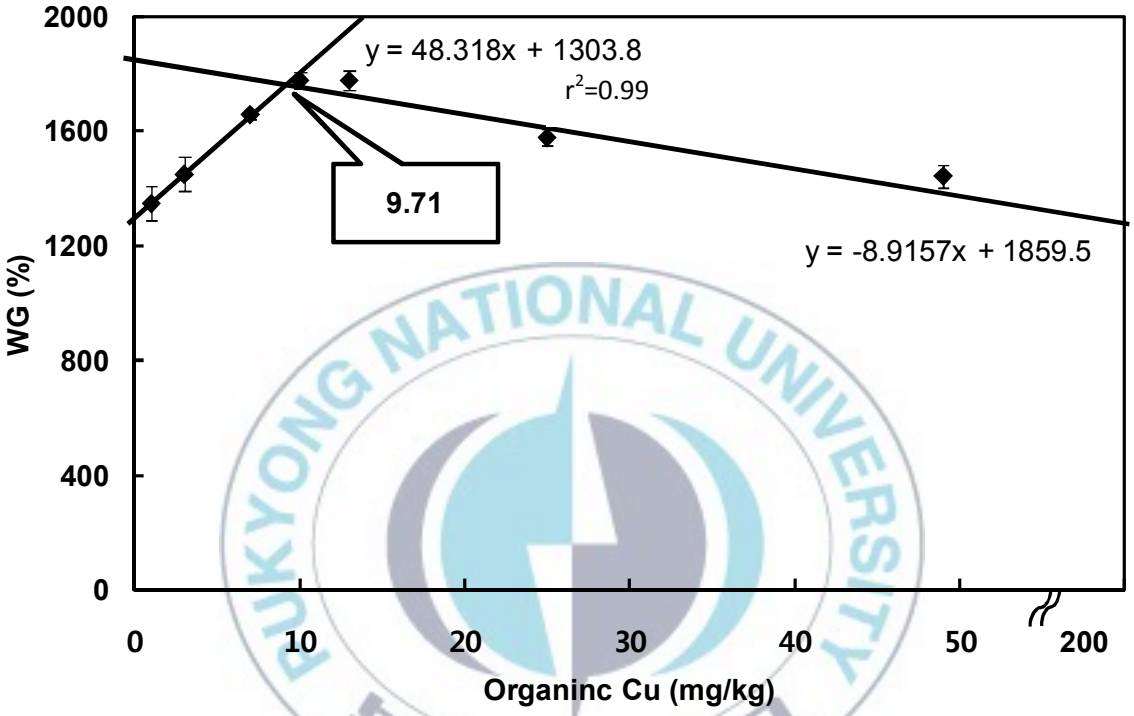

Figure 7. The relationship between dietary copper concentration and weight gain in beluga sturgeon, *H. huso*, fed different levels of organic copper. Each point represents the mean of three groups of fish. Requirement derived with the broken-line analysis method is 9.71 mg/kg diet.

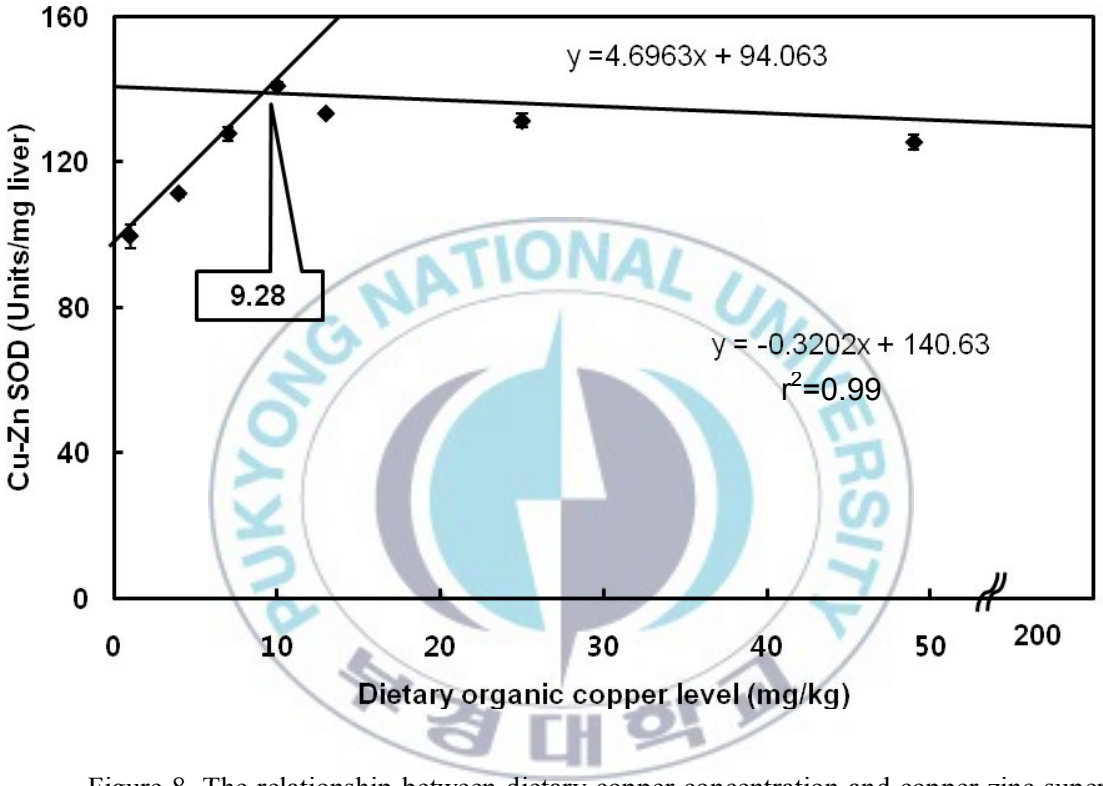

Figure 8. The relationship between dietary copper concentration and copper-zinc superoxide dismutase (Cu-Zn SOD) activity in beluga sturgeon, *H. huso*, fed different levels of organic copper. Each point represents the mean of three groups of fish. Requirement derived with the broken-line analysis method is 9.28 mg/kg diet.

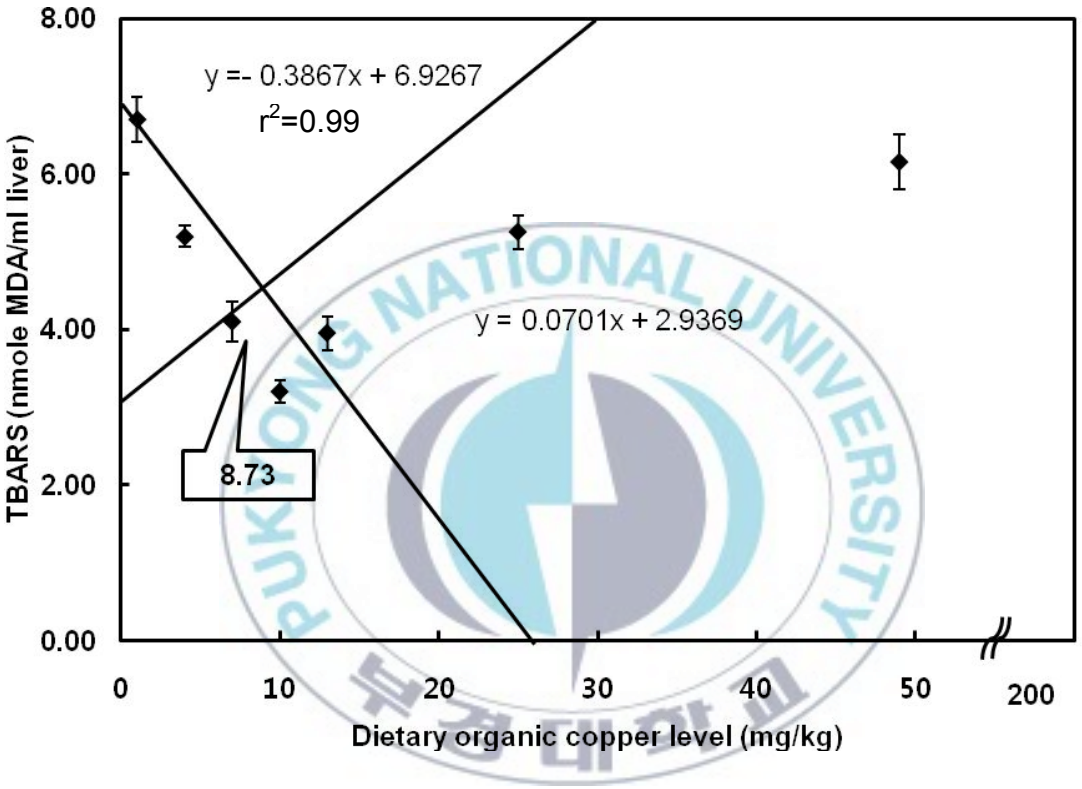

Figure 9. The relationship between dietary copper concentration and thiobarbituric acid reaction substance (TBARS) values in beluga sturgeon, *H. huso*, fed different levels of organic copper. Each point represents the mean of three groups of fish. Requirement derived with the broken-line analysis method is 8.73 mg/kg diet.

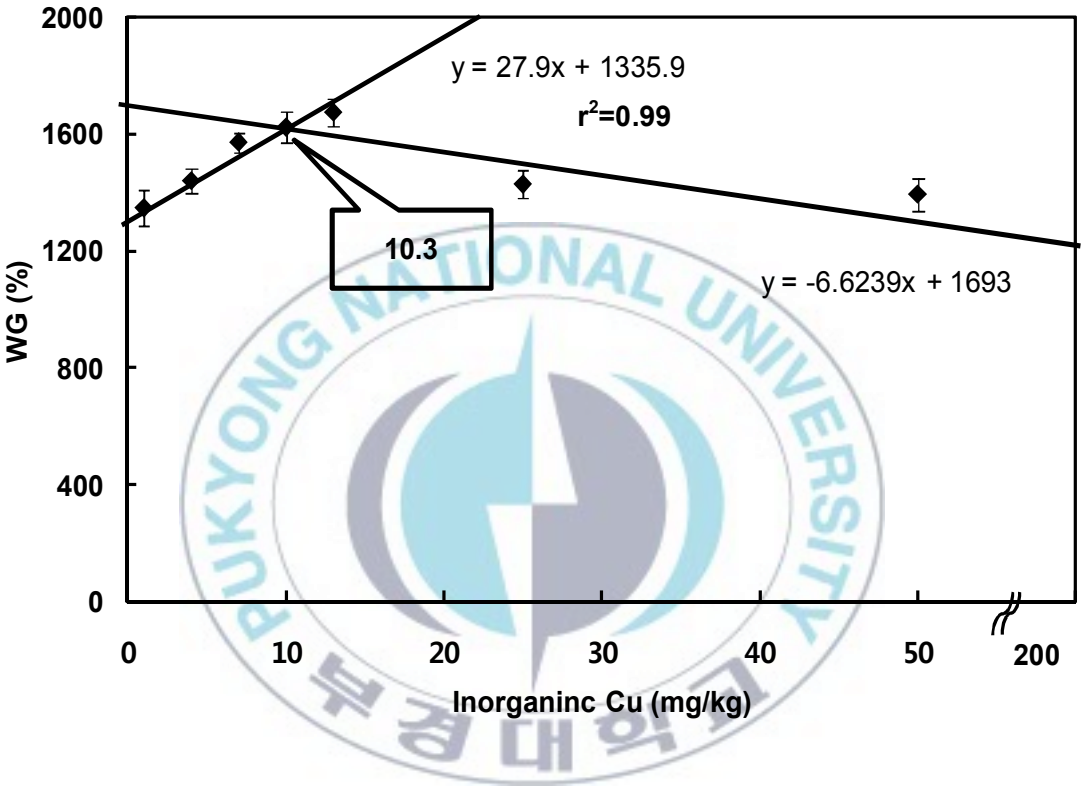

Figure 10. The relationship between dietary copper concentration and weight gain in beluga sturgeon, *H. huso*, fed different levels of inorganic copper. Each point represents the mean of three groups of fish. Requirement derived with the broken-line analysis method is 10.3 mg/kg diet.

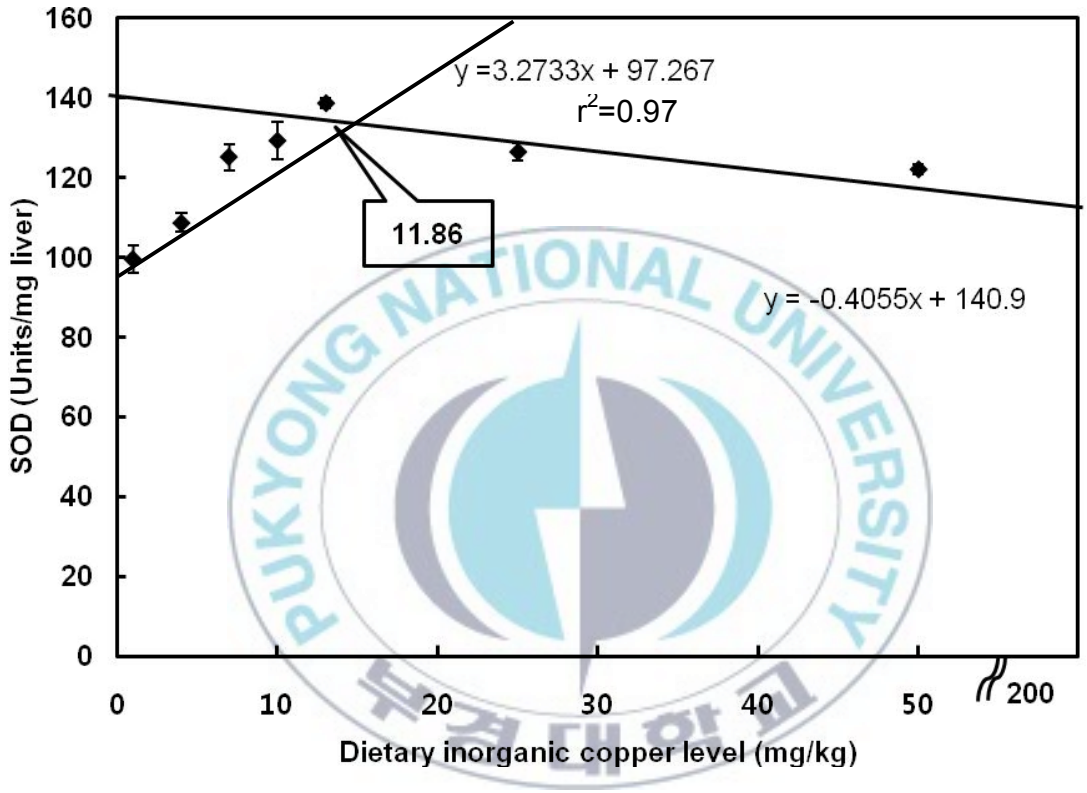

Figure 11. The relationship between dietary copper concentration and copper-zinc superoxide dismutase (Cu-Zn SOD) activity in beluga sturgeon, *H. huso*, fed different levels of inorganic copper. Each point represents the mean of three groups of fish. Requirement derived with the broken-line analysis method is 11.86 mg/kg diet.

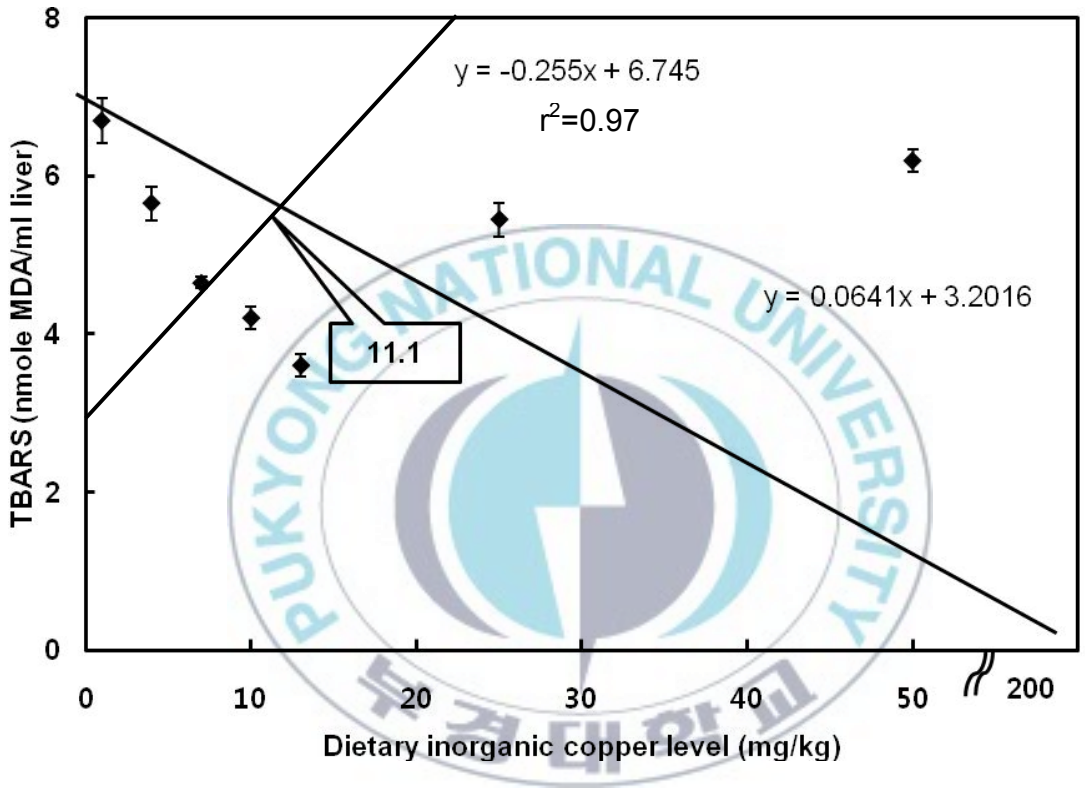

Figure 12. The relationship between dietary copper concentration and thiobarbituric acid reaction substance (TBARS) values in beluga sturgeon, *H. huso*, fed different levels of inorganic copper. Each point represents the mean of three groups of fish. Requirement derived with the broken-line analysis method is 11.1 mg/kg diet.

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