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# Influence of Different Dietary Lipids on the Activity of Metabolic Enzymes in Brown Trout (*Salmo trutta*)

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**Abstract**

The objective of this experiment was to test the effect of different dietary lipids in brown trout feeding on the activity of metabolic enzymes, such as hexokinase (HK), glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD). Four iso-nitrogenous and iso-lipidic casein-gelatin based experimental diets with varying dietary sources were formulated. The sources of dietary lipid were menhaden oil (MO diet, rich in highly unsaturated fatty acids), hazelnut oil (HO diet, predominantly oleic acid), a blend of linseed oil and soybean oil (1:1) (LO+SO diet, linoleic and linolenic acids), and soybean lecithin (LE diet, phospholipids; mostly linoleic acid). Fish with an initial individual weight of ~1g were distributed randomly in three replicate 50-L glass aquaria with a total of 30 fish per tank. Each tank received one of the four diets referenced above for 6 weeks. In all cases, the activity of all metabolic enzymes significantly increased ($P<0.05$) in comparison to their activities in initial fish. All enzyme activities had their highest values in fish fed LO+SO and lowest in fish fed HO diet. It is very important to note that total polyunsaturated fatty acid (PUFA) content of the lipids is in the same order indicating that activity of these enzymes are elevated with increasing PUFA concentrations.

*Keywords*: Carbonic anhydrase, hexokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, lipids, brown trout.

**Kahverengi Alabalıklarda (*Salmo trutta*) Farklı Diyetsel Yağ Kaynaklarının Bazı Metabolik Enzim Aktiviteleri Üzerine Etkisi**

**Özet**

Mevcut çalışmanın amacı farklı diyetsel yağ kaynaklarının kahverengi alabalıkta hexokinase (HK), glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) gibi metabolik enzimler üzerine etkisini araştırmaktır. Tek farkları içerdikleri yağ kaynağı olan dört iso-nitrogenous ve iso-lipidik kazein-jelatin tabanlı yem formüle edilmiştir. Diyetsel yağ kaynakları olarak, ringa balığı yağı (MO diyeti, çoklu doymamış yağ asitleri bakımından zengin), fındık yağı (HO diyeti, başlıca oleik asit içeriyor), keten tohumu yağı/ soya yağı karışımı (1:1) (LO+SO diyeti, linoleik asit ve linolenik asit bakımından zengin) ve soya lesitini (LE diyeti, forfolipid yapısında ve çoğunlukla linoleik asit içeriyor) kullanılmıştır. Başlangıç ağırlıkları yaklaşık 1g olan balıklar, 50 litrelik cam akvaryumlara 30'ar adet, üç tekerrürlü olarak konulmuştur. Balıklar deneysel yemlerle 6 hafta boyunca beslenmişlerdir. Altı haftalık deneme besleme denemesi sonunda, bütün gruplarda metabolik enzim aktiviteleri başlangıç değerlerine göre önemli artış göstermiştir ($P<0,05$). Bütün enzimler en yüksek aktivitelerini LO+SO diyeti ile beslenen balıklarda gösterirken, en düşük aktivitelerini HO diyeti ile beslenen balıklarda göstermişlerdir. Çalışmamız, enzim aktivelerindeki artışların diyetsel yağ kaynaklarındaki çoklu doymamış yağ asitlerinin artışına paralel olarak gerçekleştiğini göstermiştir.

*Anahtar Kelimeler*: Carbonic anhydrase, hexokinase, lucose-6-phosphatedehydrogenase, 6-phosphogluconate dehydrogenase, yağlar, kahverengi alabalık.

## Introduction

Turkish aquaculture production with an annual amount of ~167,000 tons is based on 3 species: rainbow trout (46.8%), sea bass (31.5%) and sea bream (16.8%) (TUIK, 2011). Brown trout is highly regarded species in Turkey and it can be considered as a new candidate to increase the diversity of cultured fish in the country. However, studies on cultured brown trout remain relatively limited in comparison to other cultured species.

Fish oil (FO) is a common lipid source in fish diets due to its high proportion of long-chain, n-3 fatty acids, which are nutritionally essential to teleosts

(NRC, 1993). Because of predictable insufficient FO availability for fish feeds, alternative sources must be assessed (Menoyo *et al.*, 2005). Studies on fish fed vegetable oil rich diets containing high-oleic sunflower oil (Torstensen *et al.*, 2000), rapeseed oil (Bell *et al.*, 2001), palm oil (Bell *et al.*, 2002), soybean oil (Grisdale-Helland *et al.*, 2002), and linseed oil (LO; Bell *et al.*, 2003) revealed that these dietary lipid sources have no detrimental effects on growth. Nevertheless, use of vegetable oil sources in fish nutrition still needs to be evaluated in terms of flesh quality and metabolic use (Menoyo *et al.*, 2005). A significant loss of n-3 highly unsaturated fatty acids (HUFA) in the flesh of Atlantic salmon was observed when FO was replaced with >66% of vegetable oils (Bell *et al.*, 2003). Contradictory reports on the effects of vegetable oils on lipogenic and lipolytic enzymes (Torstensen *et al.*, 2000; Regost *et al.*, 2003; Menoyo *et al.*, 2003, 2005) suggest that different vegetable oils may have different effects on fish metabolism and that different species respond in different ways. In fish, the pentose phosphate pathway, where the cytoplasmic reducing equivalents (NADPH) are provided, is affected by many factors (Ekinci and Senturk, 2013). To study the mechanistic basis of fish nutrition, it is essential to understand the biochemical mechanisms enabling efficient enzymatic catalysis under different nutrient conditions. In addition determination of factors affecting enzymes activities has gained considerable attention by nutritionists, pharmacologists, medicinal chemists and those who has interest to design therapeutic agents because alteration of enzymatic activity may result in both useful and hazardous outcomes in the metabolism (Balaydin *et al.*, 2012; Cavdar *et al.*, 2012; Ekinci and Beydemir, 2009; Cakmak *et al.*, 2011). Using lipid sources in the fish feed has been shown to be practical and economical way of nutrition (Mercan *et al.*, 2013). Therefore, the objective of this research was to evaluate the influence of dietary lipid sources on the activity of NADH related metabolic enzymes HK, G6PD, 6PGD in brown trout.

## Materials and Methods

### Chemicals

Chemicals used for enzyme activity measurements were purchased from Sigma-Aldrich (Munich, Germany). All other chemicals were of analytical grade and obtained from either Sigma-Aldrich or Merck (Munich, Germany).

### Experimental Diets

Four experimental casein-gelatin based diets with different sources of lipids were formulated to be isonitrogenous (65%) and isolipidic (15%). The first diet contained fish oil (menhaden oil; MO) and provided 20-22$^{o}$C long chain polyunsaturated fatty acids (LC-PUFAs) (12.3% 20:5n-3 and 14.1% 22:6n-3). The second diet, composed of hazelnut oil (HO), was characterized by high level (76.4%) of oleic acid (18:1; OA). The third diet, supplemented with a blend of linseed oil and soybean oil (1:1) (LO+SO), was dominated by linoleic acid (18:2n-6; LA) and linolenic acid (18:3n-3; LNA) (32.9 and 29.4%, respectively), but no LC-PUFA. The fourth diet, supplemented with soy-refined lecithin (LE), contained high level (55.6%) of LA. Menhaden oil, soybean oil, linseed oil and soy-refined lecithin were purchased from MP Biomedicals (Aurora, OH). Hazelnut oil was donated by Ordu Yağ Sanayii Ltd. Şti. (Ordu, Turkey). Total PUFA contents of MO, HO, LO+SO, and LE diets were 46.6, 14.3, 63.9, and 61.9%, respectively. Diets were prepared with a laboratory pelleting machine after 35-40 g of distilled water was added into 100 g mixture of ingredients, and freeze-dried. Diets were then crushed to the approximate size of 0.7-2 mm and kept at -20$^{o}$C until used. Detailed composition of experimental diets can be seen in Arslan *et al.* (2012).

### Fish Husbandry and Maintenance

Juvenile brown trout (~1 g initial weight) obtained from The Inland Aquaculture Research and Application Center, Faculty of Fisheries, Ataturk University were randomly distributed in three replicate 50 L glass aquaria with a total of 30 fish per tank. Each tank received one of the four above experimental diets. Well water was supplied at a rate of 1 L min$^{-1}$, and a semi-recirculation system with partial sedimentation and bio-filtration was used to maintain stable conditions. Each aquarium was equipped with continuous aeration and the water temperature was 13.0±0.2$^{o}$C (mean±SD). The fish were fed four times a day at (~5% body weight/day) for six weeks. At the end of the feeding trial, two fish from each aquarium were sampled and stored at -80$^{o}$C for enzyme analysis (Arslan *et al.*, 2012).

### Enzyme Assays

Whole body fish samples were washed three times with 50 mM Tris-HCl+0.1 M $Na_2SO_4$ (pH 8.0), and each was homogenized by liquid nitrogen, transferred to the same buffer, and centrifuged at 4°C, 15,000 g for 60 min. Supernatant was used in further studies.

### Hexokinase Activity

HK activity was determined by NADH formation following the absorbance at 340 nm. The assay medium contained 10 mM Tris-HCl, pH 7.4, 5 mM D-glucose, 10 mM $MgCl_2$, 1 mM ATP, 1 mM NAD+, and 1 unit/ml G6PD in a final volume of 1 ml.

**Glucose-6-Phosphate Dehydrogenase Activity**

G6PD activity was measured by Beutler's method (Beutler, 1971). One enzyme unit was defined as the enzyme amount reducing 1 mol $NADP^+$ per 1 minute. The activity buffer contained: 50 mM $NaH_2PO_4$, 5 mM EDTA, pH: 7.8.

**Glucose-6-Phosphate Dehydrogenase Activity**

6PGD enzymatic activity was measured by Beutler's method (Beutler, 1971). One enzyme unit was defined as the enzyme amount reducing 1 µmol $NADP^+$ per 1 minute. The activity buffer contained: 50mM $NaH_2PO_4$, 5mM EDTA, pH: 7.8.

**Protein Determination**

Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method (Bradford, 1976), with bovine serum albumin as a standard.

**Statistical Analysis**

Results were expressed as mean±SD (n=3). Each experimental unit (aquarium) was considered a replicate. For enzyme activity analysis, two fish from each aquarium were used. Data were subjected to one way analysis of variance (ANOVA) to test the effect of dietary lipid sources on metabolic enzyme activity and subsequent comparison of means by Duncan's multiple range test. Independent *t* test was used to compare activity of each metabolic enzyme for each experimental group with those for initial fish. Differences were considered statistically significant at $P<0.05$.

## Results

Dietary treatments had significant effect on growth performance of juvenile brown trout ($P<0.05$). Fish fed LO+SO diet had the highest growth, while HO fed fish exhibited the poorest growth performance. The fish fed MO and LE diets had similar growth rates to one another (Arslan *et al.*, 2012). Although some concern exists that feeding diets with vegetable oil may affect fish health (Bell *et al.*, 1991; Thompson *et al.*, 1996), survival was not affected by the dietary treatments averaging 86.0±11.5% in the current study. Dietary treatments had significant effect on the activity of metabolic enzymes. Changes in the enzyme levels in brown trout fed diets with different lipid sources are presented in Table 1. In all cases, the activity of all metabolic enzymes significantly increased ($P<0.05$) in comparison to their activities in initial fish. The highest level of activity for each metabolic enzyme was observed in fish fed LO+SO diet and the lowest activity was detected in those fed HO diet. The level of each metabolic enzyme activity increased with the increasing level of PUFA content of the fish. However, activities of HK, G6PD and 6PGD were certainly in the same order as PUFA content (LO+SO > LE > MO >HO), but the LO+SO group showed exceptionally higher activities of these enzymes. This shows that some factors other than PUFA content may also affect the enzymatic activities.

## Discussion

Compound diets are formulated to meet the specific nutritional requirements of fish. Lipids play an important role as a source of dietary energy for fish. They are well utilized by fish and necessary for normal growth and development. They also provide the essential fatty acids for normal functions of organisms. In brown trout, diets with different lipid sources have been rarely tested. This study attempted to establish a relationship between differing lipid nutrition and activity of metabolic enzymes HK, G6PD and 6PGD in brown trout. Although few studies focused on lipid nutrition in brown trout (Arzel *et al.*, 1994; Bayir *et al.*, 2011) there is no information about enzymatic consequences.

In this experiment, we have demonstrated that different dietary lipid supplementation altered the activity of the metabolic enzymes. Transport and the first step of glucose utilization within the cells are catalyzed by HK, which participates in blood glucose homeostasis. G6PD and 6PGD produces cellular NADPH, which is required for the biosynthesis of fatty acids and cholesterol. As seen from Figure 1, these three enzymes are directly or indirectly involved in catalyzing NADPH production. Thus, they could be considered as NADPH related enzymes.

**Table 1.** Changes in some metabolic enzymes in brown trout fed diets with different lipid sources

| Metabolic enzymes | | Experimental diets | | | |
|---|---|---|---|---|---|
| | Initial | MO | HO | LO+SO | LE |
| HK (U/mg pro) | 12.26±0.11* | 30.61±0.71$^c$ | 26.91±0.78$^d$ | 41.49±1.44$^a$ | 33.07±0.52$^b$ |
| G6PD (U/mg pro) | 10.69±0.56* | 26.38±0.45$^b$ | 18.89±0.69$^d$ | 34.94±1.01$^a$ | 24.11±0.77$^c$ |
| 6PGD (U/mg pro) | 7.3±0.19* | 20.71±0.72$^c$ | 16.43±0.58$^d$ | 32.42±0.91$^a$ | 24.84±0.27$^b$ |

Values are mean ± S.D. Means with different superscript letters in a row are significantly different ($P<0.05$). Asterisk indicates initial enzyme activity is significantly lower than those after dietary treatments ($P<0.05$).

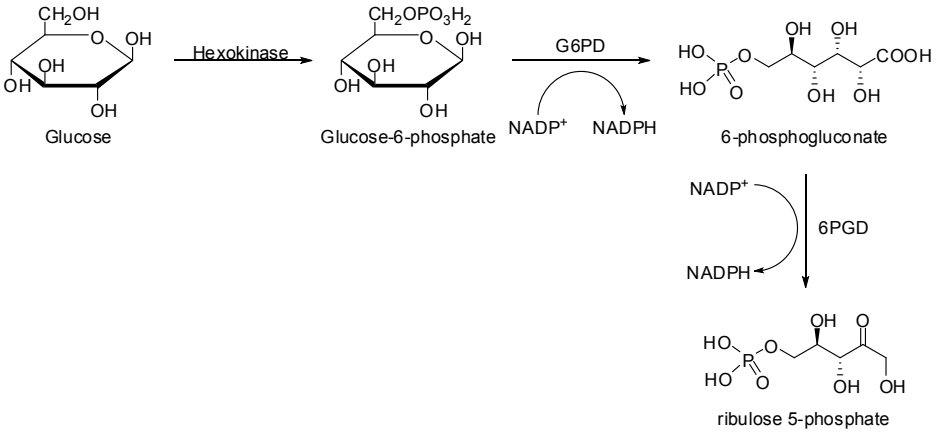


**Figure 1.** The reactions catalyzed by HK, G6PD and 6PGD enzymes.

HK activities upon different diets are shown in Table 1. Hexokinases play a vital role in the cellular uptake and utilization of glucose. As such, they are of fundamental importance to all cells. By catalysing the ATP-dependent phosphorylation of glucose to yield glucose-6-phosphate, hexokinases control the first committed step of glucose metabolism, thereby sustaining the concentration gradient that permits facilitated glucose entry into cells and initiating all major pathways of glucose utilization. Hexokinases are thus ideally positioned to influence not only the magnitude, but also the direction, of glucose flux within cells (Robey and Hay, 2006). The activity of HK significantly increased ($P<0.05$) in fish fed all experimental diets as compared with that of the initial fish, and reached the highest value in LO+SO fed fish and the lowest in HO diet, in accordance with the level of PUFA in fish (Table 1).

Changes in G6PD activity in fish fed diets with different lipid sources are also presented in Table 1. Glucose 6-phosphate dehydrogenase is the first enzyme in the pentose phosphate pathway. The principal physiological function of pentose phosphate pathway is the production of NADPH and ribose 5-phosphate, which are essential for reductive biosynthesis and nucleic acid synthesis (Kuo *et al.*, 2000; Aksakal *et al.*, 2011; Ciftci *et al.*, 2008). In addition, NADPH also participates in cell-membrane protection and cell detoxification from xenobiotics through the gluthatione reductase-peroxidase system and the mixed-function oxidases (Barroso *et al.*, 1999; Díez-Fernández *et al.*, 1996). G6PD activity exhibited a similar manner to that of HK activity. In other teleosts, it has been reported that the activity of G6PD was depressed by elevated levels of dietary lipids (Årnesen *et al.*, 1993; Shimeno *et al.*, 1993). Wang *et al.* (2005) reported that, with regard to the two key regulatory enzymes in the lipogenic pathway, the activity of G6PD was over 6 times higher than that of malic enzyme (ME) in fish fed with 15% and 25% lipid diets, and suggested that the cytoplasmic reducing equivalents NADPH are mainly provided by the pentose phosphate pathway. These results were also in accordance with data from studies with coho salmon (Lin *et al.*, 1977a,b), seabass (Iniesta *et al.*, 1985) and rainbow trout (Walzem *et al.*, 1991; Hung and Storebakken, 1994).

6-Phosphogluconate dehydrogenase is the third enzyme of the pentose phosphate metabolic pathway, catalyzing the conversion of 6-phosphogluconate to D-riboluse-5-phosphate in the presence of $NADP^+$ (Figure 1). The reaction catalyzed by 6PGD, yields NADPH, which protects the cell against oxidant agents by producing reduced GSH (Bianchi *et al.*, 2001; Lehninger *et al.*, 2000) and a step in the pentose phosphate pathway of glucose metabolism. For this reason, 6PGD contributes to antioxidant protection (Kozar *et al.*, 2000; Srivastava and Beutler, 1989; Ceyhun *et al.*, 2010). In the enzyme assays, 6PGD activity significantly increased ($P<0.05$) in fish fed all experimental diets in comparison to the activity in initial fish. The level of 6PGD activity also corresponded with PUFA level in fish.

Overall results indicate that activities of all metabolic enzymes HK, G6PD and 6PGD are significantly elevated upon lipid diets. Each enzyme reached the maximum activity in fish fed with LO+SO. Menoyo *et al* (2008) tested the effect of total or partial substitution of dietary fish oil (FO) by linseed oil (LO) in Atlantic salmon feeding on performance, liver and muscle fatty acid composition, selected lipogenic and lipolytic enzyme activities, and flesh oxidative stability. Activity of liver G6PD increased with increasing levels of LO. Thus, our data is in agreement with that study.

Activity of the enzymes increased in the order of LO+SO>LE>MO>HO. It is plausible to note that total PUFA content of the lipids is in the same order (Arslan *et al.*, 2012) indicating that activity of these enzymes are elevated with increasing PUFA concentrations. It has been well documented that the increasing elongation activity in fish is triggered by LO (Bell *et al.*, 1993; Tocher *et al.*, 2000). Menoyo *et al.* (2008) observed the accumulation of eicosatetraenoic acid (20:4n-3), which is the main product from the Δ6 desaturation of linolenic acid (18:3n-3) in the liver of Atlantic salmon, with increasing inputs of LO. Considering these data and

our results, the increase in the activity of HK, G6PD and 6PGD may be related to the greater concentration of intracellular NADPH needed to accomplish the elongation process.

Inside the cells, each metabolic pathway is continuously regulated in order to maintain homeostasis and, in general, few key enzymes control the metabolic flux (Brooks and Storey, 1995; Carvalho *et al.*, 2008). In the anaerobic metabolism of glucose such key enzymes as hexokinase is at the beginning of the glycolytic sequence and the glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are key enzymes of the pentose phosphate shunt. The fact that the increase in the activities of these enzymes was consistent with the increased PUFA contents evidenced that the changes in unsaturated fatty acid concentrations result in biochemical changes inside the cells, which in turn may modulate the activity of these enzymes.

## Conclusions

One of the most efficient strategies to sustain the future of intensive aquaculture is to develop the quality feed in order to meet the increasing demand of that species so that important developments would be made on the production of fish feeds. Following the expansion in the use of the extrusion technique in feed production, the extruded feeds which contain high level of fat are started to use widely in feeding of cultured fish species (Yildiz *et al.*, 2006; Autin, 1997; Lanari *et al.*, 1999). In the present study, dietary lipid sources significantly affected the metabolic enzymes. All diets caused significant increase in the activity of the enzymes. Activity of hexokinase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were significantly higher in whole body of fish fed LO+SO diet than those fed all the other experimental diets. HO diet fed fish had the lowest activity in all metabolic enzymes investigated. Nonetheless, all diets significantly increased the activity of the enzymes and might enhance the pentose phosphate pathway and NADPH production, which in turn gives rise to reductive biosynthesis, nucleic acid synthesis, cell-membrane protection and cell detoxification.

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