ÇUKUROVA UNIVERSITY

INSTITUTE OF NATURAL AND APPLIED SCIENCES

MSc. THESIS

Growth Performance and Tissue Fatty Acid Change in Rainbow Trout (*Oncorhynchus mykiss*) Feed with Different Energy Levels

Doaa Bahjat Alwan KRIEI

Department of Biotechnology

January, 2024

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We certify that the thesis titled above was reviewed and approved for the award of the degree of the Master of Science by the board of jury on 02/01/2024.

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DECLARATION

I hereby declare that all information in the thesis has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all the material and results that are not original to this work.

Doaa Bahjat Alwan KRIEI

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MASTER OF SCIENCE THESIS

Growth Performance and Tissue Fatty Acid Change in Rainbow Trout (*Oncorhynchus mykiss*) Feed with Different Energy Levels

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Advisor: Prof. Dr. O. Tufan EROLDOĞAN

Department of Biotechnology

ABSTRACT

A feeding trial was conducted in rainbow trout juveniles to assess the growth performance and feed utilization after fed the two diets with different energy levels (medium energy-ME, (18.4 kJ/kg) and low energy-LE (17.7 kJ/kg). Twenty fish (10 females and 10 males) males and females are distinguished through the head and anal fin, were randomly distributed into each cylindrical tank (300-L rearing volume) with four replicates. The fish were fed twice daily with each experimental diet until apparent satiation for 60 days. At the end of the feeding trial, the mean final weight in the LE group ranged from 385.7±21.4 to 406.0±23.6 g and the mean final weight in the ME group ranged between 424.1±22.0 g and 434.2±37.3 g. Overall, the fish fed with LE achieved the highest percentage of body weight gain of 290.34±1.99% among both treatments, with a statistically significant difference. Irrespective of gender, there was no significant difference in feed intake and FCR. No significant difference was also found in VSI which ranged from 3.83 to 4.08%. However, female fish fed with LE diet had the highest VFI than male fish fed with ME counterparts. In terms of specific fatty acids such as EPA and DHA, no significant differences were detected between experimental diets and there were no significant interactions between two factors (energy levels and gender) in EPA and DHA profile of the fillets of the experimental fish. A significant difference between genders was found in triglyceride, total protein, ALT and AST whereas the experimental diets had a significant impact on cholesterol levels in both genders. Liver histology scores in fish fed ME and LE showed no specific pathologies, normal liver structure, and no fibrous tissue increase. In the study, the expression of IL-1β and TNF-α was reduced in the liver of fish fed on ME diet, while the highest expression was found in fish fed on LE diet. Also, the expression of glucokinase increased in the liver of fish fed the LE diet. In contrast, the highest expression of IL-18 was found in the liver of fish fed the ME diet. While the lowest expression of IL-18 was in the liver of fish fed the LE diet.

Key Words: Dietary energy, Growth performance, Feed intake, Liver histology, Gene expression, Immunology

ÇUKUROVA ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

YÜKSEK LİSANS TEZİ

Farklı Enerji İçeriğine Sahip Yemlerle Beslenen Gökkuşağı Alabalıklarında (*Oncorhynchus mykiss*) Büyüme Performansı ve Doku Yağ Asidi Değişimi

Doaa Bahjat Alwan KRIEI

Danışman: Prof. Dr. O. Tufan EROLDOĞAN

Yetiştiricilik Anabilim Dali

ÖZ

Gökkuşağı alabalığı yavrularının iki farklı enerji içeriğine sahip (orta enerji-ME, (18.4 kJ/kg) ve düşük enerji-LE (17.7 kJ/kg) yemlerle beslenmeleri sonucu büyüme performansı ve yemden yararlanma açısından bir besleme denemesi gerçekleştirdi. Yirmi balık (10 dişi ve 10 erkek) erkek ve dişiler baş ve anal yüzgeç sayesinde ayırt edilir, her bir silindirik tanka (300 L yetiştirme hacmi) dört tekerrülü şekilde rastgele dağıtıldı. Balıklar deneme süresi olan 60 gün boyunca günde iki defa doyana kadar beslendiler. Deneme sonunda LE grubunda ortalama son ağırlık 385,7±21,4 ila 406,0±23,6 g arasında ve ME grubunda ise ortalama son ağırlık 424,1±22,0 g ila 434,2±37,3 g arasında değişmiştir. Sonuçta, LE yemleri ile beslenen bireyler diğer gruba göre istatistiki olarak en yüksek canlı ağırlık artışı yüzdesini (%290.34±1.99) sağlamıştır. Cinsiyete bakılmaksızın, yemlerdeki enerji seviyesinin yem alımı ve yemden yararlanma oranında (FCR) önemli bir etkisinin olmadığı görülmüştür. Deneme gruplarının viserasomatik indeks (VSI) değerleri %3.83 ile %4.08 arasında değişim gösterdi ve gruplar arasında anlamlı bir fark bulunamadı. Ancak LE yemleriyle beslenen dişi balıklar, ME yemleriyle beslenen erkek balıklara göre en yüksek visceral yağ indeksine (VFI) sahipti. Özellikle EPA ve DHA gibi spesifik yağ asitleri açısından her iki deneysel yemde bir farklılık tespit edilmedi ve deneme balıklarının fileto EPA ve DHA profilinde iki faktör (enerji düzeyi ve cinsiyet) arasında anlamlı bir etkileşim yoktu. Cinsiyetin trigliserit, toplam protein, serum alanin aminotransferaz ve aspartat aminotransferaz üzerinde anlamlı bir etksi bulunurken, yemlerin kolesterol düzeyleri üzerinde her iki cinsiyet üzerinde de bir etkisi olduğu görülmüştür. ME ve LE ile beslenen balıklardaki karaciğer histolojisi skorları, spesifik bir patoloji, normal karaciğer yapısı ve retiküler çatıda herhangi bir fibröz doku artışı görülmemiştir. Çalışmada, ME balıklarının karaciğer dokusunda IL-1β ve TNF-α ekspresyonu azalmış, en yüksek ekspresyonun ise düşük enerjili yemle beslenen balıklarda gerçeklemiştir. LE ile beslenen dişi balıklar yüksek glukokinaz ekspresyonu gösterdi. Bunun aksine, ME yemleri ile beslenen balıkların karaciğerlerinde yüksek IL-18 ekspresyonu bulunmuştur. En düşük IL-18 gen ekspresyonu ise LE yemleri ile beslenen bireylerin karaciğerlerinde bulunmuştur.

Anahtar Kelimeler: Besinsel enerji, Büyüme performansı, Yem alımı, karaciğer histoloji, Gen ifadesi, İmmünoloji

EXTENDED ABSTRACT

The use of different dietary energy levels can be required for the enhancement of fish production and health vital in aquaculture, especially under environmental stresses. In most cases, long-term administrations of high lipid or high energy diets in carnivorous species may potentially affect immunological function, regulating metabolism and physiology in fish. Therefore, it is, in fact, very crucial to understand the long-term feeding effects of different dietary energy in species cultured. Thus, the aim of the present study was to investigate the effects of long-term feeding of two dietary energy levels (medium energy-ME, (18.4 kJ/kg), as control and low energy-LE diets (17.7 kJ/kg)) on growth, blood chemistry and pro-inflammatory response of rainbow trout between male and female. In the present study, rainbow trout individuals were obtained from a local fish farm. The experimental fish were acclimatized to the experimental conditions under a normal photoperiod cycle and fed a commercial rainbow trout diet. Twenty fish were randomly distributed into cylindrical tanks connected to a freshwater RAS system where the daily water exchanged was %400 of the total tank volume. Throughout the experiment, fish were given both ME and LE energy diets twice daily until satiation for 60 days. In this phase of the experiment, the temperature of the RAS system was kept at 14.0±1ºC. At the end of the trial, the performance parameters of the fish were taken and recorded. The growth performances of the fish were not affected by different dietary treatments. The mean final weight was significantly higher in fish fed the ME diet compared to those fed the LE diet. No significant differences were observed in feed intake and feed conversion ratio between gender and diets. Overall, the growth parameters of the fish were not significantly different among the groups. Thus, the results of the present study showed that the energy difference would not have severe effects on energy metabolism, at least for the growth performance parameters, in the present study. Thus, the diet composition of these groups of fishes is crucial and the energy content of the formulation is an important factor that could change the metabolism of the species. The experimental diets and fillet fatty acid profiles were identical, with only slight differences in MUFA. However, some specific fatty acid content was significantly affected by the diets. Female trout had higher LC-PUFA content than males. No significant differences were found in specific fatty acids like EPA and DHA. MUFA content increased in male fish fed ME and LE diets, while LC-PUFA, n-6 PUFA, and n-6 LC-PUFA levels increased in female fish. The study found significant differences in blood chemistry parameters between genders and experimental diets. Glucose, creatine, and alkaline phosphatase were significantly different, while triglyceride, total protein, ALT and AST showed gender-specific effects. The liver histology scores revealed no specific pathologies in fish fed ME and LE, with no significant changes observed. The overall structure and morphology of liver tissues were normal, and no fibrous tissue developed. The study found that IL-1β expression significantly decreased in the liver of females fed the ME diet, while its highest expression was found in males fed with ME. The

lowest IL-1β expression was found in male fish fed with LE, while the highest expression was achieved in female fish (3-fold) fed with the same diet. In male fish fed with LE, their immune system is less active, leading to a decrease in IL-1β production. Glucokinase expression was significantly higher and up-regulated in female fish fed with LE, possibly due to an attempt to enhance glycogen synthesis for energy storage. Also, fish fed with LE showed an increasing trend in TNF-α expression compared to those fed with ME diets. On the contrary, the highest expression of IL-18 was found in the liver of fish fed the ME diet. While the lowest expression of IL-18 was in the liver of fish fed the LE diet. The study suggests that medium-energy diets may stimulate IL-18 production.

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LIST OF SYMPOLS/ ABBREVIATIONS

ALB : Albumin
ALP : Alkaline phosphatase
ALT : The alanine aminotransferase
ANOVA : Analysis of Variance
AST : Aspartate transaminase
BM : Blood Meal
cDNA : Copy DNA
CHOL : Cholesterol
CoA : Coenzyme A
CREAT : Creatin
DHA : Docosahexaenoic acid
DNA : Deoxyribonucleid acid
DO : Dissolved Oxygen
E : Energy
EPA : Eicosapentaenoic acid
FAO : Food and Agriculture Organization of the United Nations
FAs : Fatty Acids
FCR : Feed Conversion Ratio
FI : Total Feed İntake
FM : Fish Meal
GDFA : Glass-Distilled Formic Acid
GLC : Circulating Glocose
GLU : Glucokinase
HE : High Energy
HF : High Feed
HFD : High Feed Dietary
HP : High Protein
HIS : Hepato-somatic index
HUFA : Highly unsaturated fatty acids
IL-18 : Interleukine 18
IL-1B : Interleukine 1B
LC-PUFA : Long-chain polyunsaturated fatty acid
LE : Low Energy
ME : Medium-Energy
MiR : Micro RNA

MUFA	: Monounsaturated Fatty Acids
n-3 LCPUFA	: Long-chain omega-3 polyunsaturated fatty
NBF	: Neutral Buffered Formalin
NRC	: National Research Council
P	: Protein
PCR	: Polymerase Chain Reaction
PUFA	: Poly-unsaturated fatty acids
RNA	: Ribonucleic Acid
SFA	: Saturated Fatty Acid
SGR	: Specific Growth Rate
TAGs	: Trigliserides
TG	: Triglyceride
TNFα	: Tumor necrosis factor
TP	: Total Protein
VFI	: Visceral fat index
VSI	: Viscerosomatic index
WG	: Weight Gain

1. INTRODUCTION

According to Food and Agriculture Organization of the United Nations, in the next few years, fisheries production will level off (Mathiesen et al., 2012). Aquaculture is the fastest-growing food industry, and it is responsible for half of total fish consumption. Presently, 181 countries culture more than 600 fish species (Mathiesen et al., 2012). Additionally, more than 70% of aquaculture production is based on feed input, which typically accounts for 50–60% of the total operating costs. To promote sustainable aquaculture practices, reduction in feeding costs is an ongoing priority. Finding a suitable replacement for expensive and finite ingredients such as fish meal (FM) is one way to minimize costs (Webster et al., 1997). FM and fish oil are the main supply of secure protein and fat, especially in carnivorous fish feeds, which normally account for 30–50% of the diet (Tidwell et al., 2005). According to an FAO report in 2012, FM and fish oil consumed in aquaculture were, respectively, 68% and 89% of the global production (Mathiesen, 2012). Hence, many studies worldwide are focused on FM replacement with alternative protein sources. FM is an important ingredient in rainbow trout diets, as a carnivorous fish, this species requires a nutrient-dense diet and it is known to utilize alternative animal protein sources such as MBM (meat and bone meal), poultry by-product meal and blood meal. These alternative animal protein sources are considered promising feed ingredients because of reduced carbohydrate content in comparison with plant proteins. Additionally, MBM has a high amount of protein, a relatively lower price and a good amino acid profile and is also considered a promising alternative to replace FM in the salmon diet (Meeker, 2006). Nonetheless, MBM has some constraints, such as lower digestibility in comparison with FM, lysine and methionine deficiency, and also presents elevated ash content (Bureau et al., 1999). Accordingly, in rainbow trout (*Oncorhynchus mykiss*), red drum *Sciaenops ocellatus* and sea bream, *Sparus aurata*, diets up to 300 g FM per kilo feed are commonly replaced with MBM (Robaina et al., 1997; Bureau et al., 2000; Kureshy et al., 2000). MB has been reported have a positive protein digestibility for carp (54%), and rainbow trout (75%). In this study (Robaina et al., 1997; Bureau et al., 2000; Kureshy et al., 2000), it was demonstrated that rainbow trout exhibited greater protein digestibility than carp for being a carnivorous species and for presenting more protease in its digestive system in comparison with carp, an carnivorous species. Thus, it was suggested that rainbow trout could better digest MBM (Watanabe et al., 1993). It is known that growth and feed efficiency in fish are related to the physiological and biochemical capacity of digestion and transmission of nutrients, but many factors have a role in the digestion, absorption and transfer of nutrients in the intestine. The ability of fish to digest nutrients depends on the adequate amount of enzymes present in the digestive system (Suárez et al., 1995).

Rainbow trout is one of the most important aquaculture species worldwide, in particularly in Europe, North America, Japan and Australia (FAO, 2014). According to the Iran Fishery Organization announcement in 2012, fish production was 131 thousand tons with rainbow trout being the highest consumed species nationally. As this species is carnivorous, it exhibits higher protein requirements and more aggressive and/or complete FM replacement rates with alternative protein sources such as MBM are known to impair fish performance and physiology. The searches for alternatives without these negative effects are valuable to the industry.

With respect to Turkish rainbow trout production, the production has been achieved in both marine and inland waters of Turkey. According to the Glass-Distilled Formic Acid (2021) statistics, the total production of 44.533 tonnes comprised 56.35% of total aquaculture output in 2000, whereas production amount increased to 144.182 tonnes in 2020 and comprised 34.21% of total aquaculture output (Diken and Kaknaroglu 2022). Dietary energy supply should meet the energy requirements that encompass the amount of energy expended for the maintenance of vital. As a result, the main macronutrient differences between the three HE diets-high protein HP, high fat HF, and high digestible carbohydrate, and the LE diet were in terms of nutrients. The HF diet was the only one with higher analyzed gross energy contents; the other diets were comparable because the cellulose in the LE diet was replaced by an equal quantity of extra macronutrient. The primary source of expensive fish production is aquafeed, namely the dietary protein component of feed used for carnivorous species. Fishmeal was the primary source of protein for aquafeed during the feeding years. It is thought to be the most suitable for aquatic creatures. Owing to their scarcity and rising cost, proteins generated from plants are thought to be a good substitute. However, the majority of them are unbalanced or lacking in essential amino acids.

In the context of using high lipid diets (high energy diet-HE, lipid content above 22%), the cold water species generally need HE diets during winter conditions, particularly growth-out periods between 50 g to marketable size. However, if the rainbow trout is produced and processed for fume, then the energy content of the diet must be around 19-21% termed low energy diet (LE) (Personal communication with Pinar Demir Söker, Skretting – Turkey). Thus, diets containing HE and LE energy provide essential fatty acids for growth and muscle quality. However, there is no relevant study to assess the long-term feeding of HE energy diets on lipid metabolism and stress status of the rainbow trout. Above mentioned studies indicated that rainbow trout specifically select diets containing different energy concentrations in different culture conditions. Additionally, studies suggested that long-term HE caused leptin resistance, selectively lowering lipid catabolism (Liu et al., 2018). Moreover, HE diets also alter lipid deposition in the liver (Tang et al., 2019). However, a comprehensive model is needed to explain long-term HE diet feeding compared with LE diets in rainbow trout growth-out period is of interest to the industry.

2. LITERATURE REVIEW

Aquaculture is currently one of the fastest-developing food-producing industries globally and it has multiplied nearly 12-fold over the past three decades and now provides consumers with a regular supply of top-notch seafood (Lazzarotto et al., 2018). According to FAO (2022) global aquaculture production in 2020 reached a record 122.6 million tonnes and 54.4 million tonnes were farmed in inland waters resources and 68.1 million tonnes came from marine and coastal aquaculture. Aquaculture production is estimated to increase further by 15% by 2030 (FAO, 2022). However, this growth is largely depending on protein feedstuff to feed aquaculture (Suarez et al., 2013). Globally, commercial feeds, particularly extruded aqua-feed, will play a pivotal role in the increase of aquaculture. Aqua-feed is the main variable expense (more than 70%) of the aquaculture production thus it must be taken into account all culture types (FAO, 2016). Even after the COVID-19 pandemic, the share of feeding in the production reached almost 75% due to difficulty reaching terrestrial-based protein sources and general economic situation all over the world. Thus, there is a need for a holistic approach and to consider nutritional quality, cost of the ingredients along diet quality for sustainable aquaculture.

In this regard, the management of feeding in fish farming is currently more important than ever before due to the fact that its economic effect as well as its effects on fish health and welfare (Luis et al., 2017; Tacon et al., 2022). The components of properly-balanced diets such as protein: energy ratio (P:E) and ingredient inclusion have become a more important issue when a profitable production is considered. On the other hand, well-balanced diets containing all vital nutrients in adequate concentrations can promote recovery from disease or assist fish in conquering the outcomes of environmental stress (Lanser et al., 2017). During the early period of development of the aquaculture industry, feeds for herbivorous species such as tilapia, and carp were based on largely plant ingredients whereas those of carnivorous species such as cold-water species Atlantic salmon (*Salmo salar*) and rainbow trout based on marine ingredients including fish meal and fish oil. However, due to the overexploitation of the ocean and sea sources and public concerns for the conservation of global fisheries stocks, the traditional aqua-feed formulation has drastically and heavily changed to plant-based and/or by-product-based, which could be termed “Aqua-feed V2.0” (Cottrell et al., 2021; Colombo and Turchini, 2021). However, with regard to recent economic and ecological trends, Aqua-feed V3.0 will use raw materials/ingredients produced through the circular economy (Colombo and Turchini, 2021). Even though the Aqua-feed V3.0 was launched in the feed industry, it will take time to reach production of this kind of aqua-feed in the future sustainable development. Yet, current aqua-feed produced mainly for carnivorous species contains high inclusion levels of plant ingredients. For instance, a high inclusion rate (70-75%)of plant-based ingredients in Norwegian salmon feeds is the case (Ytrestøyl et al., 2015; Aas et al., 2019). Long-term feeding with feeds containing plant-based ingredients and high lipid may have negative effects on performance parameters and body composition of cold-water carnivorous species ie. Atlantic salmon and rainbow trout. Thus, it is very important and pertinent to understand the physiological effects related to protein: energy ratio and long-term effects on energy dynamics in cultured fish.

2.1. Energy Requirements of Fish and Dietary Energy Utilization

Macronutrients including protein, lipids and carbohydrates are required by fish to maintain all life processes including swimming activity, growth, reproduction and other metabolic activities. The nutrients consumed are catabolized to harness chemical free-energy required for use in anabolism and other life-sustaining mechanisms in culture animals. However, animals including fish cannot simply metabolize energy per se instead they metabolize specific nutrients that have different roles and metabolic fates in the body (Van Milgen, 2002 from NRC 2011). Energy requirements of fish are dependent on a range of things such as fish size, species, feeding methods and environmental conditions as well as diet composition. Fish have a low maintenance energy requirement in comparison with other terrestrial animals for the following reasons: (i) fish do not need to maintain the body temperature (poikilothermic), (ii) fish spend less energy to maintain body position inside the water column (Goodsell et al., 1996; Trotter et al., 2001), and (iii) fish have a lower energetic expenditure for the elimination of waste of protein catabolism, ammonia, compared with other terrestrial animals (Brett and Groves, 1979).

As macronutrients such as lipids and carbohydrates, are the main energy sources of animal feed. Among these macronutrients, protein is one of the pivotal resources in three purposes for fish: (i) provide energy (ii) provide essential amino acids and (iii) is used for useful proteins- enzymes, hormones and structural proteins. Thus, protein is the most expensive nutrient that must be provided by the proteinous ingredients in formulated diets. Dietary protein requirements in fish are typically expressed in dietary percentage or g protein required per kg body weight per day. The requirement for dietary protein of fish is actually a requirement for the amino acids inside the dietary proteins.It depends on and changes across the life stages of the fish. Younger fish require higher percentages of dietary protein than older fish, as younger fish grow faster and need more nutrients to support their growth. Protein mobilization differs from the mechanisms used for carbohydrates and lipids; proteins are actively deposited and stored throughout the body in muscles, organs, and other tissues (Olive Teles et al., 2021). This protein accretion is fundamental for fish growth and development. An excess of dietary protein is used as an energy source in intermediary metabolism or is converted to glucose or lipids as energy deposits, and the end product ammonia is largely excreted via the gills (Dabrowski and Guderley, 2002). Nutritional protein supply is likewise regarded to affect lipid deposition and fatty acid bioconversion capacity and regulate serum and liver lipid stages in higher vertebrates as well as in fish (Terasawa et al., 1994; Potter, 1995; Aoyama et al., 2000).

The partitioning of nutrients such as amino acids and fatty acids between nutrition pools and into and out of the bloodstream system are presented as terms within differential equations based on rules of bioenergetics (Figure 1). Further, fish require a well-balanced mixture of both essential and non-essential amino acids that constitute protein (Wilson 2002; Olive Teles et al., 2021). There should be also a well-balanced protein-to-energy ratio in order to reach better growth performance and dietary protein-sparing effect in fish. A good knowledge of protein: energy ratio, using non-protein digestible energy sources, carbohydrates and lipids, is necessary to reduce feed costs and enhance growth (Bautista 1986, Molina-Poveda, 2016).

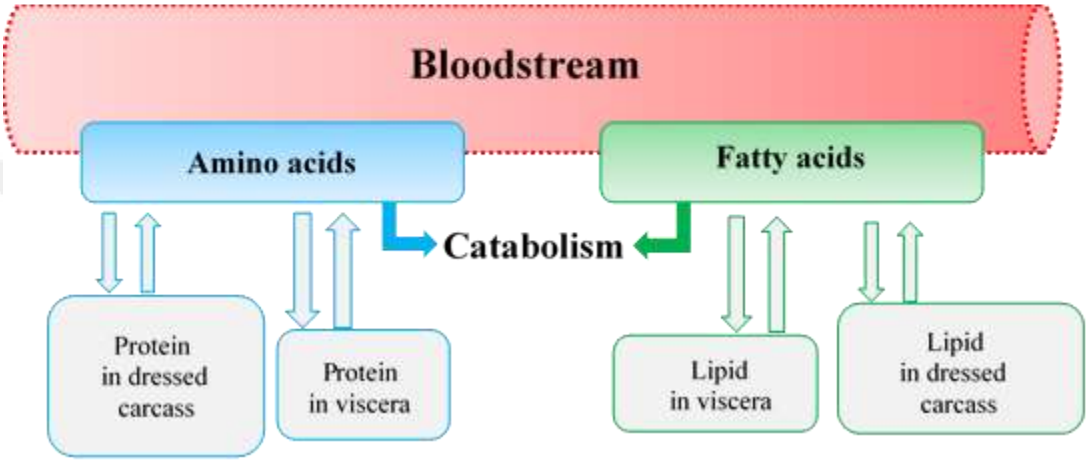

Figure 2.1. Flow of nutrients between each pool (amino acid, fatty acids, protein and lipid in the viscera and dressed carcass) is determined using differential and stoichiometric equations described in Dumas et al. (2010).

When considering protein to energy ratio (P:E), the fish can regulate its intake according to dietary energy concentration. For instance, if the diet has low P:E, animals may stop feeding before meeting their protein requirements, resulting in key performance indicators such as growth and feed conversion ratio. In the opposite situation (high P:E), fish eat an excess of dietary protein, which will end up with protein catabolism for energetic use (Bureau et al., 2002; Wilson 2002; Olive Teles et al., 2021; Basto-Silva et al., 2021).

As protein is the most expensive nutrient to be met via ingredients in manufactured diets, lipids and carbohydrates are used as non-protein energy sources to direct the dietary protein toward the deposition of body protein rather than energetic use (Hemre et al., 2002; Li et al., 2019). Carbohydrates are reasonably priced in the market for aqua-feed. Despite the fact that carbohydrates are not essential for fish, they are used in aquaculture diets to spare dietary protein and benefit their pellet-binding properties (NRC, 2011). However, excessive dietary carbohydrates can potentially elevate blood glucose levels, as fish lack the robust regulatory mechanisms seen in terrestrial animals (Hemre et al., 2002; NRC, 2011). This hyperglycemia can negatively impact fish health and performance, requiring careful dietary balancing to optimize protein sparing without compromising metabolic regulation.

2.1.1. Lipid Requirements and Lipid Deposition in Fish

Compared with dietary carbohydrates, lipids are also preferably used as a primary energy source in the diets of carnivorous fish species. Even though lipids in the diet, particularly fish oil, have a pivotal role in growth, and health and provide energy to the fish, excessive dietary crude lipid levels may cause some adverse effects, especially undiserable levels of lipid depositions in various parts of the body. However, fish have adaptive mechanisms to modify lipid metabolism in response to crude lipids in diets such as low-fat and high-fat diets. The lipid dynamics in fish (absorption, transportation, deposition and mobilization) had been previously reviewed via Sheridan (1988; 1994). The most important sites of deposition are perivisceral lipids, muscle, and liver.

Dietary lipids are digested and absorbed within the small intestine and fatty acids are eventually transported to muscle and distinctive tissues including liver and intraperitoneal fat. Excess fat is mainly deposited in organs in the form of triglycerides (TAG). Phospholipase via β-oxidation pathways, stored lipids and TAGs are broken down into fatty acids and glycerol under the catalysis of utilization as fuel (Leaver et al., 2008; NRC, 2011). The sequential cleaving of two-carbon units breaks down lipids into acetyl-CoA, which is then used for energy production. As mentioned above, fish store lipids in various several depot organs, such as mesenteric membranes, the liver and muscle tissue. Lipids provide essential fatty acids, energy, eicosanoids and components of cell membranes as phospholipids. They also assist in the uptake of lipid soluble vitamins (Storebakken, 2002).

Overall, lipids are stored in cultured animal tissue mainly in muscles and livers along with the intraperitoneal part of the body, which changes depending on the species. In the case of muscle deposition, lipid is an important energy supply for unique physiological situations along with prolonged muscular exercising (Jeukendrup, 2010). Muscle lipid additionally contributes to improving the sensory houses, which includes dietary texture and juiciness (Johansson et al., 2000; Katikou et al., 2001; Robb et al., 2002; Timber et al., 2004). The distribution and content material of muscle lipid varies widely and depends on fish species (Katikou et al., 2001). However, the high lipid content of the muscle and/or intraperitoneal fats can create negative health problemss in fish species when they are fed with high lipid diets for a long-term. For instance, the replacement of fish oil by alternative land animal lipid sources has been reported to show some side effects such as the modification of the fatty acid composition, higher susceptibility to lipid peroxidation, and/or altered secretion of lipoproteins, and increased body fat deposition (Kousoulaki et al., 2015; Ruiz et al., 2022).

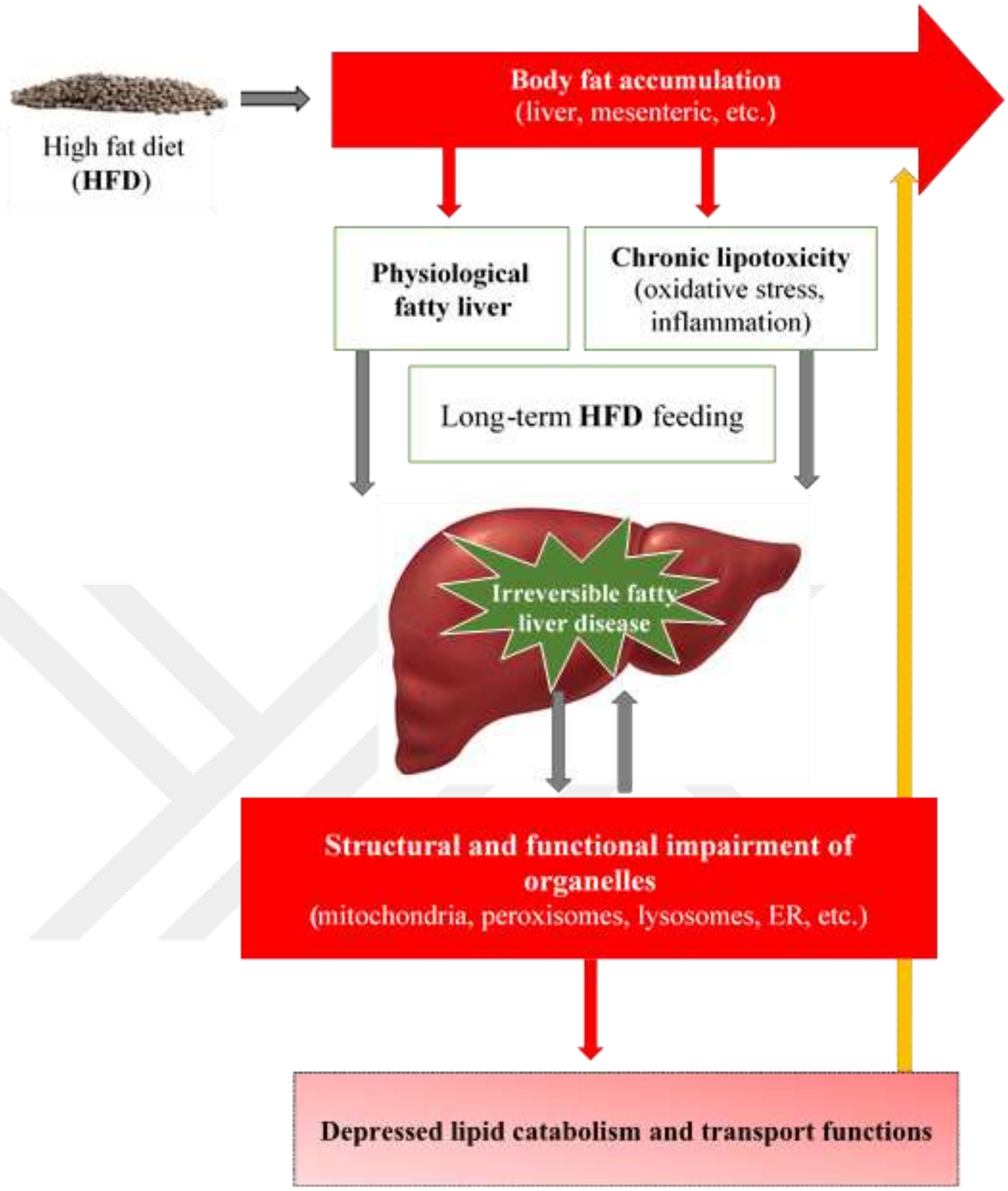

Figure 2.2. Development of fatty acid liver in fish with long-term feeding of a high-fat diet (HFD).

The liver is an important organ for lipid deposition in fish, despite the fact that the contribution of the liver to total body lipid deposition varies greatly among species (Holdway and Beamish, 1984; Kikuchi et al., 2009; Zeng et al., 2010). In general, the lipid content of the liver in Atlantic salmon is typically less than 5%, which is significantly lower than the lipid content of muscle (Bou et al., 2017; Shapar and Johari, 2019), and maximum other farmed fish have intermediate lipid contents. Lipid deposition in liver represents complex mechanisms including hepatic secretion, oxidation, transport and uptake of lipid (Lu et al., 2013) and many key enzymes and transcription factors are involved. The primary lipid class within the muscle is phospholipids, whereas TAGs are the primary lipid storage depot within the liver. However, long-term feeding with high-lipid diets often result in ectopic lipid accumulation within the tissues of farmed fish, along with the liver and belly adipose tissue, and set off metabolic disturbances. Lipid deposition is a complicated manner concerning lipid delivery, uptake, synthesis and catabolism. In the liver, the lipid stores encompass fatty acids (FAs) from three sources: weight loss program, de novo lipogenesis and circulating non-esterified fatty acids. Because of their protein-sparing effects, high-lipid diets are increasingly being used for cost-effective aquaculture farming.

Deposition in fish, like in other animal models, is affected by age, sex, seasonal variation, and, especially, meal abundance. Liver lipid content of 800 g adult Atlantic halibut was significantly higher than that of 50 g juvenile fish, and hepatic lipid accumulation in the older fish was not affected by energy intake (Hazra et al., 1998). Male and female zebra fish have different liver lipid metabolism, with females having better lipogenic activities and fat deposition than males (Solar et al., 2020). The lipid content of the liver varies gonad development and spawning period. As an example, in female Arctic charr (*Salvelinus alpinus*), liver lipid content increased from 3.5% to 14.7% during ovarian development but it decreased to 3.1% in post-spawning period. In terms of seasonal variation, the liver lipid content in large yellow croaker and silvery pomfret (*Pampus argenteus*) was as high as 23.4% in cold conditions but it varied between 12.4% and 4.9% in summer (Geng et al., 2015). Other species have also been reported to experience seasonal variations (Hazra et al., 1998; Shirai et al., 2001), and feeding schedules also immediately impact liver lipid levels. In conclusion, these studies suggest that lipid deposition in fish liver changes in a dynamic manner depending repdoructive cycle, environmental and feeding conditions.

The lipid content of the liver in farmed fish is frequently higher than that of wild counterparts. When lipid deposition in the liver exceeds its normal physiological stage, this can result in fatty liver syndrome (Musso et al., 2009). Excessive fatty liver, or fatty liver ailment, in cultured fish, can lead to a immune suppression making fish vulnerable to pathogen infection that can result in disease (Bransden et al., 2005; Lu et al., 2014). Currently, the frequent occurrence of fatty liver has reached a critical stage in aquaculture for various species. The widespread use of high-fat diets (HFD) in many farmed fish species is one of the important reasons for severe fat deposition, with long-term HFD feeding increasing liver lipid accumulation. These adverse results may additionally be exacerbated by feeding expanded LC-PUFA, for instance, excessive fish oil and/or plant-based oils (Simó-Mirabet et al., 2018; Ruiz et al., 2022). Higher dietary LC-PUFA can increase the risk of peroxidation, which impairs the integrity of mobile membranes and DNA (Du et al., 2008). Lately, research advised that long-time period HFD triggered leptin resistance, selectively reducing lipid catabolism (Liuet al., 2018b), and impairing lipophagic activities in Nile tilapia (*Oreochromis niloticus*), causing decreased performance of lipid droplet removal in liver (Han et al., 2020). Moreover, HFD also altered the expression profile of micro RNA (miR) and genes related to lipid metabolism in blunt snout bream (*Megalobrama amblycephala*), inducing lipid deposition in the liver (Tang et al., 2019).

The nutritional, physiological, and toxicological causes of fatty liver in fish, such as excessive feed and power consumption, critical nutrient deficiency, oxidized nutritional ingredients, and toxicants in feeds and/or water, were eventually reviewed (Du et al., 2014). The model for HFD-brought on fish fatty liver was primarily based on the aforementioned studies.

2.1.2. Lipid Deposition in Adipose Tissue

Large variances in lipid contents of fish have been observed among the tissues, with the liver being the highest lipid content in general and muscle always being the lowest. The muscle tissues are also variable in terms of lipid storage abilities, with red muscle being richer in lipids than white muscle. Lipid storage in the myoseptum accounts for 40% of the lipid content in white muscle in Atlantic salmon (Zhou et al., 1995; Nanton et al., 2007), with the remainder stored in muscle cells as intracellular lipid droplets (Nanton et al., 2007). Abdominal lobe tissue contains both muscle and adipocytes, with the latter predominating in large salmonid species (Nanton et al., 2007). Adipose tissue in various parts of the fish plays an important role in product quality. Subcutaneous and perivisceral adipose tissues influence carcass and filet yield, while muscle depots affect the organoleptic quality of the flesh (fillet). The lipid intake promotes lipidoses in liver and abdominal adipose tissues, suppresses the growth of fish and weakens their disease resistance and immunity (Chatzifotis et al., 2010; Zhou et al., 2020). Conversely, some studies suggested that HFDs are capable of promoting the growth of carnivorous fish though this finding is mostly due to increased fat deposition in the liver and abdominal adipose tissues (Jonathan et al., 2013; Zhao et al., 2016). As a result, improving the utilization of lipids in carnivorous fish and maintaining liver metabolic homeostasis and health based on an HFD takes on a critical significance in the sustainable development of the intensive aquaculture industry.

Likewise, Fang et al. (2021) have confirmed that astaxanthin alleviated the negative impacts of a high-lipid diet on weight gain (WG), specific growth rate (SGR), oxidative stress, and inflammatory response of *Trachinotus ovatus*. Compared to fish fed diets containing 20.2% and 23.2% crude lipid, rainbow trout fed diets containing 26.1% crude lipid grew at a significantly faster pace (Liu et al., 2021). However, the optimum lipid demand for this species cultured in an indoor flow-through system is 15%–20% (Jonathan et al., 2013). One of the advantages of finfish aquaculture over fisheries is the ability to control product quality at various stages of rearing. This control has been made possible by the development of artificial feeds and breeding programs that allow improvement in the growth and phenotypic characteristics of various aquacultured fish species.

Among the cultured species, particularly cold-water species, peripheral adipose tissues have a pivotal role in mobilization and/or deposition of the lipids. In this sense, cold-water species' diets, liken Atlantice salmon, have typically formulated lower crude protein (35-42%) and higher unrefined crude lipid levels (35-40%) (Hillestad and Johnsen, 1994; Hillestad et al., 1998). As a whole, the nutrient sensing systems characterized in fish, as in mammals, are involved in the regulation of energy homeostasis through mechanisms other the regulation of food intake. Also, increasing circulating fatty acid levels induce metabolism changes in the liver and peripheral parts of fish in order to restore normal conditions (Conde-Sieira and Soengas, 2017). Therefore, in culture conditions, the long-term use of lipid-enriched diets in fish can compromise glucose homeostasis due to an impairment of insuline signaling and circulation of the glucose in the plasma. Therefore, the aim of this thesis is to assess the effects of different energy diets on growth, fatty acid metabolism and blood chemistry in male and female rainbow trout, as a cold-water model species.

3. MATERIAL AND METHOD

3.1. Experimental Design and Husbandry Conditions

The experiment was approved by the Ethical Committee judging Health Sciences Experimental Application and Research Center, Cukurova University, and all fish handling procedures in the experiment were fully compliant with the Turkish guidelines for animal care (No. 28141), as set by the Ministry of Food, Agriculture and Livestock. The study was conducted at the Faculty of Fisheries, Çukurova University, Turkey. Rainbow trout juveniles of mixed sex were obtained from a local commercial farm (Tekir Rainbow Trout Production Ltd. Co., Kahramanmaras, Turkey). The fish were transferred into an indoor recirculating (RAS) where the fish were acclimation to the experimental conditionss under a photoperiod cycle of LD 12:12 hours. Before commencement of the experiment, fish were fed restrictively twice daily at 09:00 and at 16:00 with a commercial rainbow trout diet (Optiline 2P, Skretting, Turkey as shown in Table 3.1.). The temperature of the RAS system during the accilimation period was kept at 14.0±1ºC.

At the start of the experiment, fish were individually weighed and all males were intraperitoneally tagged with PIT tags (Biomark). The selection of the males was done using the sex specific chracters as shown in Figure 3.1. At the end of the experiment, in order to confirm that we correctly marked all males, all males were dissected. Utlimatetly, we confirmed that all males marked correctly. To reduce the stress, fish were anesthetized with a phenoxy-ethanol solution (0.4 ml L^{-1}). Then twenty fish (10 females and 10 males) were randomly distributed into each cylindrical conical tank (300-L rearing volume) with four replicates. Throughout the 60 day grow-out period, 5 L/min flow rate of filtered freshwater (14±0.5ºC) was continuously supplied to each tank to ensure suitable water conditions for fish. The rearing water in each tank was supplied with adequate amounts of oxygen through air-stones from an air-blower source. Water temperature and dissolved oxygen were measured twice a day measurement.

Two experimental diets with medium energy (ME) (control diet) and low energy diets (LE) were provided from Skretting-Turkey. The energy content of the ME and LE were18.4 kJ/kg (on dry matter basis) and 17.7 kJ/kg (Table 1). Fish were fed the two experimental diets twice daily (at 09:00 and 16:00) until apparent satiation throughout the experiment. Diets were weighed daily to calculate the feed consumed.

Figure 3.1. Identification of the male and female fish in the experiment. A and B exhibited head differences, C and D exhibited anal fin characteristics.

Table 3.1. Experimental extruder diets (4 mm) provided by Skretting Turkey. This is a close formula.

Chemical analysis (%)	**ME (Select 2p)-Control***	**LE (Optiline 2p)****
Crude Protein (%)	45.0	44.0
Crude Lipid (%)	20.0	21.0
Fibre (%)	3.0	2.1
Dry matter (%)	92.9	92.8
Ash (%)	8.6	8.6
Energy (kJ/kg)	18.4	17.7
Macro Elements		
Calcium (%)	1.80	1.80
Phosphorus (%)	1.17	1.15
Sodium (%)	0.20	0.70

*Ingredients ME (Select 2p)-Control: Chicken meal/ Poultry meal, Dehulled, Extracted, Tosted Soybean Meal. Sunflower meal, Fish meal/ Fish meal, Wheat/ Wheat, Wheat gluten, Fish Oil/ Fish oil , Soybean oil, Blood meal, Vitamin&Mineral Premix/ Vitamin&Mineral Premix, Razmol/ Wheat middlings.

****Ingredients LE (Optiline 2p)**:** Chicken meal/ Poultry meal, peeled, extracted, cooked soybean meal/ Dehulled, Extracted, Tosted Soybean Meal, Fish meal/ Fish meal/ Wheat/ Wheat, Soybean oil, Sunflower meal/ Sunflower meal, Fish oil/ Fish oil , Wheat gluten. Blood meal / Blood meal, Vitamin & Mineral Premix / Vitamin & Mineral Premix.

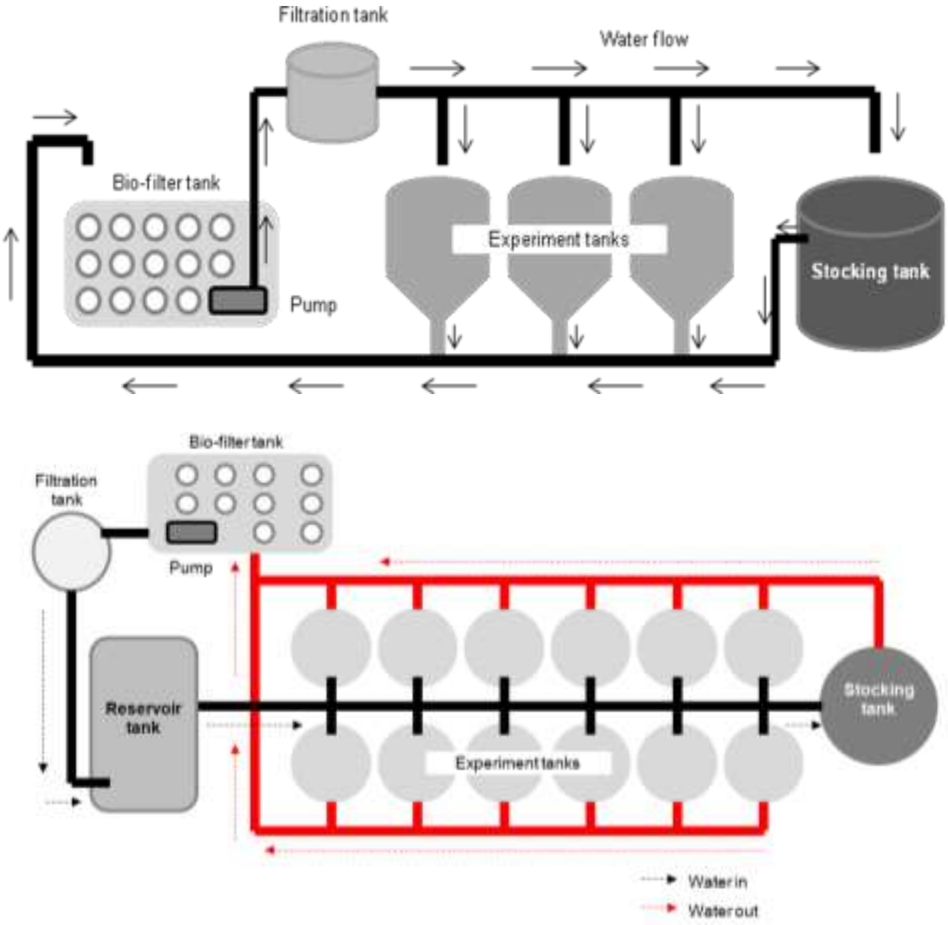

Figure 3.2. Grow-out experiment design in RAS from side-view (top) and top-view (down) adapted from Noor Izam (2021).

3.2. Sampling and Measurement Analysis

The experimental fish were fasted for two days before all samplings. All measurement was started at 9:00-09:30 in the morning. At the beginning of the experiment, ten fish from the main stock were euthanized by an overdose of anesthesia with 200 mg L^{-1} MS-222 and stored at -20ºC until analysis. All the fish in each tank were weighed at the end of the experiment. The viscera (including livers, gonads, stomach, and kidneys) were weighed and the sex of each fish was recorded. Whole body weight (g) and evisceration was performed manually. All branded individuals were dissected and 100% males and females discrimination was made to determine whether the individuals branded at the end of the experiment were males or females. Three fish from each tank (n=3, N=12) for molecular analysis and three fish for proximate analysis and body index parameters (viscera, liver, intraperitoneal fat) were sampled. The selected fish were immediately euthanized with an overdose of MS-222 immediately for tissue and organ sampling. All tissues were immediately transferred to -20ºC, but samples for molecular analysis were immediately frozen in liquid nitrogen and stored at -80ºC. The fish were then filleted and the fillets trimmed (no bones and skin).

Within 2 min following euthanasia, blood was collected from three fish per tank with heparinized syringes. Blood samples (1.5 mL) were allowed to clot at 4ºC until analyses. Then, after being centrifuged at 5000 rpm for 5 min, the serum was withdrawn and kept at -80ºC until analysis of cortisol levels, and other biochemical parameters. The list of performance indicators and physiological biomarkers used in the study is summarized in Table 4.

Table 3.2. Performance indicators and physiological biomarkers used in the study.

Category	**Performance indicator / physiological biomarkers**	**Acronym**	**Interpretation**	**Indication**	**Noof samples per tank**
Zootechnical	Proximate composition	DM	Dry matter, crude protein, lipid and ash of fillet	Metabolic macro changes	2 M and 2 FM
	Fatty acid composition	FA	Full set of specific fatty acids	Metabolic fatty acid changes	2 M and 2 FM
Metabolic	Hepato-somatic index	HSI	Liver weight over body weight	Fat metabolic disorders	2 M and 2 FM
	Visceral-somatic index	VSI	Viseral weight over body weight	Fat metabolic changes	2 M and 2 FM
	Visceral fat index	VFI	Viseral fat over body weight	Fat metabolic changes	2 M and 2 FM
	Glucokinase	Glu	Amino Acids	Unusual forms of diabetes or hypoglycemia	2 M and 2 FM
Blood chemistry / Health	Triglyceride	TG	Fatty Acids and glycerol	Metabolic status, metabolic disorder and overall physiological status	2 M and 2 FM
	Total protein	TP	Circulating protein consistinf mainly immunoglobin and albumin	Protein metabolism disorders or health issue status, metabolic disorder and overall physiological status	2 M and 2 FM
	Glucose	GLC	Circulating glocose	Metabolic status, metabolic disorder and overall physiological status	2 M and 2 FM
	Creatin	CREAT	Amino Acid	Kidney, gill functions and osmoregulatory performance	2 M and 2 FM
	Cholesterol	CHOL	Circulating cholesterol	Metabolism energy and nutritional status	2 M and 2 FM
	ALT	ALT	Proteins and	Liver function,	2 M

			Cholesterol	metabolic status and cellular functions	and 2 FM
	AST	AST	Enzymes	Liver functions, metabolic status and cellular functions	2 M and 2 FM
	Alkaline phosphatase	ALP	Enzymes	High levels of ALP in your blood may indicate liver disease or certain bone disorders	2 M and 2 FM
	Albumin	ALB	Proteins	Liver diseases, acute pancreatitis and kidney failure	2 M and 2 FM
Immune-proinflammatory cytokines	Tumor necrosis factor-α	TNF-α	Cytokine	Autoimmune and immune-mediated disorders such as rheumatoid	3 M and 3 FM
	Interleukine 18	IL-18	Cytokine	Skin redness	3 M and 3 FM
	Interleukine 1β	IL-1β	Cytokine	Bowel disease, acute and chronic myelogenous leukemia, insulin atherosclerosis, neuronal injury, and aging-related diseases.	3 M and 3 FM

3.3. Proximate Chemical Analysis and Colour Measurements

3.3.1. Moisture

Briefly, the moisture content of the samples was measured by drying samples to constant weight at 105ºC. Thermal drying using an oven at 105ºC for 24h was applied to determine the moisture content of diets and fish whole body samples. As for the whole-body fish samples, they were ground to a homogeneous consistency using a centrifugal mill**.** Then 0.5 to 1.0 g of sample was placed into a pre-weighed porcelain crucible and dried in an oven at 105ºC. The next day, the crucibles were placed in a desiccator to cool and then re-weighed. The moisture content of the samples was calculated as**:**

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

3.3.2. Ash

Ash content of diets, fish tissues and liver were determined by burning approximately 1g of sample in a porcelain crucible in a muffle furnace at 600 ºC for 5 hours**.** After incineration, the samples were removed from the furnace, cooled to room temperature in a desiccator and then re-weighed. The ash content was calculated as**:**

$$Ash, \% = \frac{ash\ weight\ (g)}{sample\ weight\ (g)}\ x\ 100$$

3.3.3. Crude Protein

Crude protein content was determined by Kjeldahl analysis (N×6.25) using an automated Kjeldahl Kjeltec 2200 Digestor (FOSS Tecator, Högans, Sweden). Briefly, 0.5 g of sample was weighed into a Kjeldahl digestion tube**.** Tablet 1 of Kjeltec catalyst and 6 ml concentrated sulphuric acid were added into the tube. The tube was then placed into the digestor machine for 2 hours at 420ºC. The tubes were removed from the digestor and left to cool inside a fume cupboard for at least 20 minutes. Then, 20 ml of de-ionised water, 20 ml of boric acid and 40 ml of sodium hydroxide solution were added to the digestion tube automatically by the distilling unit and mixed thoroughly during distillation while the steam valve was turned on**.** The tubes were distilled using the Kjeltec auto analyser and by using 0.1N hydrochloric acid, the titration values were recorded. All samples were analyzed in triplicates. Nitrogen content was calculated by using the formula below:

$$Nitrogen, N\ = \frac{14.01\ x\ (A - B)\ \ x\ \ M}{g\ x\ 10}$$

where A is the volume of titrant of the sample (ml), B is the titrant volume of blank (ml), g is the sample weight (g) and M is the molarity of HCI. The average percentage of nitrogen in plant protein is 16%. Hence, the factor for converting nitrogen to protein is 100/16 = 6.25. Therefore; crude protein, % = N x 6.25.

3.3.4. Crude Lipid

Lipids were extracted according to the procedure of (Folch et al,. 1957). Approximately 3.0 g samples of diets and fish fillets were weighed and put into a 200 ml homogenizing tube while being held in ice. A mixture of 120 ml methanol and chloroform with a ratio 1:2 was added to the samples and homogenized by using a homogenizer (IKA T25, Ultra Turrax) for 3 minutes. The homogenate was filtered using filter paper into a volumetric flask. 20 ml of 0.4 % calcium carbonate was added into the volumetric flask that had been weighed beforehand**.** The samples were left overnight until 2 layers were formed. The next day, the upper layer of the samples was discarded by using a separatory funnel leaving only a mixture of chloroform solution containing lipids in the second layer in the same volumetric flask. The flask was heated using a rotating evaporator at 60ºC until the entire chloroform evaporated leaving only lipids in the flask. Finally, the flasks containing lipids were heated in an oven at a temperature of 60ºC for 1 hour. Then, the weight of the flask was taken after it was completely cooled in the desiccator. The lipid content was calculated as follows:

$$Lipid\ (\%) = \frac{[weight\ of\ volumetric\ flask + lipid\ (g)]\ \ taken\ after\ it\ was\ comple)}{sample\ weight\ (g)} x100$$

3.4. Fatty Acid Composition

The total lipid of diets and fillet was extracted by chloroform/methanol method (Folch et al., 1957). Afterward, methanol was added to the lipid for methanolization with BF3. Then, fatty acids methyl ester was extracted by n-hexane, methanolic NaOH 2% (2 g of NaOH in 100 g of methanol) was added to the lipid, and the content was carefully mixed and stood in boiled water for 10 minutes. After cooling, 2.2 ml of BF3 was added to the composition and mixed again and stood in boiled water for 3 minutes, then, 1 ml of n-hexane was added to the mixture and carefully mixed, and 1 ml of saturated salt was added to the blend. This solution was strongly shaken and put in a stable location. After the two phases were clearly noticed, the upper layer was separated and kept at -20ºC until infusion to GC mass instrument (Firestone, 1998). The resultant fatty acid methyl esters (FAMEs) were separated using GC (Varian Analytical Instrument, CP 3800, Walnut Creek, CA, USA) equipped with a flame ionization detector fitted with a permanently bonded polyethylene glycol, fused silica capillary column (PBX70 SGE Analytical Science; 120 m × 0.25 mm internal diameter, film thickness 0.25 µm, Melbourne, Australia). The injection volume was 1.0 µl, and the carrier gas was helium**.** The injector and detector temperature was, respectively, 230 ºC and 260 ºC**.** A split injection approach of 20:1 was used, and the temperature was programmed to increase from 160 ºC to 180 ºC at a rate of 2 ºC/min, and held at 180 ºC for 85 min**.** Individual FAMEs were identified using external standards as reference (Sigma-Aldrich, Steinheim, Germany).

3.5. Calculation and Data Analysis

All fish were weighed individually to calculate the growth parameters reported below. All fish were fasted 48 hours before sampling to reduce contamination of the digestive tract with leftover diets and faeces. The parameters of growth and feed utilization were calculated using the following equations:

- Hepatosomatic index (HSI, %) = $W_{liver}/W_{fish} \times 100$;
- Viscerosomatic index (VSI, %) = $W_{vis}/ W_{fish} \times 100$;
- Intraperitonal fat index (IPFI, %) = $W_{intfat}/ W_{fish} \times 100$;
- Specific Growth Rate (SGR, % day^{-1}) = $100 \times (\ln W_t\ ) \ln W_0)/t$;

where W_{liver} is the wet weight of the liver and W_{fish} is the final whole wet weight of the fish; W_{vis} is the wet weight of viscera, W_{intfat} is the wet weight of intraperitoneal fat, W_t and W_0 are the final and initial weights (g), C is the daily feed consumption (g) and t the feeding duration (day).

3.6. Histological Analysis

Embedding, sectioning and staining of the liver samples were performed at the Faculty of Medicine Science in Cukurova University. The liver samples were fixed in 10% neutral buffered formalin (NBF), dehydrated in a graded ethanol series and embedded in paraffin. The fixing solution was changed with fresh NBF after 24 hours. Fixed samples were dehydrated in a graded series of ethanol, cleaned with xylol, and embedded in paraffin by using an Autotechnicon tissue processor (Leica TP 1020). Then, sections of the intestines were cut at a thickness of 5 µm using a microtome (Thermo Scientific, Shandon Finesse 325). They were stained with hematoxylin and eosin (H&E) and sealed with Entellan (Merck, Darmstadt, Germany) for morphometric analysis. Photographs were taken using a microscope (OlympusBX51 compound microscope) fitted with a microscope imager (Leica). Twenty images per animal were captured at 4× and 40× magnifications for morphometric measurements. Five slides were prepared for each intestine segment sample and 30 measurements for each slide were made to determine VL, VW, ME, ICL and OLL. Liver steatosis scores were ranked according to a non-continuous score grade from 0 to 5 (Table 2).

Table 3.3. Semi-quantitative scoring used for assessing the levels of lipid accumulation in rainbow trout fed with the ME and LE experimental diets.

Score	Histological description
0	Formation of vacuoles in the cytoplasm, involving less than 10% of the hepatocytes and including less than 25% of the area of the individual hepatocytes
1	Formation of vacuoles in the cytoplasm, involving less than 25% of the hepatocytes and including less than 25% of the area of the individual hepatocytes
2	Formation of vacuoles in the cytoplasm, involving less than 50% of the hepatocytes and including less than 50% of the area of the individual hepatocytes
3	Formation of vacuoles in the cytoplasm, involving less than 75% of the hepatocytes and including less than 75% of the area of the individual hepatocytes
4	Formation of vacuoles in the cytoplasm, involving less than 90% of the hepatocytes and including less than 80% of the area of the individual hepatocytes
5	Formation of vacuoles in the cytoplasm, involving more than 90% of the hepatocytes and including more than 80% of the area of the individual hepatocytes

3.7. mRNA Expression Analysis

Total RNA was extracted from the fish liver with TRIzol-reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. RNA quality and quantity were measured using the spectrophotometric (260/280 ratio, Thermo Fisher Multiskan GO Reader) and fluorometric (Qubit 2.0, Thermo Fisher Scientific) methods, respectively. The cDNAs were synthesized with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Thermo

Fisher). Reverse transcription was carried out in 20 µl of final volume (2 µl of 10 × RT Buffer, 0.8 µl of dNTP, 2 µl of random primers, 1 µl of reverse transcriptase and 10 µl RNA (1000 ng)). The reaction was incubated for 10 minutes at 25ºC, 37 ºC for 60 minutes, and 85ºC for 5 minutes. The resulting cDNAs were kept at -20ºC.

For RT-PCR, 2 µl of cDNAs were used. The quantitative PCR reactions were performed using SYBR Green Master Mix (Applied Biosystem). Reactions were carried out according to the manufacturer's instructions. The total volume was 25 µl (12.5 µl of Sybr Green Master Mix, 1.25 µl of 10 pmol forward primer, 1.25 µl of 10 pmol reverse primer, 2 µl of cDNA template and 8 µl of water). An Applied Biosystem 7500 RT-PCR device was used. The qPCR cycling conditions were 50ºC for 2 minutes, 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. β-actin was used as the reference gene. All primer sequences are given in Table 3. The relative expression levels were analyzed using comparative 2−ΔΔCt formula (Livak and Schmittgen, 2001) using threshold cycle (Ct) values obtained from replicates. All experiments were performed in triplicates.

Table 3.4. Sequences of primers used for real-time PCR analysis.

Genes	**Sequence (5'-3')**	**Amplification length (bp)**	**GeneBank accession number**	**References**
Actin beta	f: TGACCCAGATTATGTTTGAGACC r: AGGATCTTCATCAGGTAGTCTGT	223	NM_001124235.1	designed
IL1- β	f: ATATAGTGTTGGAGTTGGAGTCG r: ACGAAGACAGGTTCAAATGC	198	NM_001124347.2	designed
IL-18	f: TAAGTGTGTTGAGTTTGCGGTAG r: AGAACGTCTTCATCTACAACCAG	162	NM_001124617.1	designed
TNF-α	f: ACGATGCAGGATGAAATTGAG r: CTAAATGGATGGCTGCTTTTG	109	NM_001124357.1	designed
Glukokinaz	f: TGGGTTTCACCTTCTCTTTTC r: ATATCATGGTGGCAACTGTATCG	198	AF053331.2	designed

3.8. Statistical Analysis

All parameters of experimental groups were analyzed using the dietary energy levels. Besides, the data of growth performances, nutritional status, biochemical analyses, and histomorphometry measurements for all experimental groups were analyzed with two-way ANOVA. Intergroup differences of gene expression were evaluated using Duncan's test and Kruskal Wallis with Man Whitney U tests for homogeneous and inhomogeneous data, respectively. SPSS 20.0 software (SPSS, Chicago, USA) was used to analyze data. All data were presented as mean ± SD, except the gene expression data, which were reported as mean ± standard error. The level of significance was determined independent t-test, $P<0.05$.

4. RESULTS

4.1. Environmental Parameters

In the present study, standard water quality parameters were maintained within the critical limits for optimum growth of rainbow trout. During the study, there was no significant fluctuation recorded in the water parameters measured. Overall, the water temperature ranged from a minimum of 14.1ºC to a maximum of 14.5ºC. The mean temperature value recorded throughout the study was 14.2±0.06ºC. The mean value of dissolved oxygen (DO) measured was recorded as 10.13±0.43 mg/L. Thus the optimum water quality parameters for trout were maintained throughout the experiment.

4.2. Growth Performances

Overall, the growth performances of the fish of final weight, and specific growth rate (SGR) were not affected by different dietary treatments (Table 4.1.). SGR of the female fish fed with ME and LE were higher than the male counterparts ($P<0.05$) whereas there was no statistical interaction between gender and diets (Table 4.1.). After the following 60 days of the feeding trial, the mean final weight in the LE group ranged from (385.7±21.4 to 406.0±23.6 g) and the final weight in the ME group ranged between (424.1±22.0 to 434.2±37.3 g). The mean final weight was significantly higher in fish fed the ME diet compared with those fed the LE diet ($P<0.05$). Fish from the diet treatment group (LE) achieved the highest percentage of weight gain of (290.34±1.99%) among both treatments with statistically significant difference ($P<0.05$). Irrespective of gender, there was no significant difference in FI and FCR as presented in Table 4.1. Female fish fed with ME showed the highest SGR (1.63±0.10) among both treatments ($P>0.05$). However, no significant interactive differences between gender and diets were observed for SGR after 60 days of the feeding trial ($P>0.05$). The feed utilization parameters such as FCR and feed intake of the experimental fish were presented in Table 4.1. Even though no significant differences were recorded, the total feed intake and FCR of the ME group (306.7±24.17 g/fish) was slightly higher than the LE group (297.6±10.28 g/fish). Overall, feed conversion ratio (FCR) was no statistically significant difference between LE and HE ($P>0.05$). However, fish fed with diet treatment LE showed the highest FCR of (1.27±0.05) among both diet treatments. The average HSI ranged between (0.92 to 1.07) as shown in Table 4.1. There was not any significant interaction between gender and diet in terms of HSI. Also, no significant difference was found in HSI in both genders (Table 4.1.). Similarly, no significant difference was found in VSI which ranged from %3.83 to %4.08 ($P>0.05$). However, female fish fed with LE diet had the highest VFI than male fish fed with ME counterparts. Female and male fish fed with both experimental diets showed similar VFI, ranging from %4.88 to %6.89 (Table 4.1.) and regarding this somatic parameter, VFI was significantly affected by both factors (dietary energy levels and gender) ($P<0.05$) (Table 4.1.).

Table 4.1. Growth performance, feed utilization and somatic indexes of rainbow trout fed medium and low energetic diets for 60 days

Growth parameters	Experimental diets						
	LE		ME		Two-way ANOVA		
	Female	Male	Female	Male	Diets	Gender	Interaction
Initial weight (g)	167.6±6.0	182.5±7.4	162.9±2.6	184.2±2.8	n.s.	n.s.	n.s.
Final weight (g)	406.0±23.6	385.7±21.4	434.2±37.3	424.1±22.0	*	n.s.	n.s.
SGR (%)[1]	1.47±0.10^{b}	1.25±0.06^{c}	1.63±0.10^{a}	1.39±0.08^{b}	**	***	n.s.
FI[2] (g)	297.6±10.28		306.7±24.17				
FCR[3]	1.27±0.13		1.15±0.06				
HSI[4]	1.27±0.13	1.15±0.06	1.07±0.18	1.05±0.13	n.s.	n.s.	n.s.
VSI[5]	3.83±0.45	3.88±0.58	3.86±0.35	4.08±0.32	n.s.	n.s.	n.s.
VFI[6]	6.89±1.16^{a}	3.47±1.24^{b}	4.74±1.93^{b}	4.88±1.82^{b}	n.s.	*	*

Data represent means ± standard deviation. Asterisks represent statistically different values within genders in each diet (Independent t-test, P<0.05). ns = non-significant, *, ** and *** indicate p < 0.05, p <0.01 and p < 0.001, respectively.

[1] SGR : (SGR, % day^{-1}) = (Ln (final weight)–Ln (initial weight)/experiment duration) x 100
[2] FI : Total feed intake = (g feed / tank as per replicate)
[3] FCR : Feed conversation ratio (FCR) = dry feed intake (g)/live weight gain (g)
[4] HSI : Hepatosomatic index (HSI, %) = Liver weight / Fish body weight ×100
[5] VSI : Viscerosomatic index (VSI, %) = Visceral weight / Fish body weight×100
[6] VFI : Visceral fat index (VFI, %) = Visceral fat weight / Fish body weight×100

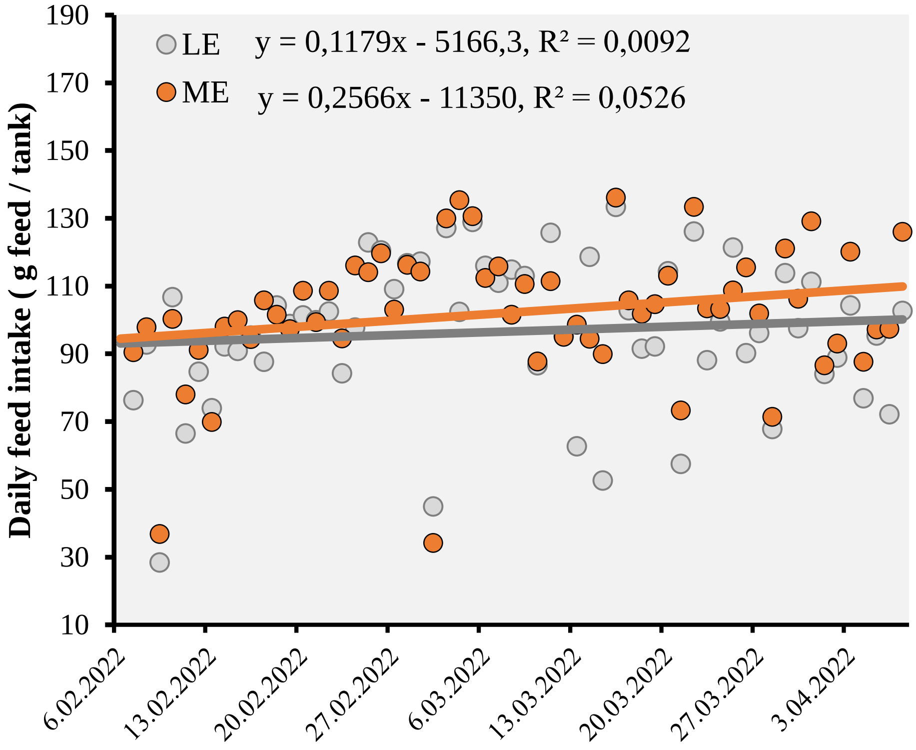

Figure 4.1. Daily feed intake (Each dot represents the average daily feed intake of each tank replicates (n=4) for the experimental groups).

4.3. Whole Body Proximate Composition

The proximate composition of the whole body is shown in Table 4.2. There were no significant differences in the mean percentage of whole body crude protein contents in both diet treatments, with fish fed with ME diet treatment recording a mean value of 21.27±0.49% of protein content ($P>0.05$). Fish fed with ME diet exhibited the higher mean lipid content of 9.10±1.32% without a significant change by genders. However, even though no significant differences were recorded, the lipid content in ME group (average lipid content was 8.6%), appeared to be approximately 11.5% higher compared with the LE group. The whole-body lipid content was a statistically not significant difference ($P>0.05$). Dry matter content was statistically lower in males on ME diet than those on LE ($P<0.05$). No significant differences were recorded in whole-body ash contents that varied from 0.44±0.04% to 0.47±0.04%.

Table 4.2. Proximate composition of fillets in rainbow trout fed medium and low energetic diets for 60 days.

	Experimental diets						
	LE		ME		Two-way ANOVA		
	Female	Male	Female	Male	Diets	Gender	Interaction
Crude protein (%)	21.20±0.90	21.20±0.61	21.27±0.49	20.13±0.15	n.s.	n.s.	n.s.
Crude lipid (%)	7.53±0.30	7.90±1.30	8.10±0.82	9.10±.1.32	n.s.	n.s.	n.s.
Dry matter (%)	30.20±0.26^{a}	31.07±0.76^{a}	28.20±0.36^{b}	30.97±0.85^{a}	**	**	*
Ash (%)	0.43±0.02	0.47±0.04	0.44±0.04	0.45±0.01	n.s.	n.s.	n.s.

Data represent means ± standart deviation. Asterisks represent statistically different values within genders in each diet (Independent t-test, $P<0.05$). ns = non-significant, *, ** and *** indicate $p < 0.05$, $p <0.01$ and $p < 0.001$, respectively.

4.4. Fatty Acid Composition of the Experimental Diets

Fatty acid profiles of the experimental diets were presented in Table 4.4. Dietary lipid levels and fatty acid profile of the experimental. diets were identical (Table 4.4.). There was only slightly difference found in MUFA in the experimental diets. However, the rest of the whole fatty acid profile in the diets were similar.

Table 4.3. Fatty acid composition (% of total fatty acids) of experimental diets

	Experimental diets	
	LE	**ME**
Lipid	20.0	21.0
14:0	3.5±0.0	3.5±0.0
14:1	0.2±0.0	0.2±0.0
15:0	0.6±0.0	0.6±0.0
16:0	23.6±0.0	23.5±0.1
16:1n7	3.6±0.0	3.8±0.0
17:0	0.7±0.0	0.6±0.0
17:1n7	0.2±0.0	0.2±0.0
18:0	7.5±0.07.8	7.8±0.0
18:1n-9	35.0±0.0	33.3±0.1
18:1n-7	2.8±0.0	2.8±0.0
18:2n-6	0.3±0.0	0.3±0.0
18:3n-6	1.0±0.0	0.9±0.0
18:3n-3	4.4±0.0	4.8±0.0
20:0	0.0±0.0	0.1±0.0
20:1n-11	0.8±0.0	0.8±0.0
20:2n-6	0.2±0.0	0.3±0.0
20:3n-6	0.8±0.0	0.7±0.0
20:4n-6	0.3±0.0	0.3±0.0
20:3n-3	1.1±0.0	1.1±0.0
20:5n-3	4.9±0.0	4.7±0.0
22:1n-9	0.2±0.0	0.1±0.0
22:5n-3	0.6±0.0	0.6±0.1
22:6n-3	7.4±0.0	7.3±0.1
24:0	0.4±0.1	1.8±0.3
24:1n9	0.5±0.0	0.5±0.0
SFA[1]	36.4±0.0	37.9±0.1
MUFA[2]	43.3±0.0	41.7±0.2
PUFA[3]	21.0±0.0	21.1±0.0
n-3 PUFA[4]	18.4±0.0	18.6±0.0
n-3 LC PUFA[5]	14.0±0.0	13.8±0.0
n-6 PUFA[6]	2.6±0.0	2.5±0.0
n-6 LC PUFA[7]	1.3±0.0	1.2±0.0
n3/n6	7.2±0.0	7.6±0.1
LC PUFA[8]	15.3±0.0	15.0±0.0

[1] SFA (Saturated fatty acid) is the sum of all fatty acids without double bounds; includes 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0 and 24:0).

[2] MUFA (monounsaturated fatty acid) is the sum of all fatty acids with a single double bound; including (16:1n-7, 18:1n-9, 20:1n-11 and 22:1n-9).

[3] PUFA (polyunsaturated fatty acid) is the sum of all fatty acids with ≥2 double bounds; includes, 18:2n-6, 18:3n-6, 18:3n-3, 20:2n-6, 20:3n-6, 20:4n-6, 20:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3.

[4] n-3 PUFA; Sum of all n-3 fatty acids.

[5] n-3 LC PUFA; n-3 fatty acids with chain lenght ≥20 carbon atoms and ≥ 2 double bounds.

[6] n-6 PUFA; Sum of all n-6 fatty acids.

[7] n-6 LC PUFA; n-6 fatty acids with chain lenght ≥20 carbon atoms and ≥ 2double bounds.

[8] LC PUFA; the sum of all fatty acids with chain length≥20 carbon atoms and ≥ 2 double bounds.

4.5.1. Fillet Nutritional Composition: Proximate and Fatty Acid Composition

The fillet fatty acid compositions of trout from all experimental groups are presented in Table 4.5. Some specific fatty acid contents were significantly affected by the dietary treatments ($P>0.05$). For instance, the LC-PUFA content of the fillets in females in both experimental groups was higher than males whereas no statistical difference was found ($P>0.05$). There were no interactive effects of gender and diets in PUFA, n-3 PUFA and n-3 LC-PUFA, as shown in Table 4.5. On the other hand, MUFA content tended to increase in male fish fed with the ME and LE diets and differences were significant ($P<0.05$). The opposite results were found for LC-PUFA, n-6 PUFA and n-6 LC-PUFA, and levels increased concomitantly in female fish ($P<0.05$) (Table 4.5.). In particular, specific fatty acids such as EPA and DHA, no differences were detected in both experimental diets and there were no significant interactions between the two factors (energy levels and gender) in EPA and DHA profile of the fillets of the experimental fish ($P>0.05$). However, a significant interaction between dietary energy levels and gender was found for 18:3n-6 (Table 4.5.).

Table 4.4. Fatty acids of fillets in rainbow trout fed fed two experimental diets for 60 days.

	Experimental Diets						
	LE		**ME**		Two-way ANOVA		
	Female	Male	Female	Male	Diets	Gender	Interaction
14:0	2.5±0.1	2.6±0.1	2.5±0.1	2.6±0.0	n.s.	n.s.	n.s.
14:1	0.0±0.0	0.2±0.2	0.0±0.0	0.0±0.0	n.s.	n.s.	n.s.
15:0	0.4±0.0	0.4±0.0	0.4±0.0	0.4±0.0	n.s.	n.s.	n.s.
16:0	23.7±0.4	24.8±0.4	23.8±0.4	23.8±1.4	n.s.	n.s.	n.s.
16:1n7	4.2±0.5	5.2±0.1	4.1±0.1	5.0±0.2	n.s.	n.s.	n.s.
17:0	0.3±0.0	0.3±0.1	0.3±0.0	0.3±0.0	n.s.	n.s.	n.s.
17:1n7	0.2±0.1	0.2±0.0	0.2±0.0	0.3±0.1	n.s.	n.s.	n.s.
18:0	7.9±0.4	7.5±0.5	8.0±0.6	7.8±0.4	n.s.	n.s.	n.s.
18:1n-9	33.6±1.0	34.8±0.8	34.7±0.6	35.0±0.7	n.s.	n.s.	n.s.
18:1n-7	3.1±0.1	2.9±0.2	2.9±0.1	2.9±0.1	n.s.	n.s.	n.s.
18:2n-6	0.2±0.0	0.2±0.1	0.2±0.0	0.2±0.0	n.s.	n.s.	n.s.
18:3n-6	0.7±0.0	0.6±0.0	0.7±0.0	0.8±0.0	n.s.	n.s.	n.s.
18:3n-3	2.9±0.0	2.9±0.1	3.2±0.2	3.1±0.2	n.s.	n.s.	n.s.
20:0	1.3±0.2^{a}	0.8±0.1^{b}	0.1±0.0^{c}	0.0±0.0^{c}	***	***	***
20:1n-11	1.8±0.2	1.7±0.3	1.8±0.1	1.6±0.2	n.s.	n.s.	n.s.
20:2n-6	1.5±0.2	1.3±0.2	1.7±0.1	1.4±0.2	n.s.	*	n.s.
20:3n-6	1.0±0.1	0.9±0.0	1.1±0.2	1.1±0.1	***	n.s.	n.s.
20:4n-6	0.4±0.0	0.4±0.0	0.5±0.0	0.5±0.1	**	n.s.	n.s.
20:3n-3	1.3±0.1	1.1±0.0	1.2±0.1	1.1±0.1	n.s.	*	n.s.
20:5n-3	2.2±0.0	1.9±0.2	2.1±0.1	2.1±0.1	n.s.	n.s.	n.s.
22:1n-9	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	n.s.	n.s.	n.s.
22:5n-3	0.9±0.1	0.9±0.0	1.1±0.1	1.0±0.1	***	n.s.	n.s.
22:6n-3	9.6±0.7	8.1±0.8	8.9±0.8	8.6±1.3	n.s.	n.s.	n.s.
24:0	0.2±0.0	0.1±0.0	0.1±0.0	0.2±0.0	n.s.	n.s.	n.s.
24:1n9	0.3±0.0	0.3±0.0	0.4±0.0	0.4±0.0	n.s.	n.s.	n.s.
SFA[1]	36.3±0.4	36.4±0.6	35.2±1.0	35.0±1.7	n.s.	n.s.	n.s.
MUFA[2]	43.3±1.2	45.6±0.9	44.4±0.8	45.3±0.6^{a}	n.s.	**	n.s.
PUFA[3]	20.7±0.8	18.3±0.9	20.7±1.2	19.9±1.8	n.s.	n.s.	n.s.
n-3 PUFA[4]	16.9±0.8	14.9±1.0	16.5±1.3	16.0±1.5	n.s.	n.s.	n.s.
n-3 LC PUFA[5]	14.0±0.8	12.0±1.0	13.3±1.1	12.8±1.3	n.s.	n.s.	n.s.
n-6 PUFA[6]	3.8±0.3^{a}	3.4±0.1^{b}	4.2±0.0^{a}	3.9±0.3^{a}	***	*	n.s.
n-6 LC	2.9±0.2^{a}	2.5±0.2^{b}	3.3±0.0^{a}	2.9±0.4^{a}	*	*	n.s.

PUFA[7]							
n3/n6	4.4±0.3^a	4.4±0.5^a	3.9±0.3^b	4.0±0.1^b	*	n.s.	n.s.
LC PUFA[8]	16.9±0.9	14.6±0.8	16.7±1.0	15.8±1.7	n.s.	*	n.s.

Values are mean and standard deviation. n = 3. Asterisks represent statistically different values within genders in each diet (Independent t-test, P<0.05).). ns = non-significant, *, ** and *** indicate p < 0.05, p <0.01 and p < 0.001, respectively.

[1] SFA (Saturated fatty acid) is the sum of all fatty acids without double bounds; includes 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0 and 24:0).

[2] MUFA (monounsaturated fatty acid) is the sum of all fatty acids with a single double bound; including (16:1n-7, 18:1n-9, 20:1n-11 and 22:1n-9).

[3] PUFA (polyunsaturated fatty acid) is the sum of all fatty acids with ≥2 double bounds; includes, 18:2n-6, 18:3n-6, 18:3n-3, 20:2n-6, 20:3n-6, 20:4n-6, 20:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3.

[4] n-3 PUFA; Sum of all n-3 fatty acids.

[5] n-3 LC PUFA; n-3 fatty acids with chain lenght ≥20 carbon atoms and ≥ 2 double bounds.

[6] n-6 PUFA; Sum of all n-6 fatty acids.

[7] n-6 LC PUFA; n-6 fatty acids with chain lenght ≥20 carbon atoms and ≥ 2double bounds.

[8] LC PUFA; the sum of all fatty acids with chain length≥20 carbon atoms and ≥ 2 double bounds.

4.6. Blood Chemistry

As shown in Table 4.6., some blood chemistry parameters were affected by experimental diets and genders. However, glucose, creatine and alkaline phosphatase were not significantly different among the groups (P>0.05). Independent t-test exhibited that a significant difference on gender was found in triglyceride, total protein, ALT and AST (P<0.05) whereas the experimental diets had an impact on cholesterol levels in both genders (P<0.05). There was only one interaction effect was found between gender and diet in AST while there were not any clear interaction trends in any other blood parameters. Overall, there was a clear effect of gender on blood parameters as seen in Table 4.5.

Table 4.5. Plasma biochemical indexes in rainbow trout fed fed two experimental diets for 60 days.

	Experimental diets						
	LE		ME		Two-way ANOVA		
	Female	Male	Female	Male	Diet	Gender	Interaction
Triglyceride	469.7±174.2^b	492.4±140.3^b	467.3±84.6^b	615.2±203.5^a	n.s.	*	n.s.
Total protein	3.9±0.9^b	4.7±0.5^a	3.9±0.4^b	4.3±0.7^{ab}	n.s.	*	n.s.
Glucose	87.4±31.1	100.3±32.4	101.3±25.3	123.6±42.8	n.s.	n.s.	n.s.
Creatin	0.27±0.1	0.27±0.1	0.26±0.1	0.34±0.1	n.s.	n.s.	n.s.
Cholesterol	316.8±96.6^b	409.3±113.7^a	330.0±19.2^b	344.8±40.2^b	**	n.s.	n.s.
ALT	13.7±10.7	26.2±6.0	17.3±9.9	27.3±9.9	n.s.	0.015	n.s.
AST	249.3±109.0^c	495.5±150.7^a	342.8±136.0^b	446.6±175.4^a	n.s.	**	**
Alkaline phosphatase	101.3±49.3	125.6±48.4	114.1±59.7	93.9±18.8	n.s.	n.s.	n.s.
Albumin	1.7±0.4^b	2.1±0.2^a	1.6±0.2^b	1.9±0.3^a	n.s.	***	**

Asterisks represent statistically different values within gender in each diet while § represents statistically different values within diets in each gender (Independent t-test, P<0.05), respectively. ns = non-significant, *, ** and *** indicate p < 0.05, p <0.01 and p < 0.001, respectively.

4.7. Hepatic Morphometric Analysis

The liver histology scores, based on the degree of steatosis were read as vacuole-formation in hepatocyte cytoplasm characterized by both micro- and macro-vesicular lesions (Figure 4.2.). Based on the observation of liver tissue as shown in Figure 4.2., histological examination revealed no signs of specific pathologies, and the overall structure and morphology of the liver tissues were considered normal. Thus, there were no clear significant pathological changes between fish fed ME and LE. Percentage of steatosis in H&E stained preparations evaluated using the scoring system according to Martinez-Rubio et al. (2012). applied to tissues by histochemical method; The reticular roof was normal in reticulin staining. No fibrous tissue increase was observed in the trichrome and no iron pigment accumulation was observed with Prussian blue.

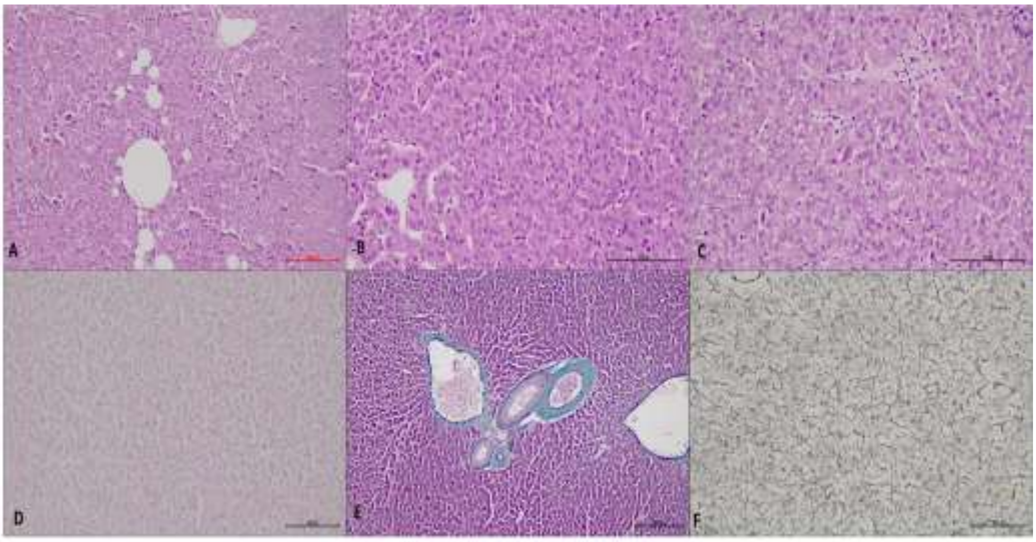

Figure 4.2. Examples of light microscope images obtained from livers of rainbow trout fed with ME and LE diets. Micrographs showing different degrees of steatosis. A, microvesicular steatosis, 0.5 score; B, moderate steatosis with a mix of microvesicular and some macrovesicular, 2.0 score; C, pronounced steatosis dominated by macrovesicular, 3.5 score. The scale bar corresponds to 40 microns.

4.8. Gene Expression

IL-1β expression is shown in Figure 4.2 showing a significant decrease in the liver ME of females (control group) at the end of experiment. while ıts expression level was highest in ME males (P<0.05). The lowest IL-1β expression was found in male fish fed with LE diet whereas the highest IL-1β expression was found in female fish (3-fold) fed with the same diet (P<0.05). On the other hand, fish fed with ME diet showed no significant difference among the genders (P>0.05) as shown in Figure 4.2. There are a few possible causes for the significant decrease in gene expression in the livers of LE-fed males. One possibility is that the fish experienced a high level of stress. Stress can damage cells and DNA, and it can also lead to a decrease in gene expression. Stress can be caused by a number of factors, including environmental pollutants. In the case of male fish fed medium energy, the stress may be due to the diet itself, the stress of being in captivity, or the presence of pathogens. Stress occurs when there is an imbalance between the production of free radicals and the body's ability to detoxify them. When fish are fed on low energy, their immune system is less active. This is because the fish need to conserve energy for other essential functions, such as growth and reproduction. As a result, the fish produce less IL-1β. The expression of genes involved in IL-1β production may be changed in fish fed on low energy. This could lead to a decrease in IL-1β production.

The expression of glucokinase at the end of the experimental period is shown in Figure 4.3. In general, the expression of glucokinase was significantly higher, and up-regulated in female fish fed with LE, which was 3-fold higher than the other treatments (P<0.05) whereas the other groups were not significantly different from each other (P>005).

Glucokinase activity might increase as a compensatory mechanism to enhance glucose utilization and maintain adequate energy levels within the liver cells. The increased glucokinase activity could be associated with an attempt by the fish to enhance glycogen synthesis for energy storage. Also, additional factors such as hormonal changes, genetic factors, and environmental conditions can also contribute to the observed increase in glucokinase activity.

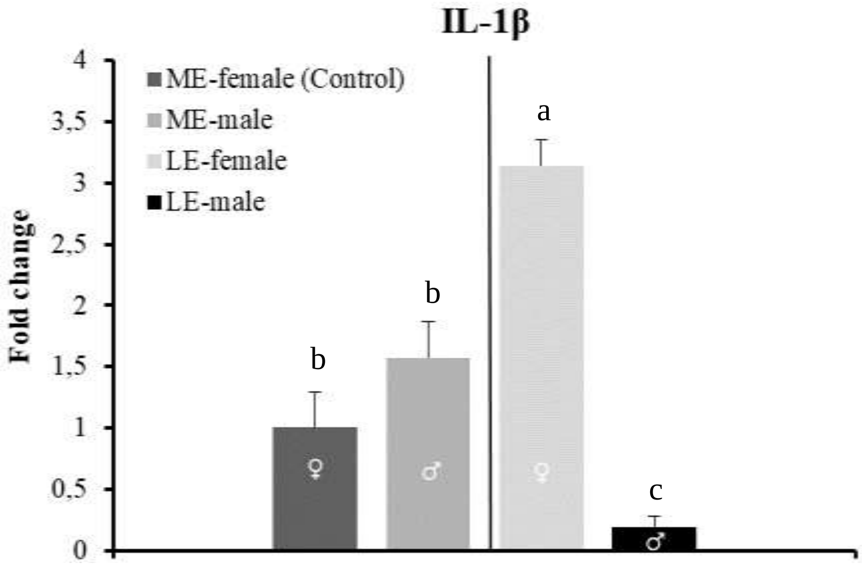

Figure 4.3. Liver relative expression of IL-1β related to proinflammatory mechanismsin rainbow trout fed different experimental diets for 60 days. Bars having different lowercase letters are significantly different at P<0.05 (ANOVA).

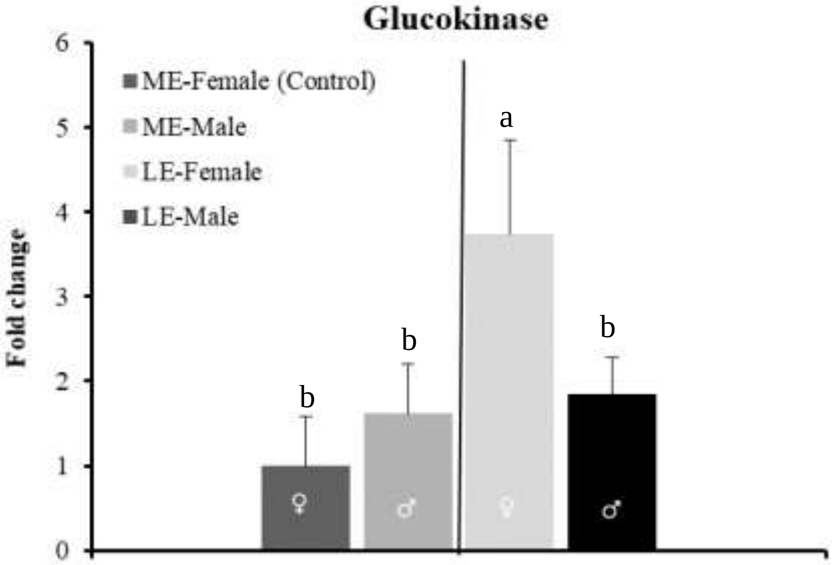

Figure 4.4. Liver relative expression of genes related to glucose mechanisms, glucokinase in rainbow trout fed different experimental diets for 60 days. Bars having different lowercase letters are significantly different at P<0.05 (ANOVA).

In the present study, the expression of TNF-α in the liver of fish fed with LE exhibited an increasing trend compared with those fed with ME diets (P<0.05) whereas the lowest expression was found in males fed with LE (P<0.05). On the contrary, the highest expression of IL-18 in the liver of male fish fed with ME was found. Again, the lowest expression of IL-18 was observed in the liver of the fish fed with LE as shown in Figure 4.4. There are a few possible causes for the significant decrease in gene expression in the livers of LE-fed males. One possibility is that the fish experienced a high level of stress. Stress can damage cells and DNA, and it can also lead to a decrease in gene expression. In the case of male fish fed medium energy, the stress may be due to the diet itself, the stress of being in captivity, or the presence of pathogens. Stress occurs when there is an imbalance between the production of free radicals and the body's ability to detoxify them. Compared to the IL-18 the highest expression in the liver of male fish fed with ME was found. Stress can lead to immune responses, including changes in cytokine production. The ME diets might contain nutrients or components that stimulate IL-18 production.

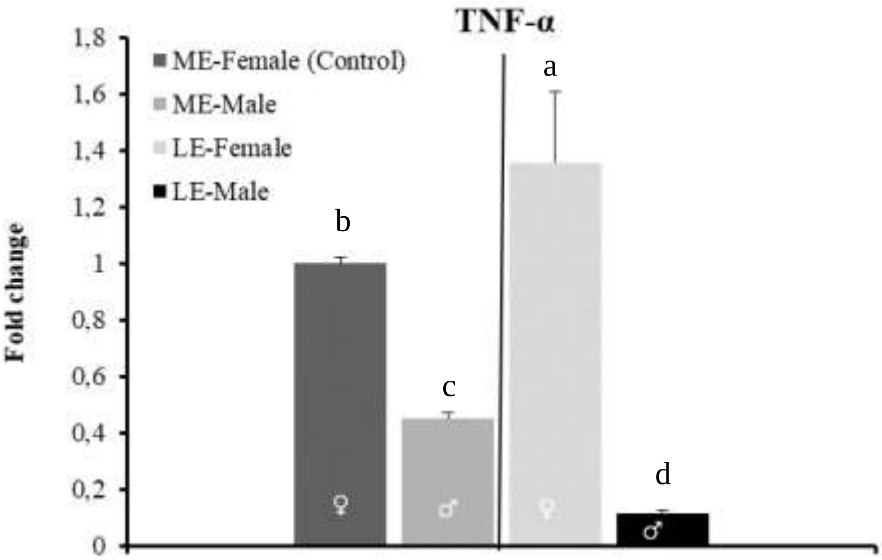

Figure 4.5. Liver relative expression of TNF-α related to proinflammatory mechanisms in rainbow trout fed different experimental diets for 60 days. Bars having different lowercase letters are significantly different at P<0.05 (ANOVA).

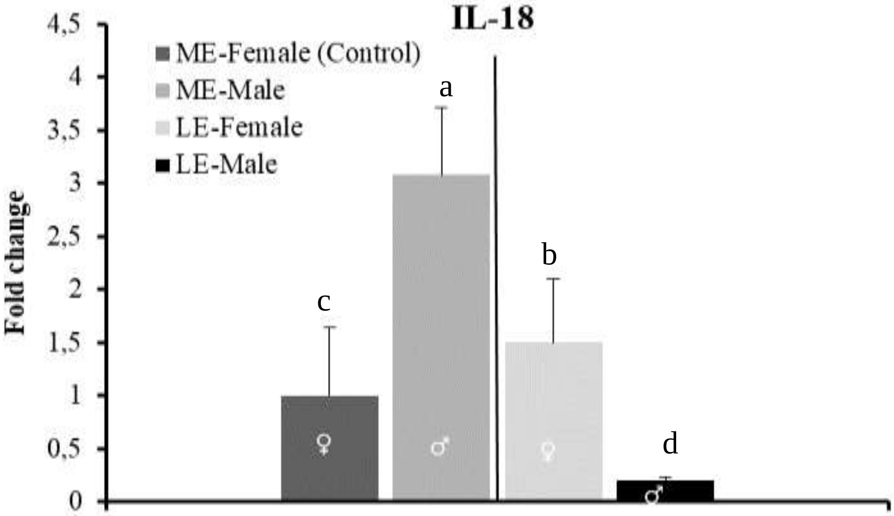

Figure 4.6. Liver relative expression of genes related to iron metabolism, proinflammatory mechanisms, IL-18 in rainbow trout fed different experimental diets for 60 days. Bars having different lowercase letters are significantly different at P<0.05 (ANOVA).

5. DISCUSSION

The objectives of this thesis study were to investigate the effects of long-term feeding of two dietary energy levels (low and medium as control) on growth performance, blood chemistry and pro-inflammatory response of rainbow trout. This study also allowed for comparisons between male and female rainbow trout responses to different dietary energy levels. Overall, the growth parameters of the fish were not significantly different among the groups. This was expected since the dietary energy levels of both commercial diets were close to each other. The high energy diets (18.4 kJ/kg) were used by the farmers that produce rainbow trout for the processing plants in Türkiye. On the other hand, the low-energy diets (17.7 kJ/kg) were used for grow-out fish in the trout industry. Thus, the results of the present study showed that the energy difference would not have severe effects on energy metabolism, at least for the growth performance parameters, in the present study. Thus, the diet composition of these group of fishes is crucial and the energy content of the formulation is an important factor that could change the metabolism of the species. It should be bear in mind that gender is also important for the energy content of the fish.

The results obtained in this study allow us to demonstrate that regardless of gender, dietary energy levels effect the final weight of the rainbow trout. According to the thermodynamics mecanisms mechanisms in animals, the part of the metabolized energy intake which is lost as heat energy can not contribute to whole-body nutrient/energy gain. This energy might be invested in reproduction which is a critical part of the production cycle for farm fish and it involves critical changes in energy acquisition in fish. Even though we could not detect any significant difference in both genders, it was obvious that female fish was slightly heavier than male counterparts. Interestingly, we found a significant difference in SGR and female fish clearly had higher SGR than male fish. When dietary nutrient/energy supply is not sufficient during gametogenesis, most of the body energy stored in the eggs (8%-15%) typically represents 30% energy of the whole body (Dygert et al., 1990). In the present study, the energy content of the ME and LE diets were respectively 18.4 kJ/kg and 17.7 kJ/kg. Early studies on the energy requirements of rainbow trout broodstock exhibited that diets containing high digestible energy (>17 Mj/kg) were enough to ensure reproductive investment and good quality gametes (Takeuchi et al., 1981; Watanabe et al., 1984). At the end of our study, the gonadal maturation was observed in both gender fed with experimental diets which shows that the energy content of the experimental diets ensured reproductive success in the present study. Thus, the growth performance data as final weight (more than double) and SGR can be attributed to the energy expenditure of the fish which reach the gonadal development leading the main energy to the reproductive performance rather than meeting energy requirements for growth.

Given feed intake observed in the present study, ME and LE diets were identical in terms of digestible energy which is generally considered a major factor controlling feed intake in fish.

The experimental fish were fed by hand according to their voluntary feed intake until they stopped eating. Thus, the fish in the present study were capable of adjusting their feed intake (average feed intake in LE and ME, 297.6 g to 306.7 g feed / fish, respectively). In this approach, fish can adjust their overall feed intake to maintain a given level of energy intake when offered diets with varying digestible energy levels (Houlihan et al., 2001). Fish are able to regulate their feed intake in accordance with dietary energy levels whether fed till satiation, as in the current study, or by demand feeders, the previous authors also noted.

All these energy balance mechanisms, with respect to feed intake and growth performance in the present study, were reflected in HSI and VSI, irrespective of genders. We found that there were no significant differences in these important morphological parameters. Notably, the females fed on LE diet shown highest VFI compared to their counterparts in the present study. As known, HSI, VSI and VFI are strongly genetically and phenotypically correlated with hepatic glycogen in metabolism, particularly in the body (Zhao et al., 2021). Based on that it can be hypothesized that high visceral fat may result in poor commercial value (Wang et al., 2017; Wang et al., 2005). Notably, HSI is positively related to dietary lipid levels (Cao et al., 2019; Han et al., 2014; Li et al., 2020a, Li et al., 2020b; Wang et al., 2017). The VSI is used to determine the rate of fat accumulation in the internal organs of fish. At the end of the experiment, VSI values were not significantly different in terms of FCR and VSI between diets.

Based on WG, the optimal dietary lipid requirement of juvenile A. schlegelii is 12.82%. However, when dietary lipid levels contain 18% or beyond, the excessive dietary lipid can reduce growth performance, cause pathological changes in the liver, and induce lipid deposition, consequently, triggering inflammation and apoptosis in A. schlegelii. The findings of this study provide further insight into the differential regulatory effects on lipid metabolism and physiological responses of marine fish fed with optimal or excessive dietary lipid levels and supply a theoretical basis for marine fish feed production. In this study, the lipid content in ME group (average lipid content was %8.6), appeared to be approximately 11.5% higher compared with the LE group. It is also important that the lipid content of the males fed with ME and LE diet was higher than females which is in accordance with the general lipid metabolism of the fish. Jøsrgensen et al (2005) exhibited that liver lipid content in female Arctic charr increased from 3.5% to 14.7% during ovarian development. On the other hand, dry matter content was statistically lower in ME than LE . Comperable results were recorded in whole-body ash contents that varied from 0.43±0.02 to 0.47±0.04%. According to the current results, there is no discernible relationship between the ration level and fillet yield, and this is consistent with the conclusions made by Wathne et al. (1995) in large Atlantic salmon.

A further indication of the present study is that rainbow trout may be more prone to changes in body proximate composition. Dry matter is closely and positively associated with lipids (e.g., Weatherley and Gill, 1983) and moisture, therefore, decreases in the fattier, fast-growing fish. The lower ash percentage in the body of fast-growing fish is probably a result of relatively low skeletal growth (negative allometry) compared to other tissues such as visceral fat. The larger visceral fraction in the fast-growing fish is likely to be explained by a higher deposition of excess energy in the viscera (Weatherley and Gill, 1983a) and confirms other studies on rainbow trout (Weatherley and Gill, 1983a; Storebakken et al., 1991).

In the present study, dietary lipid levels and fatty acid profile of the experimental diets were identical as shown in Table 4.4. Thus, there was only a slightly difference found in MUFA in the experimental diets. However, the rest of the fatty acid profiles in the diets were similar. For instance, the LC-PUFA content of the fillets in females in both experimental groups was higher than males whereas no statistical difference was found. On the other hand, MUFA content tended to increase in male fish fed with the ME and LE diets and differences were significant. However, a significant interaction between dietary energy level and interaction was shown for 18:3n-6. Surprisingly, the content of DHA (22:6n-3) was not affected by the dietary treatments, which can be explained by the abundance of n-3 PUFA in the diets supplied by the fish oil and fish meal in a smaller amount.

In the present study, the EPA and DHA content of the fish in both genders, was not significantly different from each other. However, the DHA content of male fish was found lower (15%) than male counterparts, suggesting that male fish used this specific fatty acid in sperm production. As known that EPA, specifically DHA, is recognized as a key structural component imparting high fluidity to sperm cells (Collodel et al., 2020; Roy et al., 2024). Phospholipids of most freshwater fish sperm (membranes) incorporate high DHA levels (Engel et al., 2019). Interestingly, Henrotte et al., (2010) speculate that EPA + DHA and/or the other (ARA), but both LC-PUFAs ‘fortified together’, could have brought a unit change in sperm quality. This is clearly in line with our whole body LC-PUFA finding which was significantly higher in male fish fed with LE group. However, there is a need to understand of interaction between sperm quality (specially fatty acid content) and the energy levels of the fish.

It is well documented that dietary highly unsaturated fatty acids (HUFAs), especially EPA and DHA are needed for proper growth and development of fish need and lack of these EFAs can lead to higher fish mortality (Koven et al., 1992; Takeuchi et al., 1990). Studies have found that dietary n-3 HUFA can regulate lipid accumulation in organisms, including whole-body lipids, liver lipids, and lipid-related metabolic enzyme activity in the liver (Ji et al., 2011; Tian et al., 2014; Han et al., 2013). In the present study, the LC-PUFA content of the female fish was higher than male counterparts where significant differences were observed in gender. However, the EPA and DHA content of the fish fed with either ME and LE did not differed from each other. It appears logical that a fraction of the ingested “excess” energy is deposited as lipid, and therefore increasing the feeding level leads to more fatty fish (Reinitz, 1983; Storebakken and Austreng, 1987; Storebakken et al., 1991). However, some studies have indicated that this is not necessarily so (Alsted, 1991; Tidwell et al., 1991; Azevedo et al., 1998). According to Shearer et al (1997) there is a tendency for ration to determine growth and dietary lipid to control body fat which reflects the fatty acid content of the fillet. Regardless of the results obtained, a general problem in studies within the field is the lack of awareness of the fact that body lipid increases with the size of the fish (e.g., Denton and Yousef, 1976). In our study, the size of the fish was around the marketable size which is affected by the lipid, thereby fatty acid content. Earlier studies have reported that female fish milt is vulnerable to oxidative stress thanks to the PUFA present in the spermatozoa membrane (Bozkurt and Yavas, 2021). So, it is noteworthy that the n-6 PUFA, n-6 LC-PUFA and LCP-UFA content of the fish fed with both experimental diets showed lower content compared to female counterparts. Even though we did not analyze milt of the fish, we might clearly see some of the female fish were mature and produced sperm.

The gender-significant difference in triglyceride levels was evidently observed in the current study. Indeed, in this study, the contents of serum TG and CHO increased markedly with the increase of dietary lipid levels, indicating that even though lipid content of the experimental diets slightly differed from each other even this lipid intake level could compromise the ability of the liver to secret lipid to the circulatory system. Moreover, there were no significant different effects of the energy levels of the diet on these two important blood parameters. It is believed that increasing TG and CHO concentrations in serum might be a symptom of poor health status in fish (Kikuchi et al., 2009), and both serum TG and CHO contents showed a positively connected with dietary lipid levels (Cao et al., 2019; Du et al., 2005; Li et al., 2020a, Li et al., 2020b). To the authors' knowledge, when fish fed with the optimal dietary lipid level could enhance antioxidant capacity, in turn, excessive dietary lipid intake could induce oxidative stress, which has also been confirmed in some previous studies (Pan et al., 2018; Wang et al., 2019).

In the current study, there was only one interaction effect was found between gender and diet in AST and Albumin while there was not any clear interaction trends in any other blood parameters at the end of the study. The contents of aminotransferase enzymes (AST and ALT) in serum were increased with the increase of dietary lipid/energy levels 17.7 kJ/kg (LE) and 18.4 kJ/kg (ME), particularly in male fish, which can be attributed to that excessive dietary lipid intake of male fish could cause tissue damage. When the liver is damaged or stress factors (environmental stress and dietary stress), are increased in the body, the release of aminotransferase enzymes into the circulation increases (Vadboncoeur et al., 2023). Furthermore, AST and ALT are functioning in parenchymal cells in the liver and both of their activities in serum are usually used for diagnosing liver function and health (Metón et al., 1999; Wang, 2014; Schomaker et al., 2020). A deficiecy in dietary lipid levels could also result in a liver injury by increasing AST and ALT concentrations, which have been confirmed in *Ctenopharyngodon idella* (Jin et al., 2013). Aminotransferase enzymes (AST and ALT) are found in high concentrations in liver cells, whereas they are lower in blood serum (Kusatan et al., 2004). In another study, cypermethrin was reported to raise plasma AST levels, but ALT levels did not change significantly (Velisek et al., 2012). Another damage indicator is alkaline phosphatase in the present study was not markedly impacted by dietary energy level. There was not any clear trend in both male and female fish. As it is known that alkaline phosphates belong to the non-plasma-specific enzyme which is located within tissue cells (intestine liver etc.) and has no known physiological function in plasma (Hemre and Sandnes, 1999; Jiang et al., 2015). Meng et al (2019) postulated that triploid rainbow trout fed with different levels of dietary lipid (from 6.6% to 29.4%) showed no significant effects on plasma ALT, AST and alkaline phosphates. Our findings also support this, except ALT and AST.

Changes in liver histology of the rainbow trout fed with ME and LE were observed but the overall structure and morphology of the liver tissues were considered normal as shown in Figure 4.2. There was no observed lipid accumulation among the treatments and the number and size of clear lipid vacuoles within hepatocytes between male and female individuals. However, these lipid vacuoles were uniform within each section from the same fish and group, being in line with findings in salmon fed with different EPA and DHA levels (Lutfi et al., 2023). During the evaluation of the histological examination, none of the samples scored 5 (excess lipid accumulation within the groups and/or any huge enlargement of all vacuoles). It is worth noting that the fish in the present study fed almost the same lipid levels however the energy levels were slightly differing from each other. Thus, it was evident that both diets did not severe effect on the histology of the liver. According to these findings, measurement of liver health scores and external welfare markers (liver color, bleeding injury, etc.) also revealed a tendency toward improved well-being with higher dietary EPA and DHA intake as stated in Morkore et al (2020).

The most notable effect of experimental diets upon the expression levels of pro-inflammatory cytokines including TNF-α, IL-1β, IL-18 and glucokinase which were up-regulated with the increasing of dietary energy levels. Also, IL-1β, IL-18 and TNFα are key mediators of the inflammatory response (Baud et al,. 2001; Bird et al,. 2002; Fast et al,. 2007) and the expression of such genes is induced by lipopolysaccharide-LPS (Fujiki et al,. 2000; Saeij JPJ et al,. 2003). These findings suggested that long-term feeding medium energy contents (ME) in diet could cause inflammation, whereas when fish fed with LE level could enhance the anti-inflammatory ability.

In this respect, among the most important cytokines, interleukin-1β, which has potent proinflammatory effects, stimulates and initiates the immune response associated with innate immunity to microbial invasion and tissue injury (Ederet et al., 2009). As known, it is produced by a wide range of cell types after activation of the pattern recognition receptors (PRRs) (Rebl et al., 2010). In our study, even though the energy levels were not drastically changed, hepatic IL-1β activity was significantly higher in liver LE female at the end of the experimental period. These findings suggested that the LE diet induced chronic stress, which probably could increase the inflammatory response of experimental fish.

One of the most important adipokines secreted from adipose tissue is tumor necrosis factor alpha (TNF-α). The TNF-α is a pro-inflammatory cytokine whose expression is elevated following injury or infection. Expression of TNF-α has been used as a reliable indicator for up-regulation of the innate immune response (Secombes et al., 2001) and for tissue damage (Cho and Willuweit, 2001). In the present study, an increase of TNF-α expression in the liver of fish fed with LE exhibited an increasing trend compared with those fed with ME diets. This means that it enhances the anti-inflammatory ability. The elevated expression of TNF-α found in this study provides evidence of cellular damage occurring in rainbow trout. These findings suggested that the LE diet caused an increase in adipose tissue mass. Moreover, the expression and production of TNF-α increases with the augmented adipocyte size (Morin et al., 1995). TNF-α affects adipocyte function in rainbow trout and gilthead sea bream and inhibits the differentiation of rainbow trout preadipocytes (Albalat et al., 2005b; Bouraoui et al., 2008; Saera-Vila et al., 2007). Thus TNF-α may be a potent regulator in reducing the adipose tissue mass. In addition, an important TNF-α isoform type II TNF-α3 is present in rainbow trout, which is the most responsive gene at early time points of post-lipopolysaccharides stimulation (Hong et al., 2013). TNF-α directly alters lipid metabolism through inhibition of fatty acid uptake and lipogenesis as well as stimulation of fatty acid release via lipolysis. TNF-α plays an important role in the control of lipid metabolism in rainbow trout (Albalat et al., 2005a, Albalat et al., 2005b).

Interleukin 18 (IL-18) is a pleiotropic proinflammatory cytokine whose levels increase during or following infection. IL-18 is produced by different cell types in the periphery; and centrally it is present in microglia, ependymal cells, and neurons of the medial habenula (Alboni et al., 2009, 2010, 2011). In this study, IL-18 was significantly increased in the male fish fed with ME was found. These results suggest that the ME diet caused the increased inflammatory response in experimental fish. IL-18 regulates and manages Th1 cell differentiation (Secombes et al,. 2008). As a stimulator of T helper 1 cell and of natural killer cells, IL-18 is a powerful mediator of inflammatory-associated tissue damage and has antimicrobial and antitumor activity (Dinarello et al,. 1998; Kawakami et al,. 1997). IL-18 also participates in the pathogenesis of autoimmune diseases including diabetes mellitus, rheumatoid arthritis, psoriasis and multiple sclerosis (Nakanishi and Suk, 2001), (Wildbaum et al,. 1998; Fassbender et al,. 1999). IL-18 is produced as an inactive precursor and is secreted as an active cytokine upon maturation by the interleukin-1β converting enzyme, ICE or Caspase 1 (Akita et al,. 1998; Ghayur et al,. 1997; Ushioet al,. 1996).

The glucokinase (GK) enzyme, also known as hexokinase (HK) IV (or D), is one of the four glucose-phosphorylating isoenzymes described initially in the vertebrate liver, characterized by a low affinity for glucose, sigmoidal kinetics and lack of inhibition by glucose-6-phosphate. The others, being called HK I (or A), II (or B) and III (or C), are more widely distributed (Cárdenas, et al,. 1998; Wilson et al,. 1995) hexokinases. Fish is a quite particular group regarding the study of glucokinase. Glucose appears to be a minor energy fuel in fish (Moon et al,. 2001; Polakof et al,. 2012). The presence of hepatic GK was later confirmed at a biochemical level in different fish species such as rainbow trout, gilthead sea bream, European sea bass (*Dicentrarchus labrax*), perch (*Perca fluviatilis*) and common carp (Panserat et al,. 2000; Enes et al,. 2006). In our results, appears the increased glucokinase gene expression in the liver of fish fed with LE, although the ME diet glucose content was n.s. Although glucokinase expression is high when there is a high level of glycemia in all fish species, the reverse is not true, at least in gilthead seabream. Studies have shown that there are differences in glucose tolerance tests between fish species; between carnivorous salmonids (low tolerance) and omnivorous cyprinids (tolerant); between coldwater fish and warm water fish (Polakof et al,. 2012; Wilson et al,. 1994). Thus hepatic glucokinase is probably neither the limiting step explaining the low dietary glucose utilization nor the major factor maintaining glycemia at low values in rainbow trout. Finally, the regulation in vivo by the quality of dietary lipids (different levels of highly unsaturated fatty acids, i.e. DHA) and dietary proteins (different levels of target amino acids such as glutamate, alanine and aspartate) also did not seem to affect dramatically GK expression and activities (Tapia-Salazar et al,. 2006; Kirchner et al,. 2003).

6. CONCLUSION

- Overall, fish when fed the diet supplemented with appropriate lipid levels could obtain the best growth performance and antioxidant capacity, promote lipolysis by activating the expression levels, and maintain normal physiological functions via improving antioxidant, anti-inflammatory and anti-apoptotic abilities.
- In conclusion, We demonstrated that serum AST and ALT levels significantly changed in a dose-dependent manner. Additionally, research indicates that the liver tissues of rainbow trout exhibit notable alterations in the expression of IL-1β, TNF-α, and glucokinase genes.
- Rainbow trout is a good source for protein and fat because of their high contents of protein and high proportions of essential fatty acids. Reduced growth performance in low lipid levels may be due to insufficient digestible energy or deficiencies in essential fatty acids.
- The results provide more insight about the the role of cytokines in cutaneous tissues and various physiological and pathological processes. This has not only helped in the understanding of the etiopathology of various disease processes procedures, as well as in creating different biologicals to treat these illnesses.
- In conclusion, this study shows that IL-1β, TNF-α and IL-18 are differently expressed in rainbow trout when fed two diets. The expression of these genes could be crucial for the recruitment of relevant immune cells necessary for the initiation of the immune reactions needed to clear the infection.

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