UNIVERSIDADE D
COIMBRA

Luís António Fonseca Henriques

# EFFECTS OF GLYCEROL SUPPLEMENTATION ON MUSCLE LIPIDS IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

**Thesis presented in the scope of the Master degree in Ecology, supervised by Doctor Ivan Daniel dos Santos Martins Viegas and Professor Doctor Miguel Ângelo do Carmo Pardal, submitted to the Faculty of Science and Technology of University of Coimbra Department of Life Sciences.**

July of 2019

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# Resumo

Em aquacultura, existe um grande interesse em encontrar substitutos/suplementos que possam reduzir a dependência de farinha de peixe, e ao mesmo tempo possam ser usados como um meio para potencializar a produção. A truta arco-íris (*Oncorhynchus mykiss*), sendo um peixe com grande importância em aquacultura e apresentando uma alta resiliência em cativeiro, é um dos modelos mais estudados ao nível metabólico. O glicerol tem sido estudado como suplemento em muitos peixes com esse mesmo objectivo. Para que este ingrediente seja viável, o peixe tem que ser capaz de manter um crescimento positivo, uma boa composição nutricional e fluxos metabólicos sem prejuízo ao organismo. Tivemos como objetivo principal avaliar a utilização de glicerol na dieta da truta arco-íris como meio de suplementação. Para este objetivo, os peixes foram submetidos a um ensaio de crescimento utilizando dietas contendo 0% (CTRL), 2,5% (GLY 2.5) e 5% de glicerol (GLY 5), sendo o desempenho zootécnico avaliado. Em seguida um ensaio metabólico consistindo numa residência de 6 dias em 4% $^{2}H_2O$. Mais especificamente, avaliamos o impacto do glicerol no triacilglicerol (TAG) muscular, em termos de composição (avaliada por $^{1}H$ RMN) e fluxo metabólico (seguindo o enriquecimento de $^{2}H_2O$ por $^{2}H$ RMN) permitindo a avaliação da lipogénese *de novo* (DNL) no músculo. Analisando os parâmetros de crescimento as únicas diferenças significativas foram o aumento da taxa de conversão alimentar (FCR) e a diminuição da eficiência de ingestão (FE) no tratamento mais extremo (GLY 5). O perfil de FA mostrou apenas diferenças no MUFA entre s os tratamentos de GLY 2.5 e GLY 5, o último tendo valores mais elevados, no caso do ácido linoléico foram encontradas diferenças estatísticas entre o tratamento com GLY 5 e os outros dois tratamentos. Não houve diferenças estruturais nos ácidos gordos entre os tratamentos. Nos fluxos metabólicos mesmo que tenham sido observadas alguma variabilidade, não houve nenhuma diferença estatisticamente significativa. A suplementação de glicerol não apresentou efeitos adversos em qualquer parâmetro zootécnico ou de fluxo metabólico de TAG no músculo, provando um bom desempenho da truta arco-íris até 5% de inclusão de glicerol, sendo um bom meio para reduzir a utilização catabólica de aminoácidos, potenciando assim o crescimento e reduzir o custo da ração.

**PALAVRAS-CHAVE**: Truta arco-íris, músculo, lipogénese *de novo*, RMN, triacilglicerol, ácidos gordos, glicerol.

Effects of glycerol supplementation on muscle lipids in rainbow trout (*Oncorhynchus mykiss*)

# Abstract

In aquaculture, there is a high interest in finding substitutes/supplements that can reduce dependence on fishmeal, and which can be used as a mean to potentiate production. Rainbow trout (*Oncorhynchus mykiss*) is a highly important species in aquaculture for its resilience in a farming setting, being also one of the most fish models studied in many metabolic studies. Glycerol has been a supplement studied in many aquaculture fish used to spare carbohydrate utilization. In order for this substitute to be viable fish have to be able to maintain a positive growth, a good nutritional composition and metabolic fluxes without damaging the organism. We aimed to evaluate the utilization of dietary glycerol as a mean of supplementation in rainbow trout feed. For this objective, fish were subjected to a growth trial using diets with 0% glycerol (CTRL), 2.5% glycerol (GLY 2.5) and 5% glycerol (GLY 5) where the zootechnical performance under the dietary treatment could be assessed. This was followed by a metabolic trial which consisted in a 6-day residence in 4% $^{2}H_2O$. More specifically, we aimed at evaluating the impact of dietary glycerol on muscle triacylgycerol (TAG), in terms of composition (as evaluated by $^{1}H$ NMR) and metabolic flux (following the enrichment from $^{2}H_2O$ by $^{2}H$ NMR) allowing evaluation of *de novo* lipogenesis (DNL) in muscle. Of the growth parameters analysis only the feed conversion ratio (FCR) and food efficiency (FE) showed significant differences in the most extreme diet (GLY 5), treatment increasing FCR and decreasing FE. The fatty acid (FA) profile showed only differences in the MUFA when comparing the 2.5% and the 5% treatment, the latter having higher values, and in the case of the linoleic acid statistical differences were found between the 5% treatment and the other two treatments. Relative to the structure of the FA there were no differences in the data showed between the treatments. The metabolic fluxes, the differences showed that even if some variability was observed there were non-significant between treatments. The supplementation of glycerol did not present adverse effects in any zootechnical or metabolic flux parameters, proving a good performance of the rainbow trout up to 5% inclusion of glycerol, being a good way to reduce the catabolic use of amino acids, thus enhancing the growth and reduce the cost of the feed.

***KEYWORDS**:* Rainbow trout, muscle, *de novo* lipogenesis, NMR, triacylglycerol, fatty acids, glycerol.

Effects of glycerol supplementation on muscle lipids in rainbow trout (*Oncorhynchus mykiss*)

# Abreviations

$^{1}H$ – Hydrogen

$^{2}H$ – Deuterium

$^{2}H_2O$ – Deuterated water

DHA – Docosahexaenoic acid

DNL – *de novo* lipogenesis

EPA – Eicosapentaenoic acid

FA – Fatty acids

fBW – Final body weight

FCR – Feed conversion ratio

FE – Feed efficiency

FER – Feed efficiency rate

FI – Feed intake

HSI – Hepatosomatic index

iBW– Initial body weight

MUFA – Monounsaturated fatty acids

NMR – Nuclear magnetic resonance

PER – Protein efficiency ratio

PUFA. – Polyunsaturated fatty acids

SFA – Saturated fatty acids

SGR – Specific growth rate

TAG – Triacylglycerol

UFA – Unsaturated fatty acids

WG – Weight gain

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# 1. Introduction

## 1.1 Overview

The 2030 Agenda, is a set of 17 Sustainable Development Goals created by the United Nations to raise sustainability, end poverty, promote prosperity and well-being for all, protecting the environment and combating climate change. Among those goals aquaculture, can be inserted in the ones relative to food consumption (e.g. Goal 3 and 12), poverty (e.g. Goal 1 and 8), and environment enhancement (e.g. Goal 6 and 14), since this activity is a good source for food supplies, can generate employment and it can help to reduce the dependence on the oceans for seafood (Fig. 1). The Food and Agriculture Organization (FAO), a specialized agency of the United Nations, leads international efforts to defeat hunger and achieve food security, especially where the population is predicted to grow rapidly in the years to come, helping to achieve the 2030 Agenda. In the State of World Fisheries and Aquaculture (FAO 2018), a FAO report, it was noted that over the years high demand for fish has led to over-exploitation of fish stocks, leading to the consequent decline in the ability of these stocks to restore their natural resources.


Fig. 1 The 2030 agenda 17 sustainable development goals (www.un.org)

Aquaculture has become a crucial activity, as high demand for seafood cannot be met with capture fishing alone. Since the 1990s, fishing productions have remained stable, but in the same period and contrary to previous ones, aquaculture productions have steadily increased (Fig. 2), being the fastest growing food production sector in the world for more than four decades (Asche et

al., 2012). Seafood is also a reliable income source and a main source of protein globally, mainly in developing countries promoting economic growth and social stability, in highly populated areas like Southeast Asia. Nowadays, about 44% of the seafood for human consumption comes from aquaculture and this number is expected to reach 55% by the year 2025 (FAO 2018).

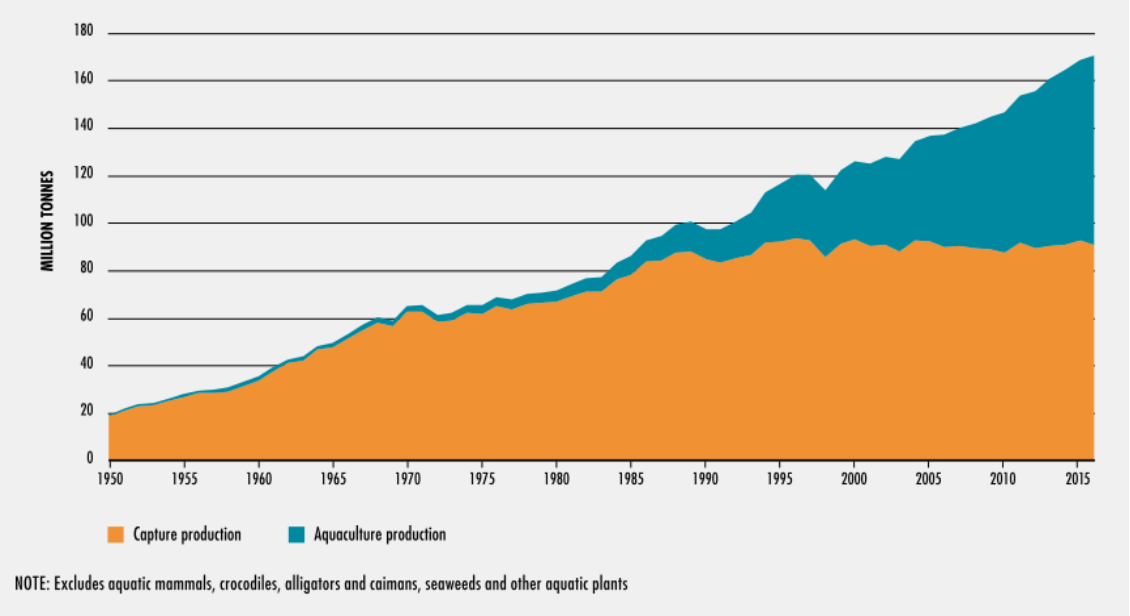



Fig. 2 World capture fisheries and aquaculture production since 1950 (FAO 2018)

In 2016, marine food production reached 171 million tons, with aquaculture accounting for more than 50% of total production, exceeding fisheries production for the first time (FAO 2018). Despite the high growth margin of other species, such as seaweed and aquatic invertebrates, the majority of aquaculture-driven species are still crustaceans, molluscs and fish. Seafood is of crucial importance for human consumption, providing about 3.2 billion people with almost 20 percent of their average per capita intake of animal protein (FAO 2018). The average world consumption of fish per person of 21.2 kg/capita/year, is expected to reach 22 kg/capita/year by the year 2025. Although consumption in Europe ranges from 55.9 kg/capita/year in Portugal to 4.8 kg/capita/year in Hungary, the average European consumes 25.2 kg/capita/year, 4 kg/capita/year more than the world average (Fig. 3). Also of paramount importance is the nutritional value of the fish as it provides a high content of vitamins (A, B and D), minerals (calcium, iodine, zinc, iron and selenium), essential fatty acids (FA) (particularly long-chain omega-3 FA) and high-quality digestible protein (Bogard et al., 2015), which can help to prevent cognitive decline (Morris al., 2015), and also reduce heart disease and high blood pressure. The omega-3 FA with which fish are known to be rich on, help to prevent inflammatory diseases due to its cardiovascular action (Grigorakis et al., 2007; Lund et al., 2013), as well as to promote infant and fetus development, which is highly recommended for pregnant women.

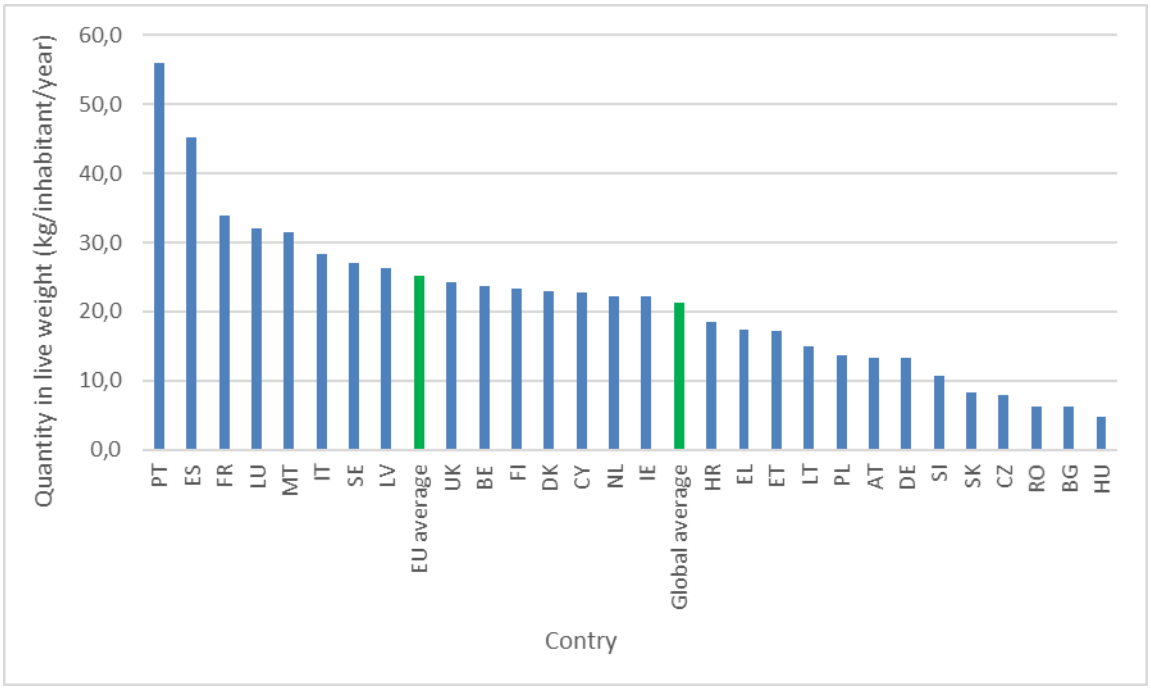



Fig. 3 Consumption of fisheries and aquaculture products (kg/capita) in Europe (2015): Portugal (PT), Spain (ES), France (FR), Luxembourg (LU), Malta (MT), Italy (IT), Sweden (SE), Latvia (LV), United Kingdom (UK), Belgium (BE), Finland (FI), Denmark (DK), Cyprus (CY), Netherlands (NL), Ireland (IE), Croatia (HR), Greece (EL), Lithuania (LT), Estonia(ET),Poland (PL), Austria (AT), Germany (DE), Slovenia (SI), Slovakia (SK), Czech (CZ), Romania (RO) Bulgaria (BG), Hungary (HU). (Source: www.ec.europa.eu/fisheries/6-consumption_en)

Despite its importance for food supply, the main problems of aquaculture are the use of invasive species, water pollution and the use of wild fish in feed for cultured fish. The incorporation is predominantly as fishmeal and fish oils, as they have a high amount of protein and lipids that are an important requirement to extremely carnivorous fish that require a constant energy to grow, develop or reproduce. Because of an even greater exploitation of fish stocks, the already overfished oceans can suffer even more stress. Fish feed reliance can also generate high amounts of ammonia through degradation of amino acids, which can lead to the risk of eutrophication and consequently damaging the environment. It is also very expensive, as up to 60% of total operating costs can come from feed costs, as any improvement can have a significant reduction in this value. Aquaculture also has its advantages. In the future it will ensure a continuous supply of food. Methods of production show a great deal of flexibility as they can go from the use of fish tanks and cages on the seafloor or hanging in the water column to artificial breeding ponds and cages in lakes or rivers. This also requires a high level of maintenance and manpower and will open up job opportunities.

Aquaculture will be common around the globe, as many communities, primarily in underdeveloped countries, depend on this meat supply and revenue. Although this industry has

disadvantages, developments have also been made to mitigate the concerns raised by aquaculture, such as the search for a good way to boost aquaculture by using a good substitute for fishmeal and fish oils. If these conditions are met, aquaculture can contribute positively to global food security (Bene et al., 2015).

## 1.2 Rainbow trout (*Oncorhynchus mykiss*)

*Oncorhynchus mykiss* (Walbaum, 1792), commonly named rainbow trout (after the many spots on its skin - Fig. 4), belongs to the family of salmonids and is a carnivorous fish. It is native to the Pacific Ocean, from Alaska to Mexico in North America, introduced worldwide for recreational and aquaculture purposes since 1874. Since the development of pelleted feed in the 1950s, its production has expanded considerably and has been maintained and grown around the globe in many countries.

Nowadays, it is farmed almost all over the world in temperate to cold waters, because it is a species with a well-studied and controlled life cycle. It has a rapid growth rate that can grow to 350 g in 10 to 12 months, tolerates a wide range of environmental parameters, easily adapts to artificial diet and has an optimal culture temperature of 21°C.

Fig. 4 Rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792).

Total world production of rainbow trout from aquaculture increased 22% from 2007 to 2016 mainly due to Iran, Turkey and Norway production. The 2016 European production of rainbow trout reached 308,130 tons, providing approximately 23% of total aquaculture production (2016), which was a decline from the 2007 total production of 31%, mainly due to the decline in production from Italy (-7%), France (-19%) and Spain (-31%) (from European Market Observatory for fisheries and aquaculture accessed at http://www.eumofa.eu/documents in April 21, 2019). Norway keeps at the forefront with 87,775 tons, followed by Italy, Denmark and France as the main European producers and although Portugal has great potential to increase its production, this is negligible compared to the major producers. The national total production is around 675 tons (Fig. 5).

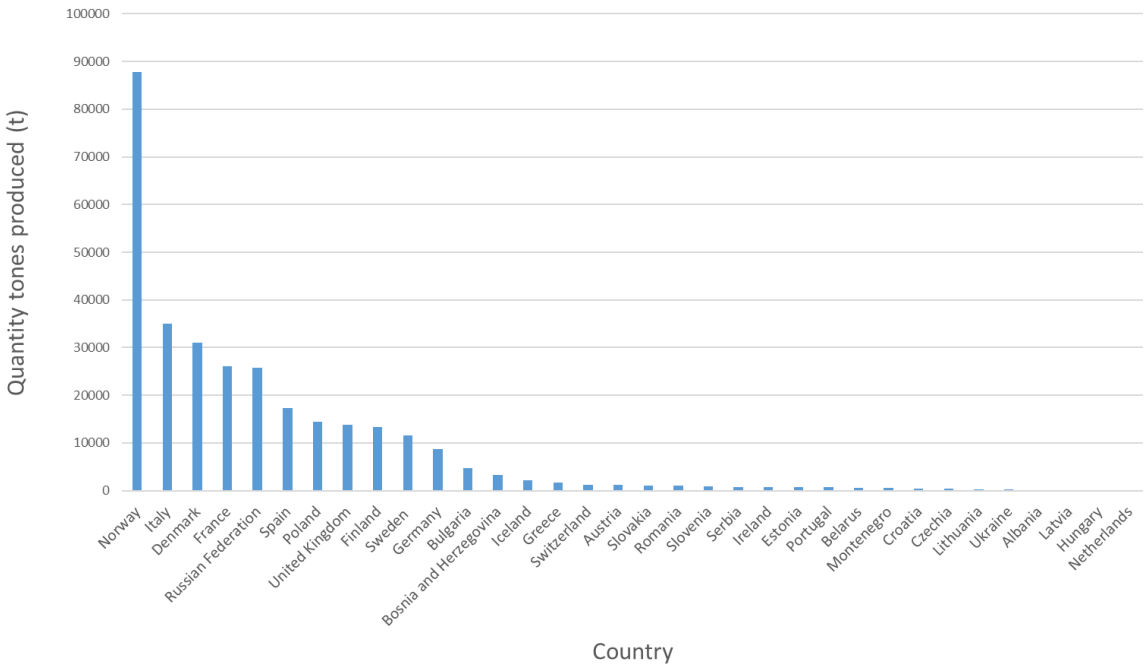



Fig. 5 Europe 2016 production of rainbow trout in tons (t) by country (January 3, 2019, from EUROSTAT accessed at https://ec.europa.eu/eurostat in DATE

Rainbow trout is a useful model for research, being a carnivorous fish, it has elevated need for dietary proteins. This elevated need for development energy can be obtained from 3 kinds of monomers, amino acids, fatty acids and glycerol. Dietary protein represents 36-38% of the diet for rainbow trout when an adequate supply of non-protein digestible energy is present (Cowey et al., 1994). Mammals were subjected to an evolutionary path where they were exposed to diets with marked carbohydrate levels, fish were not. Common pathways regulation, specific substrates and the quantities required, differ from mammal to fish (Wade et. al, 2014). There is a high interest in study carbohydrates in fish nutrition as a supplement that would spare protein catabolism for plasma glucose levels. This high digestion of dietary protein, leads to the oxidation of a large portion of amino acids, and subsequently to a high production of ammonia being excreted in form of ammonium ion across the gills and in urine (Kawshik et al., 1991). This ultimately leads to a high production of nitrogen waste, influencing water quality in aquaculture leading to pollution and eutrophication.

Efforts have been made to limit this pathway and improve protein utilization by optimizing the protein supply. In rainbow trout this has been achieved by increasing the dietary energy supply optimizing the overall protein supply meeting the quantitative needs for the indispensable amino acids (Kaushik and Seiliez 2010). The ideal indispensable amino acids profile is the one that resemble the whole body of the fish (Wilson and Cowey 1985).

Optimal ratio between indispensable and dispensable amino acids for rainbow trout appears to be 55:45 for this species (Green et al., 2002). Like in mammals the catabolism of fatty acids is the major source of energy in rainbow trout through β-oxidation in peroxisomes, which generates AcCoA being subsequently metabolized via the krebs cycle. Concerning fatty acid retention, high concentration of fatty acid in the diet leads to lipid retention, with the exception of DHA that is preferentially retained in the tissues (Thanuthong et al., 2011). Rainbow trout utilization of dietary carbohydrates is limited, and glucose intolerance phenotype is showed after carbohydrate intake (Legate et al., 2001). For long different sources of substitutes to carbohydrates have been used as a good strategy to decrease levels of fishmeal and fish oil in aquafeeds, carbohydrates incorporation this has been studied in rainbow trout (Panserat, 2009; Wilson, 1994).

In any tissue glucose is used as an energy source. When in excess the glucose will either be converted into fatty acids in liver and adipocytes or stored as glycogen in muscle and liver. All enzymes of glycolysis have been detected in rainbow trout tissues. Dietary carbohydrates such as starch are effectively digested reaching more than 90% digestibility after feed extrusion and gelatinization by rainbow trout (Krogdahl et al., 2005). Glycerol appears to be a good substitute for the use of carbohydrates as supplement in fish feed. Nevertheless its impact on lipogenic turnover, as well as its influence on the types of triacylglycerol (TAG), intended for human consumption that can cause impacts at a nutritional level are yet to be studied.

## 1.3 Glycerol

Glycerol (also known as 1,2,3-propanetriol or glycerine) is a simple water-soluble, almost colourless, odourless, viscous, hygroscopic 3-carbon compound present in all organisms. It is involved in various processes of metabolism and physiology, acting for example as a structural backbone in all TAG lipids.

Glycerol is currently produced as a by-product of biofuel production. In Europe, this production amounted to 21 million tons in 2017. Although several alternative uses have already been developed, such as cosmetics and pharmaceuticals, most of it is still disposed of without any use, making it an available and affordable compound if added value is created from it (Pagliaro et al., 2007).

Depending of the purification level glycerol can be characterized as crude glycerol, refined glycerol and commercial synthesized glycerol. Refined glycerol is used in medicine, food and cosmetic products and presents a purity level generally close to 100%. Purified glycerol has tree grades. Grade I 95% pure, is suitable for preparation of chemicals and as a synthetic preparation. Grade-II 96–99.5% pure is suitable for food products, pharmaceuticals and cosmetics, is prepared from animal fat or plant oil sources. Grade-III 99.5–99.7% pure is suitable for use in kosher foods and drinks prepared from plant oil sources, (Kenkel and Holcomb, 2008)

Glycerol is currently produced as a by-product of biofuel production about 10% of the total production 60%~80% purity. With the increase of biodiesel production as alternative to the current use of fossil fuel, so did the glycerol production increased. In Europe, this biofuel production amounted to 21 million tons in 2017. With the excessive production of glycerol other alternatives to the current uses have been researched. Since European food safety authority (EFSA) and U.S. food and drugs administration (FDA) approved it as a food additive, it has been primarily explored as a source of food supplement for farm animals as an alternative dietary energy source (Lammers et al., 2008). It was initially proposed as a potential source of energy for swine (Lammers et al., 2008), cattle (Carvalho et al., 2012; Donkin, 2008) and poultry (Freitas et al., 2017), and in recent years has fish feed supplement to substitute the use of fishmeal and fish oils. Some examples of aquaculture fish species growth that have been studied with the addition of glycerol, are catfish (*Ictalurus punctatus*) (Li et al., 2010), European seabass (*Dicentrarchus labrax*) (Rito et al., 2019), gilthead seabream (*Sparus aurata*) (Silva et al., 2012), Nile tilapia (*Oreochromis niloticus*) (Neu et al., 2012; Moesch et al., 2016; Gonçalves et al.,2015; Costa et al., 2017; Neu et al., 2013), Pacu (*Piaractus mesopotamicus*) and Silver catfish (*Rhamdia quelen*) (Balen et al., 2014), rainbow trout (*Oncorhynchus mykiss*) (Menton et al., 1986).

Table 1 – Review table of diet glycerol inclusion studies. Zootechnical parameters.

| Authors (year) | Species | Zootechnical parameters | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | fBW | FER | FCR | FI | SGR | WG | HSI | LSI | PER |
| Li et al. (2010) | Channel catfish (*Ictalurus punctatus*) | -- | ↘ | ↘ | -- | -- | ↘ | ↘ | -- | -- |
| Rito et al. (2019) | European seabass (*Dicentrarchuslabrax* L.) | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Silva et al. (2012) | Gilthead seabream (Sparus aurata) | NS | -- | -- | -- | -- | -- | NS | NS | NS |
| Neu et al. 2012 | Nile tilapia (*Oreochromis niloticus*) | NS | -- | NS | -- | NS | -- | -- | -- | -- |
| Moesch et al. (2016) | | NS | -- | ↘ | -- | NS | NS | ↘ | -- | ↗ |
| Gonçalves et al. (2015) | | -- | -- | ↗ | ↗ | ↘ | ↗ | NS | NS | ↗ |
| Costa et al. (2017) | | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Neu et al. (2013) | | NS | -- | NS | -- | -- | NS | NS | -- | -- |
| Balen et al. (2014) | Pacu (*Piaractus mesopotamicus*) | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| | Silver catfish (Rhamdia quelen) | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Meton et al. (1986) | Rainbow Trout (*Oncorhynchus mykiss*) | -- | -- | -- | -- | -- | -- | -- | -- | -- |

NS – non significant differences; (↘) – decrease; (↗) – increase; (--) – data not show.

fBW - Final body weight; FER - Feed efficiency rate ;FCR - Feed conversion ratio;

FI - Feed intake; SGR - Specific growth rate; WG - Weight gain; HSI - Hepatosomatic index

iBW- Inicial body weight; LSI – Liposomatic index; PER - Protein efficiency ratio.

Table 2 – Review table of diet glycerol inclusion studies. Blood, liver and muscle parameters.

| Authors (year) | Species | Blood Parameters | | | Liver Parameters | | | White muscle Parameters | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Glu | TAG | HDL | Glu | TAG | Enz | Gly | TAG | Enz |
| Li et al. (2010) | Channel catfish (*Ictalurus punctatus*) | ↘ | -- | -- | -- | ↘ | -- | -- | ↘ | -- |
| Rito et al. (2019) | European seabass (*Dicentrarchuslabrax* L.) | NS | -- | -- | NS | -- | -- | -- | -- | -- |
| Silva et al. (2012) | Gilthead seabream (Sparus aurata) | -- | -- | -- | -- | -- | -- | ↗ | NS | -- |
| Neu et al. 2012 | Nile tilapia (*Oreochromis niloticus*) | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Moesch et al. (2016) | | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Gonçalves et al. (2015) | | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Costa et al. (2017) | | -- | -- | -- | -- | NS | -- | -- | ↗ | -- |
| Neu et al. (2013) | | NS | ↗ | NS | -- | -- | -- | -- | ↗ | -- |
| Balen et al. (2014) | Pacu (*Piaractus mesopotamicus*) | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| | Silver catfish (Rhamdia quelen) | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Meton et al. (1986) | Rainbow Trout (*Oncorhynchus mykiss*) | -- | -- | -- | -- | -- | -- | -- | -- | -- |

NS – non significant differences; (↘) – decrease; (↗) – increase; (--) – data not show.

GLU – Glycogen; HDL – High-density lipoprotein; TAG – lipids; Enz – Enzymes.

Table 3 – Review table of diet glycerol inclusion studies, other findings of interest.

| Authors (year) | Species | General paramenters |
|---|---|---|
| Li et al. (2010) | Channel catfish (*Ictalurus punctatus*) | Fillet protein and fat generally decreased and fillet moisture increased as dietary glycerol level increased. |
| Rito et al. (2019) | European seabass (*Dicentrarchuslabrax* L.) | Data indicate that glycerol effectively competes with endogenous precursors for hepatic gluconeogenesis in carnivorous fish thereby representing a novel mechanism for reducing the catabolic utilization of amino acids |
| Silva et al. (2012) | Gilthead seabream (*Sparus aurata*) | Increased muscle glycogen, ATP levels and firmness, with no deleterious effects in terms of growth, proximate composition, fatty acid profile, oxidative state, and organoleptic properties |
| Moesch et al. (2016) | Nile tilapia (Oreochromis niloticus) | Itis possible to replace totally maize by glycerol in pelleted diets for Nile tilapia fingerlings between 10 and 30 g |
| Gonçalves et al. (2015) | | Weight gain fitted a second-degree equation and the optimum inclusion level was estimated at 5.9%, but up to 12% dietary crude glycerin did not impair growth performance of fish |
| Costa et al. (2017) | | Juvenile tilapia are able to metabolize dietary glycerol into lipids, protein and/or carbohydrates and to use it as energy source |
| Neu et al. (2013) | | Can be used in fish diets as an energy supplement without causing damage to growth performance or to the biochemical and carcass composition |
| Balen et al. (2014) | Pacu (*Piaractus mesopotamicus*) | Energy apparent digestibility coefficients ADCs were 0.97, and digestible energy were 15.2 MJ kg-1 |
| | Silver catfish (Rhamdia quelen) | Energy apparent digestibility coefficients ADCs were 0.89, and digestible energy were 13.95MJ kg-1 |
| Meton et al. (1986) | Rainbow Trout (*Oncorhynchus mykiss*) | It was concluded that free glycerol was not an effective source of dietary energy in concentrations above 6%inclusion of glycerol |

# 1.4 Lipid metabolism

## 1.4.1 *de novo* lipogenesis

Being a carboxylic acid with a long aliphatic chain FA, are found in esters such as triacylglycerol, phospholipids, and cholesterol esters. FA starts at the carboxylic group (-COOH) and ends at the methyl (-$CH_3$) of the aliphatic chain, which is designated omega (ω). They be either saturated (SFA) characterized for having the maximum amount of hydrogens bond to carbon, monounsaturated (MUFA) with one double bond or polyunsaturated (PUFA) for presenting double bonds between some of the carbon atoms. In the form of triacylglycerol, phospholipids, or cholesterol esters, they have a major role as dietary sources of fuel for animals and they are important structural components for cells. New FA are produced synthesized via *de novo* lipogenesis and the esterified to glycerol, generating TAG. Following the fatty acid synthase (FAS) mechanism. AcCoA used as a precursor is produced in the mitochondria, then AcCoA is condensed with oxaloacetate to citrate, by the citrate synthase. Then in the cytosol with the expenditure of 1 ATP, citrate is cleaved back to AcCoA and oxaloacetate (citrate shuttle). Then Acetyl-CoA carboxylase (ACC) incorporates a $CO_2$ group in AcCoA, forming malonyl-CoA. FAS condenses AcCoA and malonyl-CoA to form the end of the FA (ω). The acetyl group will be condensed by FAS and remove the $CO_2$ from the carbon skeleton that malonyl will be provided. The chain elongates by tow carbons, for every condensation. After condensation, a reduction by keto acyl-ACP reductase, a dehydration by hydroxy acyl-ACP dehydrase and a reduction by enoyl-ACP reductase, occur in a chain reaction removing water and reducing the double bond to generate a saturated chain. For each reduction, a NADPH is oxidized and for each cycle, a new malonyl-CoA molecule binds to FAS further elongating the FA. Palmitate is the first molecule to be produced by FAS after a seven cycle of the previous reactions of condensation, reduction dehydration and reduction (Lehninger et al., 2008). This reactions as a spend of 42 ATP, being quite dispendious, happening only when the basic body energy requirements are met, so that the excess of energy would not go to waste and ensuring the most efficient way of storing energy. TAG are formed my one molecule of glycerol and tree FA so reduced and anhydrous molecules, triacylglycerol are highly concentrated stores of metabolic energy.

Essential fatty acids are required for biological processes and cannot be synthesize by humans or other animals, for the lack of enzymes. The tow essential fatty acids know are alpha-linoleic acid (an omega-3 fatty acid: ω3) and linoleic acid (an omega-6 fatty acid: ω6), these are known for having anti nociceptive, anxiolytic, and neurogenic properties. Several fish species are rich in omega-3 (PUFA) such as eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA).

Glycerol is an important structural component of triacylglycerol and phospholipids. Muscle composition is a major quality aspect of fresh fish and any changes that may occur in it may have consequences for marketing and general heath. Skeletal muscle is the largest organ system in fish corresponding to the actual edible part of it. When comparing farmed fish and wild fish regarding their lipid content wild fish have a higher content of essential FA.

## 1.5 Deuterium as a tracer

Nuclear magnetic resonance techniques (NMR), can be adapted to study lipid metabolism, thanks to the use of stable isotopes. A metabolic tracer is a molecule that shares identical chemical and functionality of a natural, can be stable or radioactive isotope labelled molecule, and can follow the metabolic path of a natural molecule of interest. Deuterium ($^2H$) a stable isotope of hydrogen ($^1H$), can be used as tracer, is useful, affordable and non-radioactive. Having a nucleus composed by one proton and neutron, in NMR deuterium has a different resonance frequency from hydrogen, therefore is possible to distinguish both in the spectra of NMR.

The main ways to administer $^2H$ is using deuterated water ($^2H_2O$): by intraperitoneal injection (Muller and Seelig, 1987) or in drinking water for mammals (Jones et al., 1988); incorporation into tank water for fish (Viegas et al., 2011); or added to the medium for *in vitro* culture (Berry et al., 2015). When administering $^2H$ it is important to have in mind the percentage of it in the delivery liquid as high levels of it can be toxic causing damages. When immersed in $^2H_2O$ 5%-enriched water thank, fish incorporation, by direct or indirect hydration, of $^2H$ into plasma is fast, being incorporated in metabolic reactions involving $^1H$ addition or exchange. Incorporation reaching 1% in 15 minutes, and nearly thank water enrichment after 6 hours until an isotopic steady state point is reached (Viegas et al., 2011), from the moment $^2H$ is incorporated newly synthesized molecules involving $^1H$ will possess a percentage of $^2H$, this process is called labelling.

### 1.5.1. Triacylglycerol $^2H$ enrichment

Nuclear magnetic resonance (NMR), provides discrete positional and enrichment of lipid functional group in chemical shift, it has been demonstrated in mice (Duarte et al., 2014) and fish (Viegas et al., 2016). From the moment $^2H$ is incorporated the newly synthesized molecules are labelled so lipid synthesis through DNL and lipid metabolism (desaturation and elongation) can be assessed. Palmitate hydrogens all come from different sources. Hydrogens localized in the methyl group of FA, derive from the initial AcCoA and the $^2H$ labelling of the initial AcCoA comes from citrate synthase. FAS incorporates the enriched deuterium molecule into palmitate. At the end of on elongation cycle one hydrogen derived from $^2H_2O$ and one from malonyl-CoA are incorporated

into each odd-numbered carbon. Methyl enrichment occurs only during DNL, and enrichment in the allylic hydrogens occurs during DNL and as FA are elongated (Fig. 6)

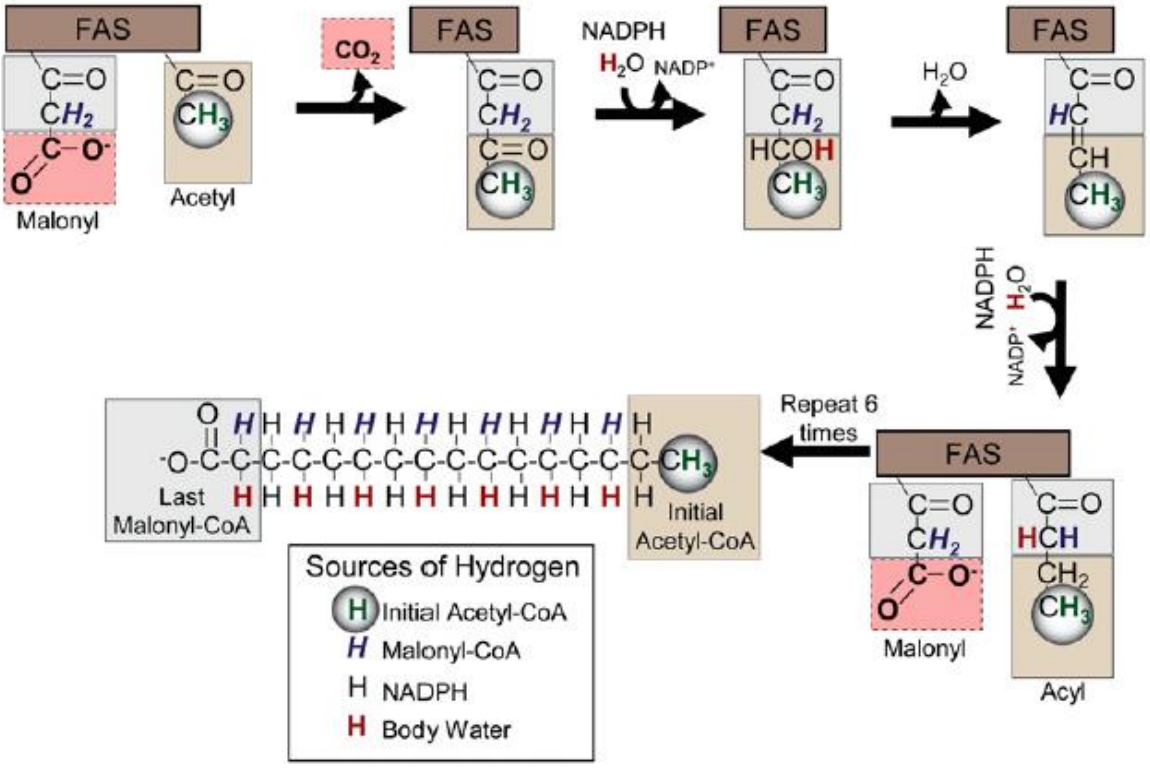



Fig. 6 Descriptive scheme from *de novo* lipogenesis in the presence of $^{2}H$ (Duarte et al., 2014)

# 1.6 Objectives

Our study aimed at evaluating the utilization of dietary glycerol supplementation in rainbow trout feed. For this objective, fish were subjected to a growth trial using diets with 0% glycerol (CTRL), 2.5% glycerol (GLY 2.5) and 5% glycerol (GLY 5) where the zootechnical performance under the dietary treatment could be assessed. This was followed by a metabolic trial which consisted in a 6-day residence in 4% $^{2}H_2O$. More specifically, we aimed at evaluating the impact of dietary glycerol on muscle TAG, in terms of composition (as evaluated by $^{1}H$ NMR) and metabolic flux (following the enrichment from $^{2}H_2O$ by $^{2}H$ NMR).

# 2. Materials and methods

This dissertation was performed in collaboration with Trás-os-Montes e Alto Douro University (UTAD). The growth trial and the metabolic trial were developed at the Experimental Research Station (UTAD - Vila Real, Portugal). All experimental procedures were conducted according to European Union regulations for use of animals for scientific purposes (Directive 2010/63/EU). This study was performed and supervised by accredited scientists in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGAV-Portugal) following FELASA category B and C recommendations.

## 2.1. Growth trial

During both the growth and metabolic trial, was used control diet (CRTL), glycerol at 2.5% w/w (GLY 2.5) and glycerol at 5% w/w (GLY 5). The glycerol (refined glycerin) in the formulation of the diet was obtained from rapeseed (Belgosuc, 050008, Beernem, Belgium). Cellulose was used at different levels, as an indigestible filler, to create dietary contrast for the addition of glycerol. All the diets were isoenergetic (21 kJ $kg^{-1}$) and isoproteic (49% of dry matter) (Table 4).

Table 4 Rainbow trout fed composition of control (CTRL), 2.5% glycerol supplemented GLY 2.5) and 5% glycerol supplemented (GLY 5).

| Ingredients, % | CTRL | GLY 2.5 | GLY 5 |
|---|---|---|---|
| Fishmeal Super Prime | 10 | 10 | 10 |
| Fish protein concentrate | 5 | 5 | 5 |
| Squid meal | 5 | 5 | 5 |
| Soy protein concentrate | 10 | 10 | 10 |
| Pea protein concentrate | 5 | 5 | 5 |
| Wheat gluten | 7,5 | 7,5 | 7,5 |
| Corn gluten | 7,5 | 7,5 | 7,5 |
| Soybean meal 48 | 8,5 | 8,5 | 8,5 |
| Rapeseed meal | 5 | 5 | 5 |
| Gelatinised starch | 9 | 9 | 9 |
| Cellulose | 5 | 2,5 | 0 |
| Fish oil | 14 | 14 | 14 |
| Vit & Min Premix PV01 | 1 | 1 | 1 |
| Lutavit C35 | 0,1 | 0,1 | 0,1 |
| Lutavit E50 | 0,1 | 0,1 | 0,1 |
| Soy lecithin | 2 | 2 | 2 |
| Antioxidant | 0,2 | 0,2 | 0,2 |
| Sodium propionate | 0,1 | 0,1 | 0,1 |
| Monocalcium phosphate | 1,3 | 1,3 | 1,3 |
| Binder | 2,5 | 2,5 | 2,5 |
| L-Histidine | 0,05 | 0,05 | 0,05 |
| L-Threonine | 0,15 | 0,15 | 0,15 |
| Chromic oxide | 1 | 1 | 1 |
| Glycerol | 0 | 2,5 | 5 |
| Total | 100 | 100 | 100 |

| As fed basis | CTRL | GLY 2.5 | GLY 5 |
|---|---|---|---|
| Crude protein, % feed | 44,24 | 44,24 | 44,24 |
| Crude fat, % feed | 17,29 | 17,29 | 17,29 |
| Fiber, % feed | 1,46 | 1,3 | 1,19 |
| Starch, % feed | 11,03 | 11,03 | 11,03 |
| Ash, % feed | 6,11 | 6,11 | 6,11 |
| Gross Energy, MJ/kg feed | 21,21 | 21,21 | 21,21 |
| C14, % feed | 0,74 | 0,74 | 0,74 |
| C16, % feed | 3,58 | 3,58 | 3,58 |
| C18, % feed | 0,89 | 0,89 | 0,89 |
| C18:1n9, % feed | 2,9 | 2,9 | 2,9 |
| LNA (C18:2n6), % feed | 0,94 | 0,94 | 0,94 |
| ALA (C18:3n3), % feed | 0,2 | 0,2 | 0,2 |
| ARA, % feed | 0,02 | 0,02 | 0,02 |
| EPA, % feed | 1,15 | 1,15 | 1,15 |
| DHA, % feed | 2,46 | 2,46 | 2,46 |
| EPA+DHA, % feed | 3,61 | 3,61 | 3,61 |
| ARA/EPA | 0,02 | 0,02 | 0,02 |

For the growth trial, each treatment had triplicates, nine thanks of 300 L each, 25 individuals were selected (n=25), with mean body weight of 20.2 ± 0.1 g, and were randomly distributed. An open circulating system was used with a natural source of water, with a flow rate of 12 L $min^{-1}$, during the experiment period. Feeding was made by hand at 9 a.m. and 5 p.m until reach apparent satiety. At the end of the $4^{th}$ and $8^{th}$ week the fish were weighted and water parameters recorded (dissolved oxygen: 8.71 ± 0.02 mg $L^{-1}$; pH: 6.68 ± 0.01; ammonia < 0.05; nitrite: 0.5 mg $L^{-1}$). The growth trial was completed between August-October 2017, during this time the fish were exposed to natural temperature (15 ± 1 ºC) and photoperiod. Total feed consumption and mortality data were daily recorded for each tank. Biomass was used to calculate and adjust the daily feed ration after 4 weeks.

## 2.1.2. Fish sampling

At the end of the feeding trial (60 days) fish were anaesthetized in a 15 L water container, with MS222 (3-aminobenzoic acid ethyl ester, 0,1 g $L^{-1}$) buffered with $NaHCO_3$ (0.2 g $L^{-1}$) and individually weighed. Samplings were made 6 h after the morning feeding (n=8) and 24 h after the morning feeding (n=8). Blood was collected, and after euthanized by slicing the cervical spine, liver, muscle, perivisceral fat and digesta were collected for growth performance and other zootechnical parameters, as well as for studying the molecular regulation of enzymes involved in the metabolization of dietary glycerol. For the zootechnical parameters presented, only the 24 h sampling was considered

## 2.1.3. Growth parameters analysis

Equations for fish growth parameters and feed utilization were given by,
Weight gain WG was calculated as:

$$\text{WG (g)} = \text{FBW} - \text{IBW}\ (eq.1)$$

Where IBM and FBW are the initial and the final body weight, respectively.

Where feed intake FI is the total amount of feed consumed per tank expressed in g of dry matter (DM) content corrected for dead fish and uneaten feed, and ABW is the average body weight of fish (g) calculated as:

$$FI = \frac{\text{IBW} + \text{FBW}}{2}(eq.2)$$

Absolute feed intake was calculated as:

$$\mathrm{FI_{abs}} = \mathrm{FI} \times \frac{\mathrm{FI}}{\mathrm{n} \times \mathrm{t}} \ (eq.3)$$

Number of fish (n) per tank corrected for dead fish.

Voluntary feed intake VFI, percentage body weight per day % BW day$^{-1}$ was calculated as:

$$\mathrm{VFI\ (\%\ BW\ day^{-1})} = 100 \times \frac{\frac{\mathrm{FI}}{\mathrm{ABW}}}{t} (eq.4)$$

Daily growth index DGI was calculated as:

$$\mathrm{DGI\ (\%\ BW\ day)} = 100 \times \frac{\mathrm{FBW}^{1/3} - \mathrm{IBW}^{1/3}}{t} \ (eq.5)$$

Where time (t) is the trial length.

Specific growth rate SGR was calculated as:

$$\mathrm{SGR\ (\%\ BW\ day^{-1})} = 100 \times \frac{ln\ \mathrm{FBW} - ln\ \mathrm{IBW}}{t} (eq.6)$$

Feed conversion ratio was calculated as:

$$\mathrm{FCR} = \frac{\mathrm{FI_{abs}}}{\mathrm{G_{day}}} \ (eq.7)$$

Where $G_{day}$ is growth per day calculated as WG / time.

Feed efficiency was calculated as:

$$\mathrm{FE} = \frac{\mathrm{G_{day}}}{\mathrm{FI_{abs}}} \ (eq.8)$$

Fish survival was calculated as:

$$\mathrm{S\ (\%)} = 100 \times \frac{\mathrm{Nf}}{\mathrm{Ni}} \ (eq.9)$$

Nf and Ni are the number of fish in each tank at the end and beginning of the trial, respectively.

The hepato-somatic index HSI was calculated as:

$$\mathrm{HSI\ (\%)} = 100 \times \frac{\mathrm{liver\ weight}}{\mathrm{FBW}} \ (eq.10)$$

## 2.2. Metabolic trial

After concluding samplings for other experimental procedures, 32 fish (12 per diet; 4 from each replicate tank) were transferred to 3 tanks (Fig. 7 c) with fresh water enriched with ~4% $^2H_2O$, in a well-aerated recirculation system equipped with an external filtering unit and UV unit for the last 6 days of feeding. Twenty-four hours after the last meal, animals were anesthetized in tank water containing 0.1 g $L^{-1}$ of MS-222 (Sigma-Aldrich) and enriched with ~4% $^2H_2O$. Ground source water was used and $^2H_2O$ was added until a concentration of 4% as previously described (Viegas et al., 2011). During duration of the trial the three thanks where interconnected, making a closed system, to prevent dilution of $^2H_2O$, the filters were watertight and had the ammonia cycle stabilized and matured beforehand. Tank water remained the same during the metabolic trial, with aeriation, recirculated freshwater passed through a mechanical filter, a biological filter, activated carbon and a UV unit. Feeding was made by hand at 9 a.m. and 5 p.m. until reach apparent satiety. $NH4^+$, $NO_3^-$, $NO_2^-$, temperature, salinity, pH, and dissolved oxygen were continuously monitored. The metabolic trial was made in duplicate. In order to ensure good metabolite signal, pools were made.

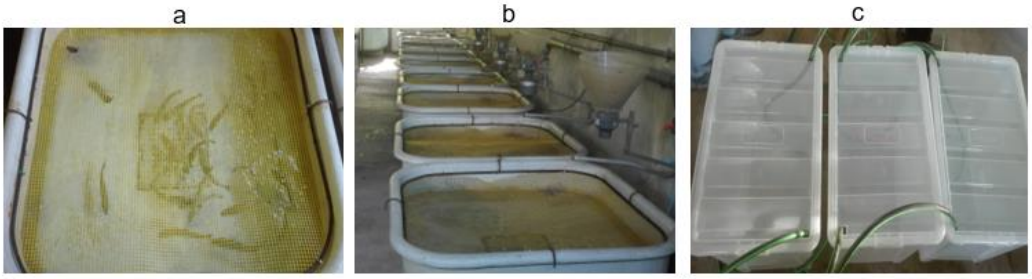


Fig. 7 Rainbow trout growth trial (a and b), and metabolic trial tanks (c).

## 2.2.1. Fish sampling

Fish were sampled after a 6-day residence in 4%-enriched $^2H_2O$, 24 h after the morning feeding (n=12 per diet). Blood collection was made through the caudal vein with heparinized 2.5 mL syringes with a 23G (assembled) needle (Fig. 8 a). Pools of two, from the same condition, were made and stored in 1.5 mL vials. After the blood collection fish were euthanized by slicing the cervical spine. Liver was the dissected, washed with a physiological saline solution (9 g NaCl/L) (Fig. 8 b), weighted, freeze clamped and grouped in pools two into falcons and stored in liquid nitrogen ($N_2$) and stored at -80°C. The intestinal tract, including pyloric ceca, was rapidly dissected from adherent adipose tissue and immediately frozen in $N_2$ and stored at -80°C. Two sample of muscle, approximately one gram each were collected, freeze clamped and grouped in pools two into falcons and frozen in $N_2$ and stored at -80°C (Fig 8 c).

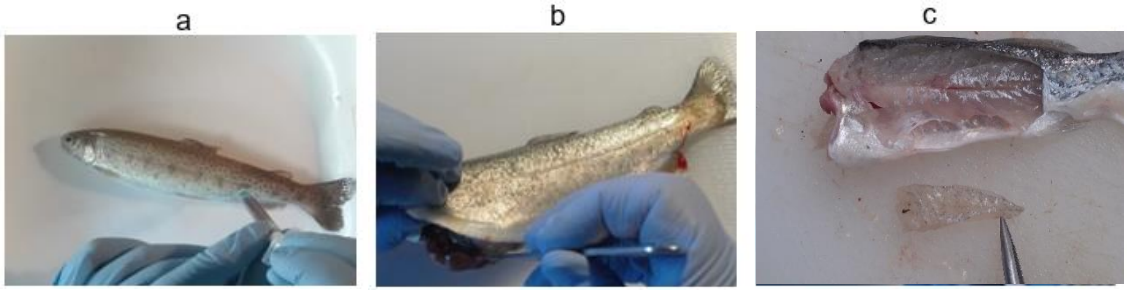



Fig. 8 Rainbow trout blood extraction (a), liver extraction (b), and muscle extraction (b).

## 2.2.2. Triacylglycerol extraction

To help achieve a better TAG extraction, the tissue was pulverized by grinding, with the help of liquid nitrogen. After the grinding was added methanol (4,6 mL/g) and vortexed. Then it was added 15,4 mL/g of methyl-tert-butyl ether (MTBE) and incubated (1 h) at room temperature in a shaker with magnet agitation Four mL of distilled water was added to induce phase separation letting the solution rest for 10 min at room temperature. The sample was then centrifuged at 1,000 g for 10 min. The upper (organic) phase was collected, transferred to a glass vial (dark glass or covered with aluminum foil) and dried in fume hood (hotte) under nitrogen stream or air-evaporate until dry, the water phase was collected to a falcon lyophilized and stored (Matyash et al., 2008).

## 2.2.3. Triacylglycerol purification (Solid phase extraction)

In this study, two solid phase extractions were performed: For the first extraction, the total lipid extract was fractioned into TAG, FA and phospholipids using solid phase extraction. Cartridges Discovery DSC-NH2 12 mL, 2 g - 52641-U (Sigma) are activated with 30 mL of hexane. Samples are suspended in 600 mL of hexane: MeOH:$CHCl_3$ (95:3:2, v/v/v) and loaded on the cartridge. TAG were eluted with 20 mL of $CHCl_3$. Then 10 mL of diethylether:acid acetic (98:2, v/v) were applied to the cartridge and the fraction with free FA was collected. The last fraction corresponding to the phospholipids was recovered after eluting with 20 mL of MeOH:$CHCl_3$ (6:1, v/v); for the second extraction, Discovery SPE DSC-Si Silica Tube 12 mL, 2 g - 52657-U are washed with 8 mL Hexane/MTBE (96:4) and 24 mL Hexane. Samples are dissolved in 1 mL of Hexane/MTBE (200:3) and added to the column. Column is eluted with 32 mL Hexane/MTBE (96:4) and fractions of 4mL each are collected, and its TAG content is identified via thin-layer chromatography (TLC). TLCs are revealed using a solution of Petroleum Ether/Diethyl Ether/Acetic Acid (7:1:0.1) and with iodine vapour. TAG different fractions are assembled is a glass vial (dark glass or covered with aluminium foil) based on the TLC information. Flasks

containing the assembled fractions are dried in fume hood (hotte) under nitrogen stream or air-evaporate until dry. Reagents specifications are listed in appendix.

## 2.3. Data analysis

### 2.3.1. $^{1}$H NMR

The samples were analysed by $^{1}$H NMR spectroscopy using a Bruker Avance III HD 500 MHz system equipped with a 5-mm $^{2}$H-selective probe with $^{19}$F lock and $^{1}$H-decoupling coil. Each TAG liver sample was resuspended with 450 µL of chloroform, 25 µL of pyrazine (standard) and 40 µL of hexafluorobenzene were added. Hexafluorobenzene served as a lock capture for probe.

### 2.3.2. $^{2}$H NMR

For $^{2}$H acquisition, the same NMR spectrophotometer in $^{1}$H NMR was used. Sample preparation was the same as previously used for $^{1}$H NMR. A spectral width of 1381 Hz was used, and broadband $^{1}$H-decoupling was continuously applied. The samples were ran at a temperature of 25ºC with 0.7 sec of acquisition time. Body waters from all the samples were run at par with $^{2}$H acquisition. Peak quantification in NMR was processed in ACD labs software v12.0 Advanced Chemistry Development, Inc (Toronto, Canadá).

Triacylglycerol data analysis:
The formulas used for lipid quantifications were adapted from (Duarte et al., 2014) and each letter from the formulas correspond to the letter in the spectra (Fig. 9).

ω3 FA:
The terminal methyl group of the ω-3 FA is slightly downfield of all the other terminal methyl groups. The distinct resonance allows the determination of the percentage of ω3 FA:

$$\%\ \text{of}\ \omega 3\ \text{fatty acids}\ = 100 \times \frac{C_{1H}}{C_{1H} + AB_{1H}}\ (eq.11)$$

Where $C_{1H}$ is the $^{1}$H area of ω3 FA and $AB_{1H}$ is the $^{1}$H area of non-ω3 FA.

PUFA and MUFA:
The percentage of mono and PUFA is given by:

$$\%\ \text{PUFA} = 100 \times \frac{G_{1H}}{(2 \times H_{1H}) + I_{1H}}\ (\text{eq. } 12)$$

Where $G_{1H}$ is the $^1H$ areas of all PUFA allylic protons, $H_{1H}$ is the $^1H$ area of all FA α protons and $I_{1H}$ is the $^1H$ area of DHA α and β protons.

The percentage of MUFA is given by:

$$\% \text{ MUFA} = 100 \times \frac{F_{1H}}{(2 \times H_{1H}) + I_{1H}} \text{ (eq. 13)}$$

Where $F_{1H}$ is the $^1H$ area of all MUFA allylic protons, $H_{1H}$ is the $^1H$ area of all FA α protons and $I_{1H}$ is the $^1H$ area of DHA α and β protons.

The percentage of UFA is therefore:

$$\% \text{ unsaturated fatty acids} = \% \text{ PUFAS} + \% \text{ MUFAS (eq. 14)}$$

And the amount of SFA is then:

$$\% \text{ saturated fatty acids} = 100 - \% \text{ unsaturated fatty acids (eq. 15)}$$

Docosahexaenoic and linoleic acid:

Linoleic acid (18:2-ω6) and DHA (22:6-ω3) have resolved $^1H$ resonances. DHA α and β protons overlap and appear slightly upfield of the other α protons. Therefore, the percentage of DHA is given by:

$$\% \text{ DHA} = \frac{I_{1H}}{(2 \times H_{1H}) + I_{1H}} \text{ (eq. 16)}$$

Where $I_{1H}$ is the $^1H$ area of DHA α and β protons and $H_{1H}$ is the $^1H$ area of all FA α protons.

The bisallylic peaks arising from linoleic acid appear as a clearly defined triplet at around 2.76 ppm. Hence, the percentage of linoleic acid is given by:

$$\% \text{ linoleic acid} = \frac{J_{1H}}{H_{1Ha} + \frac{I_{1H}}{2}} \text{ (eq. 17)}$$

Where $J_{1H}$ is the $^1H$ area of oleic acids bisallylic protons, $G_{1H}$ is the $^1H$ area of all fatty acid α protons and $H_{1H}$ is the $^1H$ area of DHA α and β protons.

Determination of body water enrichment:

Body water enrichment was calculated using $^2H$ NMR. Briefly, 10 µl of plasma was added to 1000 µl of acetone. Then, transfer to the NMR tube 490 µl of the mixture and add 40 µl of hexafluorobenzene. Following sample preparation, a $^2H$ NMR spectrum was acquired. Enrichments were calculated by comparing the ratio of the deuterium signal of acetone and water with the previously determined ratios of standards (Jones et al., 2001).

Determination of lipid methyl $^{2}H$ enrichment:

Peak A represents the methyl hydrogens of non ω3 fatty acids. This signal is corrected for linoleic acid contribution, an essential fatty acid in mammals which does not get labelled with deuterium and would otherwise leads to an underestimation of lipogenic flux. Since the percentage of linoleic acid present in the samples is calculated by eq. 20 the corrected methyl enrichment ($A_{eC}$) is given by:

$$A_{eC} = 100 \times \frac{A_{2Ha} \times \%\ 2HS \times P_{1Ha}}{(A_{2Ha} \times \%2HS \times P_{1Ha}) + \left(A_{1Ha} \times \left[1 - \times \frac{\%\ linoleic\ acid}{100}\right] \times \%1HS \times P_{2Ha}\right)} \ (eq.18)$$

Where $A_{1H}$ is the $^{1}H$ area of non ω3 fatty acids terminal methyl, $A_{2Ha}$ is the $^{2}H$ area of non ω3 fatty acids terminal methyl, $\%^{2}HS$ is the percentage of deuterium labelled standard (pyrazine), %1HS is the percentage of deuterium unlabelled standard (pyrazine), $P_{1H}$ is the $^{1}H$ area of the pyrazine standard and $P_{2Ha}$ is the $^{2}H$ area of the pyrazine standard.

*De novo* lipogenesis (DNL):

Fractional DNL is calculated as:

$$\%\ DNL = 100 \times \frac{A_{eC}}{\%\ body\ water\ enrichment} \ (eq.19)$$

Where $A_{eC}$ is the corrected methyl enrichment calculated above.

FA desaturation:

Desaturase activity was determined similar to lipogenic flux:

$$\%\ Desat.activity = 100 \times \frac{F_e}{\%\ body\ water\ enrichment} \ (eq.20)$$

Where $F_e$ is the deuterium enrichment of the monounsaturated FA' allylic protons

FA elongation:

The α protons are enriched during either DNL or chain elongation. The first pathway involves the complete synthesis of a new fatty acid chain starting from AcCoA while the second adds AcCoA to pre-existing fatty acid chains. Medium chain fatty acids pre-existing the treatment with $D_2O$ will be unlabelled, but if these fatty acids are elongated during exposure to the tracer, the subsequently added methylene hydrogens will be labelled. Comparison of enrichment at the terminal methyl end of the fatty acyl moieties with that of α protons reports the percentage of fatty acid chains that were elongated versus de novo synthesis.

Therefore, the percentage elongation is given by:

$$\%\ elongation = 100 - \frac{A_e \times 2}{H_e \times 3} \ (eq.21)$$

Where $A_e$ is the deuterium enrichment of the terminal methyl group of fatty acids and $H_e$ is the deuterium enrichment of α protons of FA.

Triacylglycerol-bound glycerol synthesis:

The percentage of newly synthesized glycerol in TAG is given by:

$$\%\ glycerol\ synthesis\ = 100 \times \frac{L_e}{\%\ body\ water\ enrichment} \quad (eq.22)$$

Where $L_e$ is the deuterium enrichment of glycerol's C1 and C3 protons.

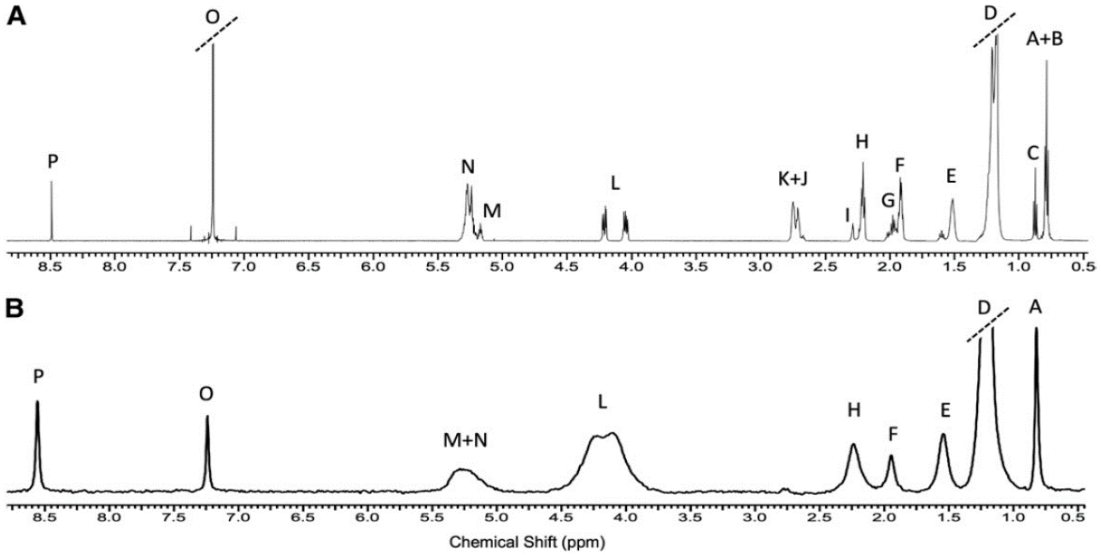



Fig. 9 $^{1}H$ (A) and $^{2}H$ (B) chemical shift assignment to the correspondent NMR spectra of TAG of European seabass after 6-day residence in $^{2}H_2O$ (Viegas et al., 2016). Non ω3 methyl (A); partial ω6 methyl (B); ω3 methyl (C); aliphatic chain methylenes (D); β methylenes (E); monounsaturated allylic hydrogens (F); polyunsaturated allylic hydrogens (G); α methylenes (H); docosahexaenoic acid α and β methylenes (I); linoleic acid bisallylic hydrogens (J); other bisallylic hydrogens (K); sn -1, sn -3 of TAG-glycerol (L); sn -2 TAG-glycerol glycerol (M); olefinic hydrogens (N); chloroform (O); and pyrazine standard (P).

## 2.4. Statistical analysis and calculations

To verify if glycerol supplemented feeds impact the metabolism and growth of the fish, it was applied a 1-way ANOVA followed by a Tukey test. Differences were considered statistically significant at $P < 0.05$. Values are presented as mean ± standard error of the mean (SEM). Statistical analysis was conducted with GraphPad Prism v7.0, GraphPad Software (San Diego, California).

# 3. Results

## 3.1. Growth trial

### 3.1.1. Growth parameters analysis

Table 5 Growth trial parameters (*Oncorhynchus mykiss*) fed with a CTRL diet, a 2.5% glycerol diet (GLY 2.5) and 5% glycerol diet (GLY 5).

| | *CTRL* | *GLY 2.5* | *GLY 5* |
|---|---|---|---|
| iBW | 20.30 ± 0.06 | 20.14 ± 0.04 | 20.23 ± 0.07 |
| HSI | 0.95 ± 0.03 | 1.08 ± 0.06 | 1.06 ± 0.04 |
| 4 week | | | |
| BW | 50.97 ± 1.97 | 50.80 ± 0.90 | 48.93 ± 1.06 |
| SGR | 3.06 ± 0.13 | 3.08 ± 0.05 | 2.94 ± 0.06 |
| FCR | 0.97 ± 0.01 [a] | 0.93 ± 0.01 [a] | 1.10 ± 0.02 [b] |
| FE | 1.02 ± 0.01 [a] | 1.07 ± 0.01 [a] | 0.90 ± 0.01 [b] |
| 8 week | | | |
| BW | 87.49 ± 2.08 | 85.27 ± 2.86 | 79.39 ± 1.38 |
| SGR | 2.43 ± 0.03 | 2.40 ± 0.05 | 2.28 ± 0.02 |
| FCR | 0.98 ± 0.02 [a] | 0.98 ± 0.02 [a] | 1.14 ± 0.02 [b] |
| FE | 1.01 ± 0.02 [a] | 1.01 ± 0.02 [a] | 0.87 ± 0.02 [b] |

Mean values ± SEM are presented (n = 3). Significant differences between dietary treatments are indicated by different letters (one-way ANOVA followed by Tukey's test; $P < 0.05$).

The results of growth trial the different treatments showed no differences iBW and HIS parameters. When comparing the results of the 4 week was no difference showed in BW and the SGR, when analysing the FCR and the FE there was a decrease in the GLY 5 treatment, meaning that the amount of food that needs to me consumed to achieve the same level of efficiency must be higher, although this does not seem to affect the body weight of the fish (Table 5).

## 3.2. Metabolic trial

### 3.2.1. Body water

Tank water was kept at 3.9 ± 0.0% $^{2}$H-enriched throughout the two replicates. Within replicate, the body water $^{2}$H-enrichment did not differed between diets, being 3.5 ± 0.1 for the first replicate experiment, and 3.7 ± 0.1 for the second. In both cases, it was not statistically different from the tank water ensuring a constant $^{2}$H delivery.

### 3.2.2. $^{1}$H NMR

After extraction and isolation by solid phase extraction, muscle TAG gave well-characterized $^{1}$H NMR spectra. The FA/glyceryl showed some differences in ratio in some spectra but was consistent overall, being approximately 3 in all treatments, demonstrating a successful TAG separation.

Table 6 Percentage of lipid species as determined from $^{1}$H NMR spectra of muscle TAG in rainbow trout (*Oncorhynchus mykiss*) fed with a CTRL diet, a 2.5% glycerol diet (GLY 2.5) and 5% glycerol diet (GLY 5).

| | *CTRL* | *GLY 2.5* | *GLY 5* |
|---|---|---|---|
| ω3 | 24.28 ± 0.62 | 25.91 ± 1.46 | 23.24 ± 0.37 |
| SFA | 18.50 ± 1.30 | 17.01 ± 2.06 | 20.64 ± 1.97 |
| UFA | 81.50 ± 1.30 | 82.99 ± 2.06 | 79.36 ± 1.97 |
| MUFA | 32.98 ± 0.56$^{ab}$ | 31.36 ± 1.17$^{a}$ | 35.56 ± 0.56$^{b}$ |
| PUFA | 48.53 ± 1.82 | 51.62 ± 3.12 | 43.80 ± 1.55 |
| DHA | 18.00 ± 0.59 | 19.12 ± 0.85 | 17.32 ± 0.71 |
| Linoleic Acid | 12.21 ± 0.14$^{a}$ | 11.90 ± 0.26$^{a}$ | 9.59 ± 0.45$^{b}$ |

Mean values ± SEM are presented (n = 6). Significant differences between dietary treatments are indicated by different letters (one-way ANOVA followed by Tukey's test; $P < 0.05$).

The results showed no differences observed between saturated FA and unsaturated FA among treatments. The percentage of PUFA showed a slight decrease in treatment with 5% glycerol no statistical differences were found, on the other case the MUFA percentage found only differences when comparing the 2.5% and the 5% treatment, showing that the 5% treatment had higher values. The SFA, DHA and UFA also did not show any statistical differences, and in the case of the linoleic acid statistical differences were found between the 5% treatment and the other

two treatments (Table 2). Relative to the structure of the FA there were no differences in the data showed between the treatments.

## 3.3.2. $^{2}$H NMR

The metabolic fluxes graph, the differences showed that even if some fluctuations are observed there were non-significant between treatments. Even if no differences are showed between treatments when comparing the different graphs we can in fact note some differences. *De novo* lipogenesis had a decrease in the GLY 5 treatment, glycerol rate had the highest values on the control diet (Fig 10), desaturation was higher in the control diet and it is approximately two times higher than the elongation ratio (Fig 11).

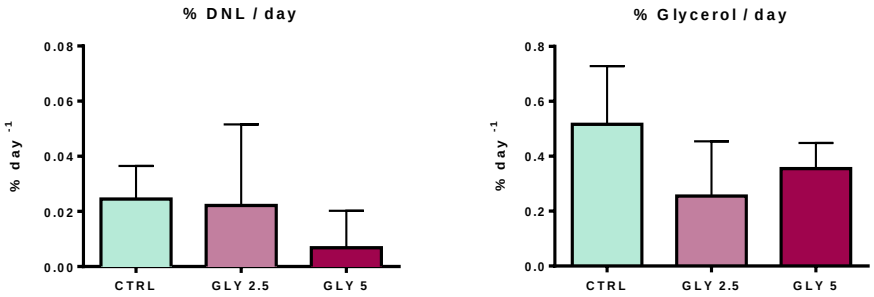
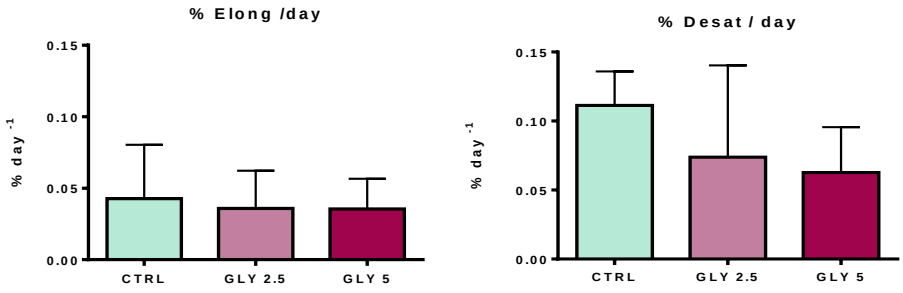



Fig. 10 Representation of *de novo* lipogenesis (DNL) of TAG-bound FA and TAG-bound Glycerol synthetic rate expressed as percent per day (*Oncorhynchus mykiss*) fed with a CTRL diet, a 2.5% glycerol diet (GLY 2.5) and 5% glycerol diet (GLY 5). After a 6 day residence in a tank with 4% $^{2}$H-enriched water. Mean values ± SEM are presented (n = 6). Significant differences between dietary conditions are indicated by different letters (one-way ANOVA followed by Tukey's test; $P < 0.05$).

Fig. 11 Representation of elongation and desaturation rate of TAG-bound FA expressed as percent per day




in rainbow trout (*Oncorhynchus mykiss*) fed with a CTRL diet, a 2.5% glycerol diet (GLY 2.5) and 5% glycerol diet (GLY 5). After a 6 day residence in a tank with 4% $^{2}$H-enriched water. Mean values ± SEM are presented (n = 6). Significant differences between dietary conditions are indicated by different letters (one-way ANOVA followed by Tukey's test; $P < 0.05$).

# 4. Discussion

## 4.1. General considerations

Muscle in fish has been known to be influenced by many factors, such as age (Peragón et al 2001), hormones (Furné et al 2012), exercise (Bernard et al 1999), feeding habits (French et al 1981), and diet composition (Alvarez et al 1999). These have been studied for aquaculture purposes to determine fish health and fillet quality - the muscle is the edible part of the fish therefore, of great importance in terms of health safety and nutritive value.

Age has one of the most detrimental influences in the muscles composition. At yearly stages of development, feeding of rainbow trout, with different levels of vitamins, has a beneficial effect in muscle growth (Panserat et al 2017). As age progresses so does the efficiency in witch food is used, influencing therefore the protein efficiency used for muscle growth (Peragón et al 2001).

Hormones influence in various ways muscle fat content (Jin et al 2014), in rainbow trout insulin has been proven to have an influence in growth, reduction of fat levels, changing in lipid oxidation and development of muscle (Castillo et al 2006; Codina et al 2008). High levels of dietary fat can impair glucose homeostasis, this is a characteristic of persistent hyperglycemia and reduces insulin sensitivity, a prediabetes insulin-resistant state found in mammals. In carnivorous fish such as rainbow trout, hyperglycemia after a high carbohydrate meal, stems from a metabolic interaction between dietary macronutrients rather than from high carbohydrate intake alone (Figueiredo-Silva et al 2012).

Exercise leads a depletion of glycogen in muscle being another factor that can majorly influence muscle factors. In rainbow trout it has been found that after prolonged swimming, a large number of the genes were up-regulated in red and white muscle, these were mainly involved in glucose use, energy generation, contraction, development, synthesis and catabolism of proteins (Magnoni et al 2013). It is known in rainbow trout that in the state of glycogen depletion, muscle is capable of glycogen synthesis and this is inversely correlated with initial depletion of glycogen (Frolow et al 2004). Exercised rainbow trout, do not mobilize reserves of TAG beyond resting levels to supply more FA to working muscles (Bernard et al 1999). Lactate has an important role in muscle, it has been found that in rainbow trout, muscle cells compartmentalize lactate metabolism (Kam et al 2006), and during starvation periods there is a mobilization of tissue reserves, using lactate in gluconeogenesis (Furné et al 2012). Sustained swimming can also improve protein deposition, resulting in an enhancement of the protein-sparing effect (Beltra et al 2012). Amino acids, dietary or from muscle protein, are major oxidative substrates in trout, and have the

capability when in periods of prolonged starvation and exercised to maintain supplies of glucose (French et al 1981).

Overall, there is an importance of dietary macronutrient interface in evolving fish nutrition strategies. Studies in rainbow trout suggested that digestible protein concentration, in diets with good nutrient base, can be reduced with a beneficial effect on tissue lipid oxidation, showing no negative effect on growth and composition of muscle (Alvarez et al 1999). Diets with limiting levels of essential amino acids lead to a negative effect in muscle growth (Belghit, et al 2014). Changes in alanine dynamics also lead to changes overall in muscle protein catabolism (Kullgren et al 2010). Depending how different sources of protein are treated and processed, there can be a influence in feed conversion and protein efficiency, this has been tested for high levels of vegetable starch content, that have been found when it is reared at low temperature, it can be used in diet of rainbow trout with no detriment effect in muscle (Capilla et al 2003).

Higher and lower fat contents also impair changing factors on muscle, in rainbow trout a high carbohydrate diet leads to a decrease of growth rate, feed efficiency and protein utilization (Kamalam et al 2012). A higher content fat diet also potentiates a higher glucose and lipid uptake in muscle. (Kamalam et al 2013) and enhance glucose utilization in both liver and muscle (Kolditz et al 2008). Low dietary protein diet on the other hand does not show gluconeogenic capacities or glucose phosphorylation capacities in rainbow trout (Kirchner et al 2005).

## 4.2. Supplementation of glycerol

Glycerol supplementation has been studied revealing effects in many parameters in many species (as reviewed in Table 1, 2 and 3). Among them, all have interest for aquaculture such as gilthead seabream (Silva et al 2012), European seabass (Rito et al 2019), channel catfish (Li et al 2010), Nile tilapia (Neu et al 2012; Neu et al 2013; Gonçalves et al 2015; Moesch et al 2016; Costa et al 2017), pacu and silver catfish (Balen et al 2014) and rainbow trout (Menton et al 1986). As in this study all studies used isoenergetic control diet, and different levels of glycerol supplementation were tested. Overall there was found a maximum glycerol inclusion percentage that did not affect survival of the fish, and proven to be a good source of dietary energy.

In channel catfish glycerol inclusion up to 10% did not affect feed consumption, weight gain, feed efficiency ratio, and liver lipid level. But, beyond that percentage, there were found adverse effect in weight gain, feed efficiency, and liver lipid content. Fillet protein and fat also generally decreased and there was fillet moisture increase as dietary glycerol level increased (Li et al., 2010). It was also found, in a study with a utilization of bolus of glycerol injection in European seabass, that glycerol effectively competes with endogenous precursors for hepatic gluconeogenesis (Rito et al 2019). In gilthead seabream study, glycerol use also did not impair zootechnical parameters and growth, organoleptic properties (aroma and color) also remained

unchanged, and an induction of increasing of muscle glycogen ATP levels and firmness, showed a low impact of glycerol on muscle metabolism (Silva et al., 2012).

For Nile tilapia glycerol supplementation has been studied in various stages of development. Glycerol supplementation in fingerlings (Moesch et al 2016), juveniles (Costa et al 2017), as well as adults (Gonçalves et al 2015; Neu et al 2012; Neu et al 2013), showed some increase in body moisture, crude protein content. A quadratic effect was showed when analyzing its productive performance, meaning that at higher level of glycerol replacement/supplementation there was a decrease in body weight, lipids and mineral content in the whole body, this showed a limit of glycerol percentage that can be used. In general terms, glycerol showed to be an interesting and safe source of energy, as tilapia are able to metabolize it into lipids, protein and/or carbohydrates. For pacu and silver catfish glycerol presented a high level of gross energy and a high rates of digestibility being a good source of energy (Balen et al., 2014).

In rainbow trout the supplementation of glycerol has been shown to have no effect either on the final carcass composition or on the efficiency of dietary protein conversion into tissue protein. Moreover, it was flagged as non-effective precursor for lipogenesis nor directly stored in liver. It was also noted that trout feed with over 6% supplementation of glycerol showed higher glucose levels and an overall decreased in feed response consumption, showing signs of hyperglycemia, been therefore labeled as a not effective source of dietary energy in rainbow trout at concentrations above 6% inclusion (Menton et al 1986). In this study glycerol supplementation up to 5% seemed to not affect in a negative way any zootechnical parameters involving growth showing, only statistical differences between treatments in FCR and FE that showing a slight a decrease in the highest concentration, meaning that the amount of food consumed by fish feed with the higher percentage diet supplementation as to be higher. The types and structure of fatty acids showed no statistical differences observed and only percentage of PUFA showed a slight decrease and that MUFA higher values. When observing the metabolic fluxes we can see some fluctuations between treatments but this were non-significant.

## 4.3. Future perspectives

After evaluating the utilization of dietary glycerol supplementation in rainbow trout feed, by the zootechnical performance and the impact on muscle TAG in terms of composition and metabolic flux, we can infer that dietary glycerol, can be used on feed of rainbow trout without any major negative effects of muscle. Above certain thresholds increase in crude protein and body moisture, and a decrease in lipids and mineral content can be observed. Despite the adverse effects observed at higher inclusion levels, glycerol presents itself as a potential energy source, with good digestibility that is an available and affordable ingredient in the global market.

Muscle lipid metabolism is largely overlooked when compared with the liver, the main lipogenic organ. The liver is a highly metabolically active tissue, while muscle is often considered as an inert tissue, just performing physiological contractility and therefore used as a reservoir for energy and storage. Because of this the metabolic dynamics behind lipid retention/accumulation, promotion of lipogenic pathways, or by their combined effects it is highly understudied, being necessary more wide and integrated point of view in fish species. So as a first future perspective of this work we need to know how the different tissue interconnect, where glycerol is stored if not on muscle. First we need to know more about lipid metabolism in liver has the major metabolic active tissue. Then, to provide a more integrated inter-tissue approach, we could study perivisceral fat as another tissue that like muscle has been overlooked. Mostly composed of adipocytes, its role was mistakenly presumed as a metabolically inert storage for excess lipid. But since its highly dynamic role has been unravelled in mammals, and recently also in carnivorous fish species (Viegas et al. 2019) with endocrine action, energy homeostasis and metabolic role in several conditions, it may be a good option to where do glycerol is being mobilized to. Blood can be also studied as a good way to look as the main transportation tissue on organisms, with these we could have a global view of the metabolism of rainbow trout.

As another future perspective we could also study other type of samples, in this work we focused on TAG for its abundance and for the major lipid, but we can also study free fatty acids, and phospholipids, that although are not abundant have, highly important physiological functions (membrane constitution). For this approach we would need to revise and adjust our protocol to be more accurate to this lipids, such as quantities on the sampling, extraction and purification, and analyses adjust sensibility for NMR our maybe change into mass spectrometry that would require new formulas to calculate composition and metabolic flux of tissue.

Supplementation was used in this study to include glycerol in the feed for the rainbow trout, this was done by substituting cellulose, which is nutritionally inert. So the next step of this study could also be the substitution of protein or fat the main source of CHO, for 0%, 2.5% and 5% as to evaluate of the overall effect of this substitution on rainbow trout, by doing we could evaluate if in fact by doing this substitution we can still maintain the basic needs of rainbow trout. Other future perspective could be complementing this study by addressing the enzymatic modulation of lipogenesis pathway in liver, muscle glycolytic pathway and fatty acid oxidation. Through the study of enzyme mRNA and protein levels, the metabolic study based on the use of stable isotopes for metabolic flux analysis, could be complemented with the assessment of the enzymatic machinery involved in those same fluxes.

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