UNIVERSIDADE D
COIMBRA

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

Dissertação no âmbito Mestrado em Bioquímica, na área de Metabolismo,
orientada pelo Dr. Ivan Viegas e Dr. John G. Jones,
apresentada ao Departamento de Ciências da Vida
da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Setembro de 2019

Acknowledgements / Agradecimentos

Durante o meu percurso académico, como exímio introvertido que sou, poucas pessoas tiveram nele impacto, mas todas as que tiveram foram o melhor que podia desejar.

Ao Ivan, que de forma paciente e altruísta me transmitiu todos os conhecimentos científicos que tinha ao seu dispor. Pelo desenvolver da minha capacidade de pesquisa e espírito critico, não me dar as respostas todas, mas apontando para onde as achar. E sobretudo pela confiança depositada em mim depositada em todas as etapas do projeto, conferindo-me um enorme senso de organização, metodicidade e zelo pelo mesmo.

À Mariana, ao Ludgero e ao João Rito, que a par com o Ivan, me acompanharam em todas as técnicas, trabalhos laboratoriais e bibliografia necessária para o meu aprendizado.

Um especial agradecimento ao Dr. John Jones, por me ter recebido no laboratório do Biocant e por ter permitido trabalhar ao lado da sua equipa.

A toda a minha família, em especial à minha mãe e ao meu pai pela força, à minha avó Albina e ao meu avô Júlio pelo carinho, e à minha esposa Sara pela cumplicidade, do fundo do coração um grande obrigado.

Mana, obrigada por seres a pessoa mais chata à face da Terra, pois foi graças à paciência que ganhei por te aturar, que consegui aos poucos ir digerindo toda a informação acerca do metabolismo lipídico.

A todos vós e a Deus eu dedico esta tese.

Financiamento / Funding

Este trabalho foi financiado pela Fundação para a Ciência e a Tecnologia / Ministério da Educação e Ciência (FCT/MEC) através de fundos nacionais e cofinanciado pelo FEDER, no âmbito do Acordo de Parceria PT2020, e COMPETE 2020, no âmbito do projeto PTDC/CVT-NUT/2851/2014 | POCI-01-0145-FEDER-016828: Glicerol como ingrediente alternativo para rações de peixe - potencial para aquacultura.

This work was supported by Fundação para a Ciência e Tecnologia (FCT; Portugal) through national funds with the co-funding by the FEDER, within the PT2020 Partnership Agreement, and COMPETE 2020 within the project PTDC/CVT-NUT/2851/2014 | POCI-01-0145-FEDER-016828: Glycerol as alternative ingredient for fish feed - potential for aquaculture.

Project “RENATURE - Valorization of the Natural Endogenous Resources of the Centro Region” (CENTRO-01-0145-FEDER-000007), funded by the Comissão de Coordenação da Região Centro (CCDR-C) and subsidized by the European Regional Development Fund (FEDER).

Financiado por:

FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA EDUCAÇÃO E CIÊNCIA

Cofinanciado por:

COMPETE 2020 PORTUGAL 2020 UNIÃO EUROPEIA Fundos Europeus Estruturais e de Investimento

Contents

List of abbreviations

^{2}H – Deuterium

$^{2}H_2O$ – Deuterated water

ACC – Acetyl-CoA carboxylase

ACLY – ATP-citrate lyase

BW – Body water

DHA – Docosahexaenoic acid

DNL – *De novo* lipogenesis

EPA – Eicosapentaenoic acid

FA – Fatty acids

FAS – Fatty acid synthase

FCR – Feed conversion ratio

FE – Feed efficiency

IDH – Isocitrate dehydrogenase

LCFA – Long-chain fatty acids

LC-PUFA – Long-chain polyunsaturated fatty acids

MTBE - Methyl-tert-butyl ether

MUFA – Monounsaturated fatty acids

NMR – Nuclear magnetic resonance

OA – Oleic acid

ppm – Parts per million

PUFA – Polyunsaturated fatty acids

SFA – Saturated fatty acids

TAG - Triacylglycerol

TLC – Thin-layer chromatography

UFA – Unsaturated fatty acids

ω3 – Omega 3 fatty acids

ω6 – Omega 6 fatty acids

Summary

Rainbow trout (*Oncorhynchus mykiss*) is a carnivorous fish, which given its resistance and high commercial value is one of the most produced fish in the world. One of the reasons for that is its nutritional value that stems from high retention of fatty acids among which are the essential fatty acids in the human diet, such as polyunsaturated fatty acids, which include omega 3 (ω3) fatty acids. The large population growth has increased the demand for food products, particularly fish. This need has led to an increase in aquaculture as a means of providing quality products affordable to a large number of people.

Studies with glycerol in both fish and mammalian have shown its value on the food industry and as such, it is used as a supplement in food for farm animals, as it is a product with high bioavailability and low production cost. The objective of this dissertation is to evaluate the impact of glycerol as a feed supplement for rainbow trout, with a primary focus on lipid turnover and composition, resorting to nuclear magnetic resonance. Deuterium is a stable isotope of hydrogen, which was incorporated in the tank water as deuterated water with a percentage of 4%. This marker is incorporated into lipid metabolism thereby allowing calculation of lipid flux through NMR.

The 2.5% supplementation of glycerol in the diet of rainbow trout presented zootechnical parameters equal to a diet based on commercial feeds. Furthermore, glycerol does not promote *de novo* lipogenesis in the liver and does not change the relative composition of the triacylglycerides.

With this study it is possible to prove that rainbow trout can metabolize glycerol without body and molecular changes up to 2.5% supplementation.

Keywords: glycerol, rainbow trout, *de novo* lipogenesis, NMR, ω3 fatty acids

Resumo

A truta arco-íris (*Oncorhynchus mykiss*) é um peixe carnívoro que, por devido á sua resistência e alto valor comercial, é um dos peixes mais produzidos no mundo. Uma das razões é o valor nutricional que resulta da alta retenção de ácidos gordos, entre os quais os ácidos gordos essenciais da dieta humana, como os ácidos gordos polinsaturados, que incluem os ómegas 3. O grande crescimento populacional aumentou a demanda por produtos alimentícios, principalmente peixe. Essa necessidade levou a um aumento da aquicultura como meio de fornecer produtos de qualidade acessíveis a um grande número de pessoas.

Os estudos com glicerol em peixes e mamíferos tem mostrado o seu valor industrial e como tal, são utilizados como suplemento em alimentos para animais de criação, pois são produtos com alta biodisponibilidade e baixo custo de produção. O objetivo desta dissertação é avaliar o impacto do glicerol como suplemento alimentar para a truta arco-íris, com foco principal no *turnover* e composição lipídica, recorrendo à ressonância magnética nuclear. O deutério é um isótopo estável de hidrogênio, que foi incorporado na água do tanque como água deuterada com uma percentagem de 4%. Este marcador é incorporado no metabolismo lipídico, permitindo assim o cálculo do fluxo lipídico através da ressonância magnética nuclear.

A suplementação de 2,5% de glicerol na dieta da truta arco-íris apresentou parâmetros zootécnicos iguais a uma dieta baseada em rações comerciais. Além disso, o glicerol não promove lipogénese *de novo* no fígado e não altera a composição relativa dos triacilglicerídeos.

Com este estudo, é possível provar que a truta arco-íris pode metabolizar glicerol sem que advenha alterações corporais e moleculares até 2,5% de suplementação.

Palavras-chave: glicerol, truta arco-íris, lipogénese *de novo*, RMN, ácidos gordos ω3

1. Introduction

1.1. Global state of aquaculture

Aquaculture is defined as the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants. Farming implies individual or corporate ownership of the stock being cultivated and also implies some form of intervention in the rearing process to enhance production, such as regular stocking, feeding and protection from predators. In order to distinguish between aquaculture and fisheries harvests for statistical purposes, aquatic organisms which are harvested by an individual or corporate body which has owned them throughout their rearing period contribute to aquaculture, while aquatic organisms which are exploitable by the public as a common property resources, with or without appropriate licenses, are the harvest of fisheries.

To feed the projected 9.7 billion people by 2050 (United Nations, 2017) in a context of climate change should be a challenge and aquaculture presents itself as a determining food production activity. Aquaculture has grown steadily in the past three decades: in 1974 only 7% of fish for human consumption came from aquaculture, arising to around 45% in 2014, while capture fishery production remained relatively static since the late 1980s (Fig. 1). Despite this, the number of wild-caught fish has not yet decreased, but in the last couple of years a milestone was reached: the consumption of wild-caught fish was overtaken by the aquaculture fish consumption. Most of the captures are performed in marine environment (seas and oceans), rounding 81.5 million tonnes, being 87% of the total captures in 2014 compared with inland captures (rivers and lakes). On the other hand, for aquaculture, inland farming contributes to 80% of the total production and produces nearly double the weight in relation to marine aquaculture. Leading the numbers of fish production from aquaculture are countries such as China, holding 60% of global fish production from aquaculture followed by India, Vietnam, Bangladesh and Egypt (FAO, 2018).

The state of the world’s marine fish stocks has not improved overall, despite some noteworthy progress in some areas. Assessed commercial fish

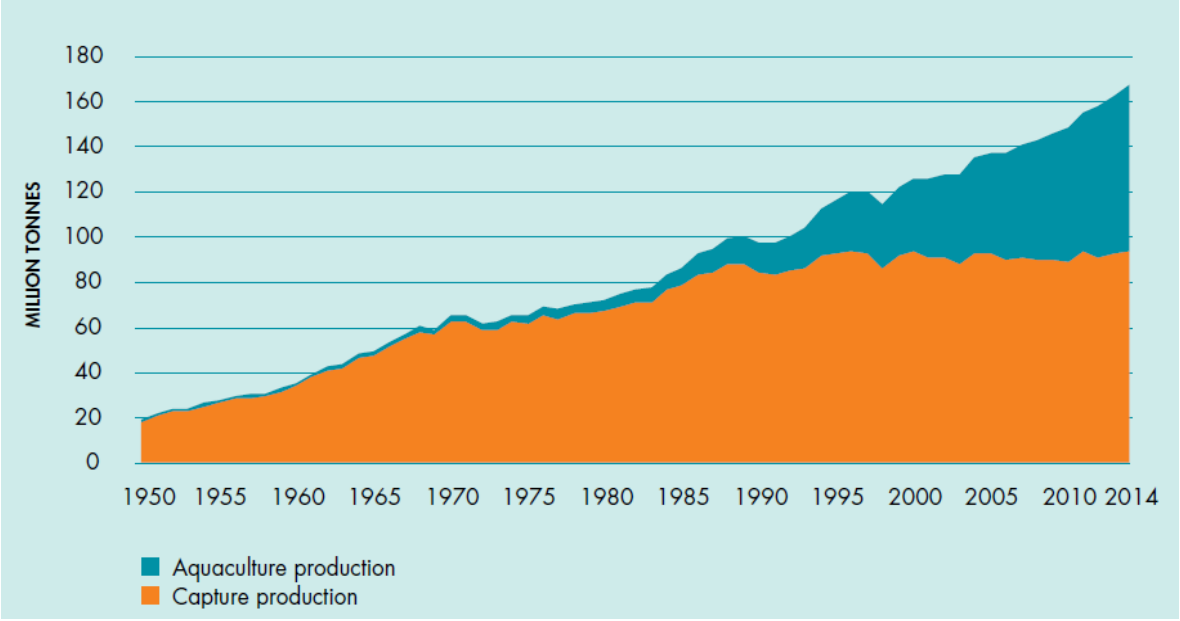

Figure 1 – World capture fisheries and aquaculture production (FAO, 2016).

stocks within biologically sustainable levels decreased from 90% in 1974 to 68.6% in 2013, based on FAO's analysis. Thus, 31.4% of fish stocks were estimated as fished at a biologically unsustainable level and therefore overfished. The underfished stocks decreased almost continuously from 1974 to 2013. The share of world fish production utilized for direct human consumption has increased significantly. In recent decades, in 2014 more than 146 million tonnes was destined for food products while 21 million tonnes was destined for non-food products, of which 76% was reduced to fishmeal and fish oil (FAO, 2016).

Fishmeal and fish oil are still considered the most nutritious and digestible ingredients, hence vastly used for farmed fish feeds (Williams et al., 2003). The utilization of by-products is becoming an increasingly relevant industry, with a growing focus on their controlled handling hence reducing waste. Fishmeal and fish oil are expensive due to their high demand for carnivorous fish diet, which lead to a better optimization and adjustment of their quantities in compound feeds for aquaculture and in recent years, their use it has starting to show a clear downward trend (Green, 2016). Strictly carnivorous fish utilise high protein and lipid diets. The ratio of protein to lipids in carnivorous fish feeds have been studied (Green *et* al., 2002) taking into account the price and the high amount of ammonia generated as a final product of oxidized

dietary protein as a source of energy. In the last decade there have been many studies (Caballero *et* al., 2001; Sarker *et* al., 2016) focusing on the ingestion and use of high lipid diets, aiming at the replacement of dietary protein for lipids as the source of energy, enabling higher growth rates to be achieved. Although it is important to find the adequate ratio for the feed components, the question that all the components used for the production of these feeds come from fisheries, still arises. The research for alternative food sources to fishmeal and fish oil is a valuable asset to reduce the overfished stocks, and to promote environmental quality and economical viability.

1.2. Aquaculture of rainbow trout

Rainbow trout (*Oncorhynchus mykiss*) is a carnivorous fish species of the family Salmonidae with high commercial value. Salmonids are amongst the most highly cultured fish groups worldwide. Rainbow trout commercial weight is around 2-3 kg, being able to reach 25 kg and 120 cm in the end of their lifespan (11 years). The habitat of rainbow trout consists of cold waters, characteristic from seas and upper rivers sections. Rainbow trout is native to the rivers and lakes of the Pacific coasts of North America and Asia. The primary producing areas are in Norway, Iran, Chile, Japan, Turkey and Australia. The species can withstand vast ranges of temperature and pH variation, but the optimal growth

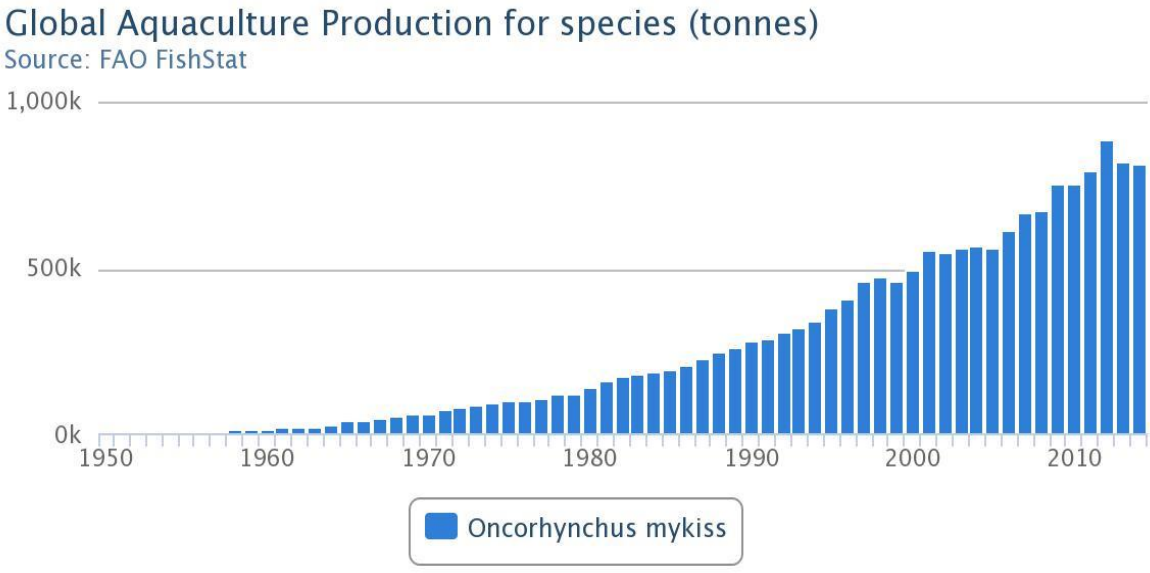

Figure 2 – Global aquaculture production for *Oncorhynchus mykiss* (rainbow trout).

parameters are between 12-21ºC and 6.5-8.5, respectively (Woynarovich *et* al., 2011).

The production of rainbow trout has grown since the 1950s, especially in Europe and more recently in Chile, with around 800.000 tonnes yield per year since 2008 (Fig. 2). Chile is currently the largest producer and it stands out from all the other producers, contributing with around half of the total yield (Colihueque, 2010).

1.2.1. Rainbow trout as a model for fish nutrition

In nature, salmonids are a predatory fish, feeding on small crustaceans, aquatic insects, and smaller fish, thus classified as carnivorous. The nutrition of salmonids, like rainbow trout, is well-documented as it is a predominant species reared and reviewed. Fish require a higher level of dietary protein than terrestrial vertebrates, representing a very interesting model of animal nutrition because up until now, few strict carnivorous animals have been extensively studied (Myers and Klasing, 1999; Verbrugghe *et* al., 2012).

Similar to all organisms, rainbow trout need energy for vital functions maintenance, growth, physical activity, renewing tissues and the development and subsequent use of the reproductive system. Oxidative catabolism of organic compounds (carbohydrates, lipids and proteins) under aerobic conditions provide these energy needs. Metabolism and energy balance are altered in response to a prandial or postprandial state. In a pre-prandial state, energy is drawn from body reserves provided during times of feeding. When in a post-prandial state, energy is provided by the macromolecules derived from the digestion of ingested foods. Growth occurs when the energy supplied by food exceeds maintenance and body reserve needs. There are four types of monomers: amino acids, fatty acids and glycerol, and monosaccharides. Amino acids arise from protein digestion, fatty acids and glycerol from the digestion of dietary lipids and glucose resulting from digestion of polysaccharides. Once absorbed, they are either catabolized in exergonic reactions therefore producing energy that is used to meet energy needs, or incorporated into cell structures

after biosynthesis. Fish, mammals and birds share the pathways of energy production, where the potential energy of organic molecules is released by the transformation of the covalent links between two carbon atoms of these molecules to CO_2 and H_2O. Acetyl-CoA (AcCoA) is a key intermediate compound in cellular metabolism, and its formation converges from fatty acid oxidation, breakdown of amino acids and pyruvate from glycolysis. Subsequently AcCoA is incorporated into the Krebs cycle with the production of CO_2, GTP and reducing equivalents. Reducing equivalents are oxidized by the electron transport chain to molecular oxygen, which is accompanied by the capture of electron transport chain in the phosphorylation of ADP, which generates the cellular fuel, ATP. Although these are common pathways their regulation by nutrients, hormones and signalling pathways is often specific and both the specific substrates and the quantitative requirements used for energy production differs from fish to mammals (Wade *et* al., 2014).

Rainbow trout oxidizes a large proportion of the amino acids to energy production by the digestion of dietary protein. The liver is the main site of this process. The oxidation of amino acids is catalysed via amino transferases and glutamate dehydrogenase, a transdeamination process, of which nitrogen is a by-product. The amino acid is transformed into an α-ketoacid and the amine group is transferred to α-ketoglutarate synthesizing glutamate. The α-ketoglutarate is then regenerated by the deamination of glutamate by glutamate dehydrogenase. Glutamate dehydrogenase activity is higher in the liver of fish than in mammals (Walton and Cowey, 1982). In fish oncoming ammonia from the deamination of amino acids is converted into the ammonium ion which is excreted across the gills and in the urine (Kaushik *et* al., 1991). Urea excretion can be found in rainbow trout as a result of the turnover of arginine and nucleic acids, rather than the end product of the urea cycle (Fournier *et* al., 2003). Krebs cycle is well regulated by the activity of citrate synthase, which controls the entry of AcCoA into the cycle, and in rainbow trout is also regulated by entry at the level of α-ketoglutarate, due to its major role in amino-acid catabolism. Subsequently, isocitrate dehydrogenase (IDH) that produces α-ketoglutarate through the oxidative decarboxylation of isocitrate having $NADP^+$ and NAD^+ as co-factors, is a key-enzyme in the process. In fish tissues, both $NADP^+$ and

NAD^+ are used as co-factors by IDH, but the activity of $NADP^+$-IDH exceeds that of NAD^+-IDH in all instances (Storey, 1988). Rainbow trout has a high requirement of dietary protein, averaging 36-38% of the diet when an adequate supply of non-protein digestible energy is present (Cowey, 1994). Amino-acid catabolism generates nitrogen waste. Nitrogen is inversely related with water quality in aquaculture, causing pollution and eutrophication (Conley *et* al., 2009). Consequently, efforts have been made to limit this pathway in fish farms with low hydrodynamics or water renewal, as well as operations where taxation is imposed on nutrient-enriched effluent waters. In rainbow trout this has been directly achieved by increasing energy dietary supply in the form of lipids and indirectly achieved by optimizing the overall protein supply meeting the quantitative needs for all the indispensable amino acids (Kaushik and Seiliez, 2010).

Improvement in protein utilization was made through optimizing the protein supply regarding the demand of indispensable amino acids. The ideal indispensable amino acids profile is the one that closely resemble that of the whole body of the fish (Wilson and Cowey, 1985). The optimal ratio between indispensable amino acids and dispensable amino acids appears to be 55:45 for this species (Green *et* al., 2002).

Like in mammals, the catabolism of fatty acids is a source of energy in rainbow trout through β-oxidation in peroxisomes and eventually all cells mitochondria. Following activation, fatty acids are transported to the mitochondria in the form of fatty acylcarnitine esters formed by the action of the carnitine acyltransferase complex, which are converted back into fatty acyl-CoA derivatives, which then enter a round of dehydrogenation, hydration and cleavage in order to produce AcCoA and NADH. β-oxidation reactions are tightly regulated by hormones and nutrients. The entry of fatty-acyls into mitochondria controlled by carnitine-acyltransferase 1 and the second dehydrogenation step in the β-oxidation that is mediated by the hydroxy-acyl CoA dehydrogenase, which seems to be the key steps (Polakof et al., 2011a). Fatty-acids after β-oxidation cycles generate AcCoA which is subsequently metabolized via the Krebs cycle to produce NADH providing ATP through the processes of electron transport and oxidative phosphorylation. Concerning fatty

acid retention, high concentration of a fatty acid in the diet leads to low lipid retention, with the exception of DHA that is preferentially retained in tissues (Thanuthong *et* al., 2011).

The incorporation and study of carbohydrates in rainbow trout diet, although it is generally accepted that they do not have a specific requirement for dietary glucose, is a good strategy to decrease levels of fishmeal and fish oil in aquafeeds (Panserat, 2009; Wilson, 1994). Rainbow trout has a limited ability to utilize dietary carbohydrates, showing a glucose intolerance phenotype after carbohydrate intake, similar to another carnivorous fish (Legate *et* al., 2001). Glucose metabolism is strongly dependent of the feeding status of the animal. Animals, in many tissues, use dietary glucose as an energy source through glycolysis and further oxidation in the Krebs cycle by converting pyruvate to AcCoA by the pyruvate dehydrogenase complex. In fed animals the excess of AcCoA prevenient from high dietary glucose in food is either converted into fatty acids in liver and adipocytes or stored as glycogen in muscle and liver. Dietary carbohydrates such as starch (digestible saccharide) are efficiently digested by rainbow trout after feed extrusion and gelatinization, reaching more than 90% digestibility (Krogdahl *et* al., 2005). Dietary carbohydrates are effectively digested and absorbed by the gut, and although all enzymes of glycolysis have been detected in rainbow trout tissues, in the liver the metabolism of dietary glucose is a limiting step, leading to postprandial persistent hyperglycemia when fed with more than 20% carbohydrates (20 g of carbohydrates per 100 g of diet) (Moon, 2001). Carbohydrates have always been studied in fish nutrition as a supplement that would spare protein catabolism for plasma glucose levels, however unlike mammals, which evolutionary were exposed to diets with marked carbohydrate levels, fish were not. Several metabolic studies with carbohydrate diets in fish are coupled with glucose intolerance results, even with low percentages (Hemre *et* al., 2002; Lopez-Olmeda *et* al., 2009). In this scenario glycerol presents as a good feed supplement, but the impact of its lipogenic turnover in fish is yet to be studied and the fluctuation in the quantities and types of triacylglycerols (TAG) in fish intended for human consumption, can cause impacts at a nutritional level.

1.3. Glycerol

Glycerol (1,2,3-propanetriol) is a simple polyol compound, water-soluble, clear, almost colourless, odourless, viscous, hygroscopic liquid that can be found in all lipids known as TAG, acting as a structural backbone. Glycerol is virtually nontoxic to human health nor environment, when administered at physiological levels (Frank *et* al., 1981) and it can be categorized into three main types, the crude glycerol, the purified/refined glycerol and commercially synthesized glycerol. Traditionally, glycerol is obtained from four different processes: soap manufacture, fatty acid production, fatty ester production and microbial fermentation. Industrial produced glycerol (purified/refined glycerol) has a purity level generally close to 100% due to its use in sensitive areas like medicine, food and cosmetic products (Hazimah *et* al., 2003). Purified glycerol is also graded depending on its preparation and usage. Grade-III (Kosher or USP/FCC grade, 99.5–99.7% pure) is prepared from plant oil sources, suitable for use in kosher foods and drinks; Grade-I (Technical grade, ~99.5% pure) is prepared by synthetic process and used as a building block for various chemicals but not applicable to food or drug formulation; Grade-II (USP grade, 96–99.5% pure) is prepared from animal fat or plant oil sources, suitable for food products, pharmaceuticals and cosmetics (Kenkel and Holcomb, 2008).

Glycerol is well known in fuel industry, being a major by-product of biodiesel manufacturing process. The price of glycerol is therefore determined by biodiesel demand and production. Due to its environmental benefits and the fact that it is made from renewable biological sources, biodiesel production has grown worldwide in recent years, increasing the quantity of glycerol in the market (Ayoub and Abdullah, 2012). Generally the percentage of crude glycerol produced versus the amount of biodiesel produced is about 10%, and it has a purity level among 60% to 80% (Cardona *et* al., 2007). Biodiesel is the primary end product from the transesterification of triacylglycerols from vegetable oil animal fats with an alcohol in the presence of an acid catalyst (Kosmider *et* al., 2011). The most common used alcohol for this process is methanol, since it is of lower cost. Crude glycerol resulting from biodiesel industry contains impurities such as moisture, ash, soap and chloride. These impurities also include residual methanol, especially when the alcohol is used in excess to

drive chemical transesterification and total recovery of entire methanol is not achieved. A recent regulatory letter issued by FDA indicates that methanol levels higher than 150 ppm could be considered unsafe for animal feed (Donkin, 2008), causing central nervous system injury, weakness, headache, vomits, blindness or Parkinsonian-like motor diseases (Dorman *et* al., 1993). Therefore, if glycerol is used to form consumer products such as food or drugs, it must be refined far beyond the purity of just a biodiesel by-product.

1.3.1. Glycerol as food supplement for farmed animals

Glycerol (E422) has been present in the food industry for some time and recently it has been re-evaluated by the European food safety authority (EFSA) (Mortensen *et* al., 2017). According to EFSA glycerol has a low acute toxicity, does not show any genotoxic activity and is not carcinogenic in mice and rats on doses up to 1%. Considering that none of the animal studies available identified an adverse effect for glycerol, glycerol is authorized as a food additive in regulated quantities.

The absorption of glycerol depends on the percentage of purity and the different animal models. Crude glycerol may contain residual fatty acids, which are known to have a high digestibility, especially in fish (Sarker *et* al., 2016). A study conducted in juvenile tilapia presents higher apparent digestibility coefficients for crude glycerol than for purified glycerol (Meurer *et* al., 2012). The inclusion of glycerol in the diet of juvenile tilapia up to 15% did not affect its absorption, which ranged from 74.4% to 82.5% (da Costa *et* al., 2017). In mammals, intestinal absorption of glycerol ranges from 70% to 89% in rats (Höber and Höber, 1937) and in pigs it might be more than 97% (Bartelt and Schneider, 2002).

Once absorbed, a glycerol molecule can be metabolized as a glycolytic, gluconeogenic or lipogenic substrate for subsequent metabolism. In order to integrate these pathways, glycerol is converted into glycerol-3-phosphate by glycerol kinase. Glycerol-3-phosphate dehydrogenase oxidizes glycerol-3-phosphate to dihydroxyacetone phosphate, integrating either glycolysis or

gluconeogenesis. In one hand, dihydroxyacetone phosphate metabolized from glycerol can replenish the blood glucose levels by gluconeogenesis or in the other hand, it can be oxidized via glycolysis and then further oxidized in the Krebs cycle by being converted to pyruvate and ultimately to AcCoA. Glycerol-3-phosphate is also the precursor of the TAG backbone, where a multienzymatic complex composed by 5 enzymes condense and dehydrate the glycerol-3-phosphate and 3 fatty acid molecules. The enzymes that catalyse reactions of glycerol metabolism are tissue specific. In mammals, liver and kidney express more abundantly glycerol kinase and glycerol-3-phosphate dehydrogenase (Elia *et* al., 1993). Small concentrations of glycerol kinase and glycerol-3-phosphate dehydrogenase have been identified in skeletal muscle (Newsholme and Taylor, 1969) and intestinal mucosa (Frank *et* al., 1981). Glycerol phosphate dehydrogenase is mainly found in the liver, skeletal muscle and adipose tissue (Lin, 1977). Recent studies in European seabass (*Dicentrarchus labrax*) using ^{13}C show that intraperitoneal glycerol is readily used as a gluconeogenic substrate, contributing about 20% and 50% of systemic glucose in fed and fasted conditions (Rito *et* al., 2019), leading to the deduction that the enzymatic machinery required to metabolize glycerol is also present in fish.

The amount of weight gained by farm animals plays an important role in the formulation of novel feeds. Concerning this matter, crude glycerol has been extensively studied in farmed birds (Min et al., 2010), mammals, such as pigs, cows (Carvalho *et* al., 2012), and farmed fish such as Nile tilapia (*Oreochromis niloticus*) and silver catfish (*Ictalurus punctatus*). Most mammals fed a glycerol-rich diet do not have a negative impact in terms of weight gain when compared to a non-supplemented diet representing a readily available energy source. Nursery pigs were used in order to evaluate the influence of diet (control, 5% and 10% glycerol supplementation) on weight gain, and at 21 day-old all pigs had the same weight, and no difference in performance according to diet was observed, concluding that glycerol can be used as a feed supplement for young pigs (Lammers *et* al., 2007). It has been demonstrated that levels up to 10% of dietary crude glycerine do not affect performance of catfish (Balen *et* al., 2014) and Nile tilapia (Neu *et* al., 2013). Above 15% supplementation with crude

glycerol, it is known that catfish have impaired growth (Li *et* al., 2010), also highlighted in pigs by Lammers *et* al. (2007).

Apart from the impact that glycerol may have in growth performance, a closer look into metabolic fluxes and meat composition determines if the meat quality changes. Young bulls fed a 6%, 12.5% and 18% glycerol, had linear increase in the oleic acid (C18:1-ω9) content in their muscle. The saturated fatty acid content linearly decreased in the muscle as a function of crude glycerol supplementation. Increased lipogenic turnover in carnivorous fish fed with glycerol is also present. A metabolic study in Nile tilapia juveniles showed a high lipogenic activity when fed with purified glycerol as a supplement. In 10% and 15% purified glycerol treatments, muscle and liver TAG had higher levels. Given the higher activity of enzymes involved in the lipogenic process and the higher amount of TAG found in these tissues, it can be concluded that glycerol is used as a lipogenic substratum in Nile tilapia juveniles (Costa *et* al., 2015).

1.4. Lipids metabolism

1.4.1. *De novo* lipogenesis

Fatty acids (FA) are carboxylic acids with hydrocarbon chains (aliphatic chain), which is either saturated, monounsaturated or polyunsaturated. The molecule starts at the carboxylic group (-COOH) and ends at the methyl ($-CH_3$) of the aliphatic chain, which is designated omega (ω). De novo lipogenesis (DNL) is a process that synthesizes FA, which are then esterified with glycerol, generating TAG. Since the first lipid molecules to be produced from DNL are palmitate, we will concentrate on the fatty acid synthase (FAS) mechanism. AcCoA is a precursor of FA synthesis and it can be generated by catabolic pathways in mitochondria. DNL does not occur in mitochondria due to lack of the necessary enzymes, so the AcCoA is condensed with oxaloacetate by the action of citrate synthase forming citrate. Citrate is then transported to the cytosol via the tricarboxylic carrier, where it is cleaved back to AcCoA and oxaloacetate with expenditure of 1 ATP by ATP-citrate lyase (ACLY). This cycle

is called citrate shuttle. The first DNL reaction (condensation) requires AcCoA and malonyl-CoA. Acetyl-CoA carboxylase (ACC) incorporates a CO_2 group in AcCoA, forming malonyl-CoA. AcCoA and malonyl-CoA bind to FAS by a thioester bound and losing the CoA. The first AcCoA, will be the end of the FA (terminal ω). FAS will condense the acetyl group and the malonyl that will provide the carbon skeleton in a growing chain of new FA, and remove the CO_2 previously added by ACC. For every condensation, the acyl chain elongates by two carbons. After the condensation, the last 3 enzymes from the FAS, β-keto acyl-ACP reductase (reduction), β-hydroxy acyl-ACP dehydrase (dehydration) and enoyl-ACP reductase (reduction), will chain react reducing the ketone group, removing the oxygen in the form of water and reducing the double bond, generating the saturated acyl chain. For each reduction reaction, one NADPH is oxidized. For each cycle of four reactions, a new malonyl-CoA molecule binds to FAS, further elongating the FA. One palmitate is produced by FAS after seven cycles of condensation, reduction dehydration and reduction (Lehninger *et* al., 2008). The synthesis of a palmitoyl-CoA molecule requires 58 ATP, thus being a pathway with a substantial consumption of reducing equivalents and energy. FA can only be catabolized to return part of the used energy to produce them. Although it seems that energy is being wasted, it is the contrary because FA are only synthesized when the basic body energy requirements are met, meaning that all the excess energy would be loss, ultimately saving energy. FA have low weight and they are efficient in terms of “storage” space compared to another molecules.

1.4.2. Fatty acid elongation and desaturation

For the purposes of this work we define 2 groups, saturated fatty acids (SFA) and unsaturated fatty acids (UFA). In this last category, we can distinguish monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). SFA are composed by simple bonds between the aliphatic carbons. UFA can have a double bond in the aliphatic chain and are called MUFA, or two or more double bonds therefore named PUFA. FA nomenclature indicates the number of the aliphatic chain carbons and the number and

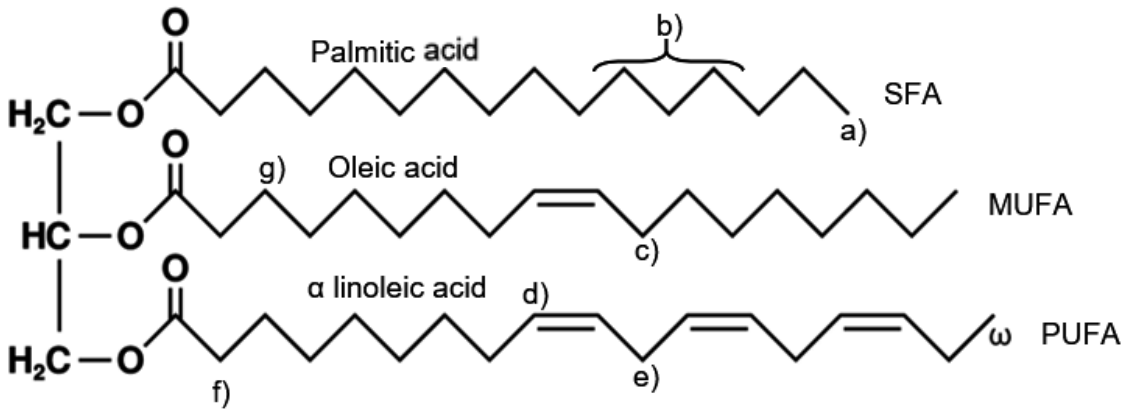

Figure 3 – Triacylglycerol schematic with palmitic acid, oleic acid and α linoleic acid chemical structures. Functional groups are represented by letters: a) methyl group; b) aliphatic chain methylenes; c) allylic group; d) olefinic group; e): bisallylic group; f): α methylene; g): β methylene.

position of double bonds. As an example, oleic acid (OA) (18:1-ω9) has a double bound between the 9th and 10th carbon counting from the methyl end (Fig. 3).

UFA and long-chain fatty acids (LCFA) are produced from palmitate by elongases and desaturases, with exception of small quantities of stearic acid (18:0) which can also be formed by FAS. Longer chains can be saturated (arachidic acid, 20:0), or monounsaturated (palmitoleic acid, 16:1; oleic acid, 18:1). Mammals cannot convert the OA to linoleic acid or α-linoleic acid since they do not have the enzymes to form double bonds between the carbon C-10 and the methyl end group, so these are essential FA. Omega-3 *de novo* biosynthesis is not present in vertebrates due to the lack of the necessary enzymatic components to form double bonds between the carbon C-10 and the methyl end group (Fig. 4), so these are essential FA. To date only plants, photosynthetic marine microalgae, heterotrophic protists, and bacteria account for natural ω3 LC-PUFA production (Nichols, 2003; Pereira *et* al., 2003). However, in a recent study with multiple metazoans (e.g. Rhabditida, Palpata and Heterobranchia), methyl-end desaturases (ωx desaturases) characterization showed that both Δ12 and Δ15 desaturase activities are present in organisms of this subkingdom, giving them the ability to produce ω3 PUFA *de novo.* One of the ωx desaturases described in cnidarians, molluscs, annelids, and crustaceans was able to produce a variety of ω3 LC-PUFA from

their corresponding ω6 precursors. Enzymatic activity confirms LC-PUFA-synthesizing by Δ15, Δ17 (*Δ9e*, fig. 4), and Δ19 (*C20e*, fig. 4) activity, previously unknown outside microbes (Kabeya *et* al., 2018).

Vertebrates, such as rats and rainbow trout, have important physiological needs for docosahexaenoic acid (DHA), and since they cannot *de novo* synthesize ω3, it can only be obtained in the diet or by further biosynthesis from an ω3 precursor. The biosynthetic process requires a Δ6 desaturation of 24:5-ω3 to 24:6-ω3, and is called "Sprecher pathway" (Sprecher *et* al., 1977). An independent pathway of the Sprecher pathway, described in Senegalese sole

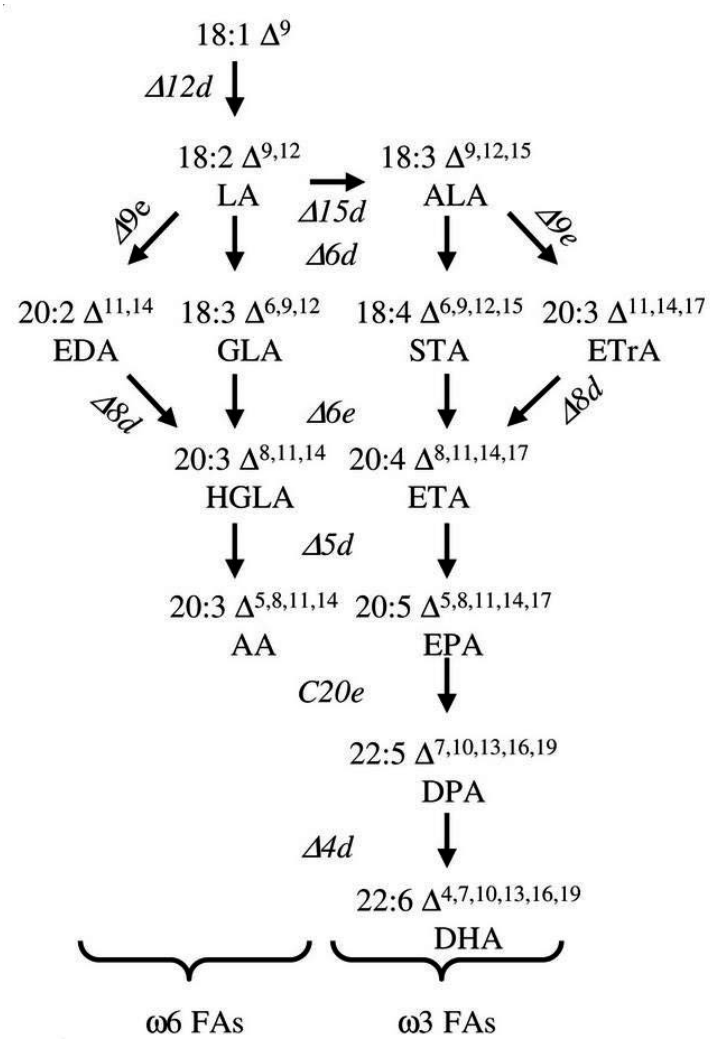

Figure 4 – ω3 LC-PUFA and ω6 LC-PUFA biosynthetic pathways. The Δ6 pathway (11, 12, 14) is represented by Δ6 desaturase (Δ6d)– Δ6 elongase (Δ6e)– Δ5 desaturase (Δ5d), whereas the alternative Δ9 elongase pathway (13) is represented by Δ9 elongase (Δ9e)– Δ8 desaturase (Δ 8d)– Δ5 desaturase (Δ5d). EPA is converted to docosahexaenoic acid by the action of C20 elongase (C20e) and Δ4 desaturase (Δ4d) (Damude *et* al., 2006).

(*Solea senegalensis*), presents a fatty acyl desaturase with Δ4 activity. This Δ4 desaturase enable the biosynthesis of DHA through a more direct route, synthesizing it from EPA (Morais *et* al., 2012).

1.4.3. Lipid metabolism in rainbow trout

The liver is considered the main lipogenic organ in many fish species, including salmonids (Henderson and Sargent, 1985). Rainbow trout liver FA composition when fed a marine diet is present in Fig. 5. Whole body FA composition, has a great percentage variability. Palmitic acid (16:0), oleic acid (OA) (18:1-ω9), linoleic acid (18:2-ω6) and DHA (22:6-ω3) are the major contributions in their respective groups (Caballero *et* al., 2001; Görgün and Akpinar, 2007). Liver and muscle total FA compositions tend to mimic the diet FA composition, but some fatty acids fluctuate between these two tissues. OA (18:1-ω9) is synthesized by the liver according to its presence in the feed, having an intermediate percentage between the diet and the percentage in the liver. Low OA intake levels show an induction of its biosynthesis. On the other hand, when levels of OA intake are high, they match the liver levels. PUFA levels greatly decrease in both tissues with exception of DHA, which levels are doubled despite of its low quantity in the feed (Caballero *et* al., 2001; Panserat, 2008). Eicosapentaenoic acid (EPA) can be a substrate for DHA production, and given the crucial importance of DHA in fish, it can be hypothesized that the higher expression of *Elovl2* (*C20e*, fig. 4) in fish fed a plant-base diet links to a preference given to the biosynthesis of DHA, instead of EPA (Lazzarotto *et* al., 2018). Analysis of gene expression by real time PCR showed that the removal of dietary fish oil is associated with induction of metabolic pathways involved in *de novo* lipid biosynthesis and elongation of very long fatty acids, proven by long-chain fatty acid-CoA ligase and desaturase (Panserat *et* al., 2008). Gene expression of Δ6-desaturase is higher in fish fed the plant-based diet, probably linked to a high dietary intake of linolenic acid and the absence of LC-PUFA in vegetable oils. In short, rainbow trout have the capacity to synthesize LC-PUFA from dietary precursors and DNL and lipid metabolism are tightly regulated by dietary protein and lipid levels.

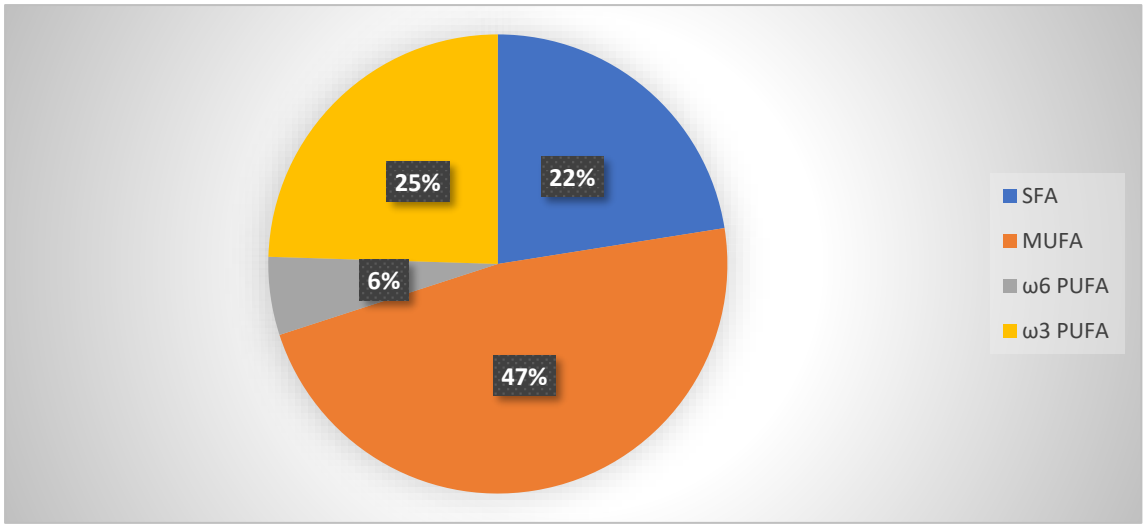

Figure 5 – Rainbow Trout SFA, MUFA, ω6 PUFA and ω3 PUFA approximate composition percentage in liver (Caballero *et* al., 2001).

In response to insulin infusion when fed a high-carbohydrate diet ACC and FAS activity are upregulated. Protein activity, protein abundance and mRNA abundance may or may not correlated with each other. *FAS* and *ACLY* mRNA have increased abundance at the 0.35 IU/kg^{-1} d^{-1} insulin dose while protein abundance and activity did not differ from control. The increase in protein abundances for 0.7 IU/ kg^{-1} d^{-1} insulin is not matched by the correspondent mRNA abundances. FAS and ACC abundance and enzymatic activity correlate on both insulin concentrations. Insulin increased DNL in carbohydrate-fed trout reinforcing the hypothesis that this pathway may act as an important outflow for excess glucose (Polakof *et* al., 2011b). High carbohydrate intake suppressed the expression of ACC, compared with a protein diet that induced higher FAS and ACLY expression. FA elongation is also potentiated by a high protein diet with higher expressions of *Elovl2* and *Elovl5* (represented in fig.4 as *Δ6e*). Overall, we conclude that unlike mammals, the expression of genes corresponding to FA synthesis in rainbow trout is more responsive to dietary protein intake than dietary carbohydrate intake during acute stimulations (Dai *et* al., 2016).

1.5. Deuterium as a tracer

Existing nuclear magnetic resonance (NMR) stable tracer methodologies can be adapted to the study of lipid metabolism. A metabolic isotope tracer is a molecule chemically and functionally identical to the natural molecule of interest and provides information on the tracee's metabolism following the tracer's fate (Wolfe and Chinkes, 2004). A metabolic tracer can be a stable or radioactive isotope labelled molecule.

Deuterium (^{2}H) is a useful, affordable, non-radioactive and stable tracer, isotope of hydrogen that has a nucleus composed of one proton and one neutron and it is easily delivered into the body. Deuterium has a different resonance frequency from hydrogen/proton (^{1}H) in NMR thus being possible to differentiate both in NMR spectra. High levels of ^{2}H are toxic because the strength between their bond to carbon is stronger than the ^{1}H bond and this causes slower metabolism rate. The administration of ^{2}H may be by intraperitoneal injection (Muller and Seelig, 1987) incorporation into drinking water (Jones *et* al., 1988) for mammals or immersion into swimming water for fish (Viegas *et* al., 2011) and as an in vitro culture medium (Berry *et* al., 2015). Once immersed, fish ^{2}H incorporation from deuterated tank water ($^{2}H_2O$) 5%-enriched into plasma (body) water is fast, reaching more than 1% in 15 minutes, reading half of the enrichment of the tank water within 1 hour and approaching the tank water enrichment after 6 hours (Viegas *et* al., 2011). The ^{2}H present on the deuterated water will be incorporated in metabolic reactions (direct or indirect hydration) involving ^{1}H from regular water, reaching an isotopic steady state point. Newly synthesized molecules involving these reactions will possess a percentage of ^{2}H, this process is called labelling.

1.5.1. Triacylglycerol ^{2}H enrichment

The resolution of lipid functional groups by nuclear magnetic resonance (NMR) in the chemical shift provides discrete positional enrichment as demonstrated in mice (Duarte *et* al., 2014) and fish (Viegas *et* al., 2016). Once administered the tracer, lipid synthesis through DNL and lipid metabolism

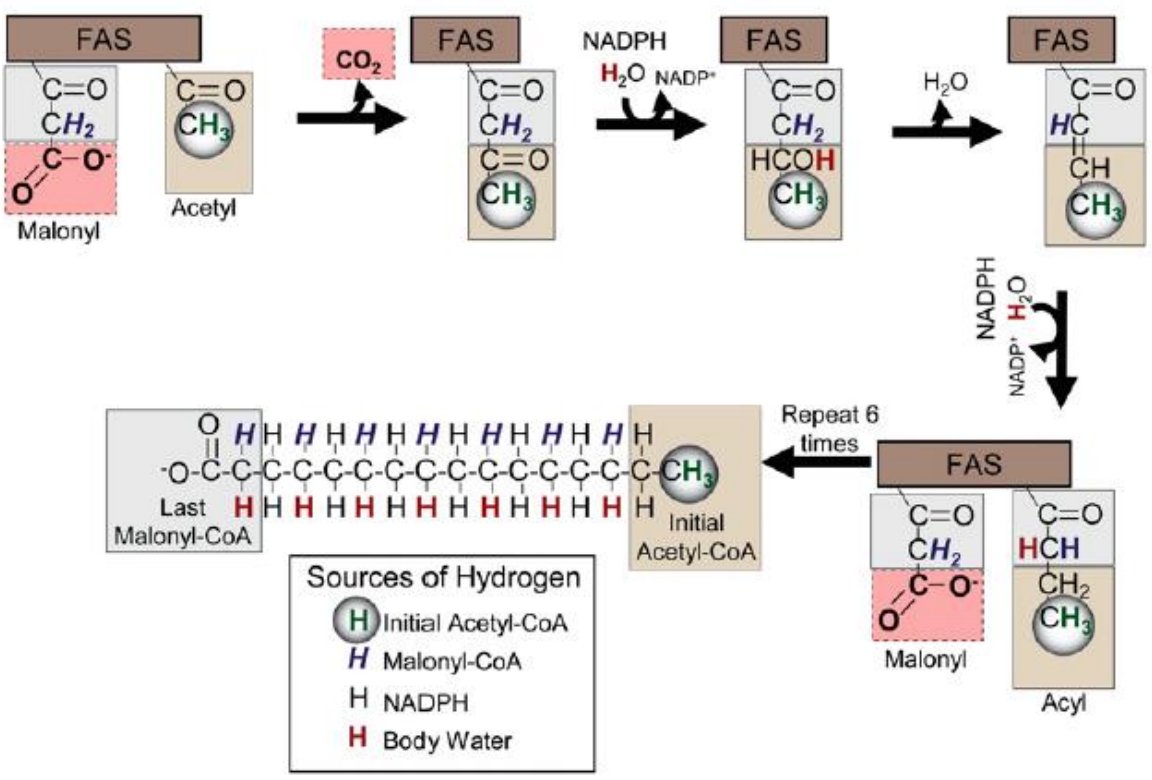

Figure 6 – Descriptive scheme from *de novo* lipogenesis in the presence of ^{2}H (Duarte *et* al., 2014).

(desaturation and elongation) can be assessed using $^{1}H/^{2}H$ NMR. Every hydrogen in the new palmitate comes from a different source. Hydrogens derived from the initial AcCoA unit (green) are localized in the methyl group of the FA. The ^{2}H labelling in the initial AcCoA comes from the citrate when aconitase backward scrambles the isocitrate molecule adding $^{2}H_2O$ in the process. The incorporation of deuterium into palmitate is regiospecific and occurs through the actions of FAS (Fig. 6). In the end on one elongation cycle, one hydrogen derived from $^{2}H_2O$ and one from malonyl-CoA are incorporated into each odd-numbered carbon of the nascent FA moiety. In each of the even-numbered carbons of the FA moiety, the two hydrogens come from NADPH. Thus, methyl enrichment occurs only during DNL, but enrichment in the allylic hydrogens occurs during DNL and as FAs are elongated (Fig. 6).

Fish are not able to synthesize *de novo* ω3 FA and so in this work only non ω3 FA synthesis will be studied and described.

2. Objectives

The objective for this project was to evaluate the impact of dietary glycerol in growth levels and lipid turnover of rainbow trout (*Oncorhynchus mykiss*).

In the first instance it was evaluated the zootechnical performance of the diets by performing a growth trial, using well establish growth parameters. Diets had different levels of inclusion of glycerol (0%, 2.5% and 5%).

Secondly, a metabolic trial was performed and information about liver lipid composition and overall liver quality was gathered, evaluating TAG composition using ^{1}H NMR. Metabolic fluxes of liver lipids were accessed by TAG glycerol turnover and synthetic rate of fatty acid (DNL) using deuterated water - $^{2}H_2O$ and ^{2}H NMR.

3. Materials and methods

The research done for this dissertation was performed in collaboration with Trás-os-Montes e Alto Douro University (UTAD). The growth-trial was developed at the Experimental Research Station (UTAD - Vila Real, Portugal).

3.1. Diet formulation

For both trials 3 different conditions, with different glycerol content, were used: basal diet (CTRL); glycerol at 2.5% (GLY 2.5) and glycerol at 5% (GLY 5.0). All the experimental diets were isoproteic (49% DM) and isoenergetic (21 kJ kg^{-1}). Glycerol used in the formulation of the diet (refined glycerin) was obtained from rapeseed (Belgosuc, 050008, Beernem, Belgium). The incorporation of cellulose at different levels in the formulation was used to create the dietary contrast for the addition of glycerol. Therefore, cellulose was used as an indigestible filler (Table 1).

3.2. Animal Trials

3.2.1. Growth-trial

Each treatment was made in triplicates (nine tanks) with 25 fish each (n=25) (Fig. 7a, b). The individuals selected for this first trial had 20.2±0.1g, and were randomly distributed among nine fiberglass tanks (300 L per tank). Natural groundwater from UTAD, with a water flow rate of 12 L min^{-1}, was circulating through the system during this trial which lasted for 60 days. Fish were fed two times a day (9 a.m., 5 p.m.) by hand until reach apparent satiety. Biomass was used to calculate and adjust the daily feed ration after 4 weeks. At the end of the 8th week fish were weight and water quality criteria was recorded (dissolved oxygen: 8.71 ± 0.02 mg L^{-1}; pH: 6.68 ± 0.01; ammonia: < 0.05; nitrite: 0.5 mg L^{-}

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

Table 1 – Rainbow trout fed composition of control (CTRL), 2.5% glycerol supplemented (GLY 2.5) and 5% glycerol supplemented (GLY 5.0).

[1]). The trial was completed between August-October 2017, in which the fish were exposed to natural temperature (15 ± 1 ºC) and photoperiod. Total feed consumption and mortality data were daily recorded for each tank.

3.2.1.1. Fish sampling

For the growth trial, all the fish were individually weighed at the end of the trial (60 days). Fish were anaesthetised using well aerated water from the rearing system (15 L container) containing MS222 (3-aminobenzoic acid ethyl ester Sigma E 10521, 0,1 g L^{-1}) buffered with NaHCO3 (0.2 g L^{-1}). Fish were euthanized by slicing of the cervical spine and used for assessing the growth parameters described in section 3.2.1.1.

3.2.1.2. Growth parameters analysis

Parameters for fish growth and feed utilization were calculated per treatment (n=3 tanks).

Weight gain was calculated as:

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

Where IBM and FBW are the initial and the final body weight of fish in the trial, respectively.

Daily growth index was calculated as:

$$\mathrm{DGI\ (\%\ BW\ day)} = 100 \times \frac{\mathrm{FBW}^{1/3} - \mathrm{IBW}^{1/3}}{t}\ (eq.2)$$

Where time (t) is the length of the trial (60 days).

Specific growth rate was calculated as:

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

Voluntary feed intake per percentage body weight was calculated as:

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

Where 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 BW of fish (g) calculated as:

$$\frac{\text{IBW} + \text{FBW}}{2} (eq.5)$$

Absolute feed intake was calculated as:

$$\text{FI}_{\text{abs}} = \text{FI} \times \frac{\text{FI}}{\text{n} \times \text{t}} \ (eq.6)$$

Where n is the number of fish per tank corrected for dead fish.

Feed conversion ratio was calculated as:

$$\text{FCR} = \frac{\text{FI}_{\text{abs}}}{\text{G}_{\text{day}}} \ (eq.7)$$

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

Feed efficiency was calculated as:

$$\text{FE} = \frac{\text{G}_{\text{day}}}{\text{FI}_{\text{abs}}} \ (eq.8)$$

Fish survival was calculated as:

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

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

The hepato-somatic index was calculated as:

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

Where both weights were in g.

3.2.2. Metabolic trial

Once the growth trial was completed, twelve fish (n=12) from each condition, totalling 36 from all conditions, were selected at random and transferred to new tanks for the metabolic trial (Fig. 8c). Three interconnected tanks were used for this trial. The system was kept closed to prevent the evaporation of ^2H_2O. Filters were watertight and had the ammonia cycle stabilized and matured prior to the study. The water used for this trial came from the same source as the growth trial. ^2H_2O was added until the proportion of ^2H_2O (99%, Cambridge Isotope Laboratories Inc. through TracerTec), reached 4%. 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. The fish were fed at 9 a.m. until reached apparent satiety. Tank water temperature, salinity, pH, dissolved oxygen, NH_4^+, NO_3^- and NO_2^- were assessed at the beginning and end of the trial. This trial was performed in duplicate, and the samples collected in the first week were added to the ones of second week. Samples was collected at natural temperature (October 2017) after the last meal.

3.2.2.1. Fish sampling

For the metabolic trial (6-day residence in 4%-enriched ^2H_2O) all the fish were individually weighed and anaesthetised using well aerated water from the

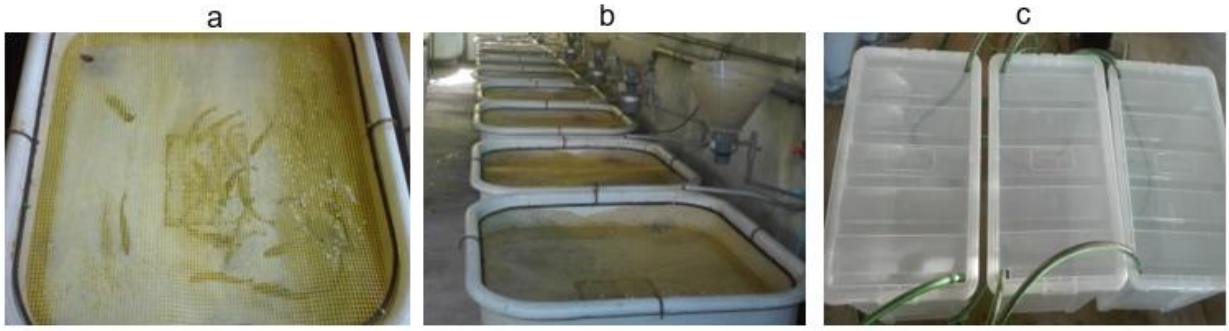

Figure 7 – Rainbow trout growth trial (a and b), and metabolic trial tanks (c).

rearing system (15 L container) containing MS222 (3-aminobenzoic acid ethyl ester Sigma E 10521, 0,1 g L^{-1}) buffered with $NaHCO_3$ (0.2 g L^{-1}) and 4%-enriched ^2H_2O. Fish were euthanized by slicing of the cervical spine. Blood was collected from the caudal vein with heparinized 2.5 mL syringes with a 23G needle (Fig. 8a, b), an aliquot was centrifuged at 10,000x*g* for 10 min and plasma stored at -20ºC. Liver was dissected, washed with a physiological saline solution (9 g NaCl L^{-1}) (Fig. 8c), weighted, freeze clamped and grouped in pools two into 18 falcons and stored in liquid nitrogen (N_2). The tissue was then pulverized with liquid nitrogen by grinding and stored at -80°C. Quantifications of glycerol, plasma glucose and TAG were performed in a fully-automated analyzer Miura 200 (I.S.E. S.r.l.; Guidonia, Italy) using its dedicated reagent kits (ref. A-R0100000601 and A-R0100000901, respectively). Quantification of plasma glycerol was performed following the instructions of the provider (Sigma, MAK117).

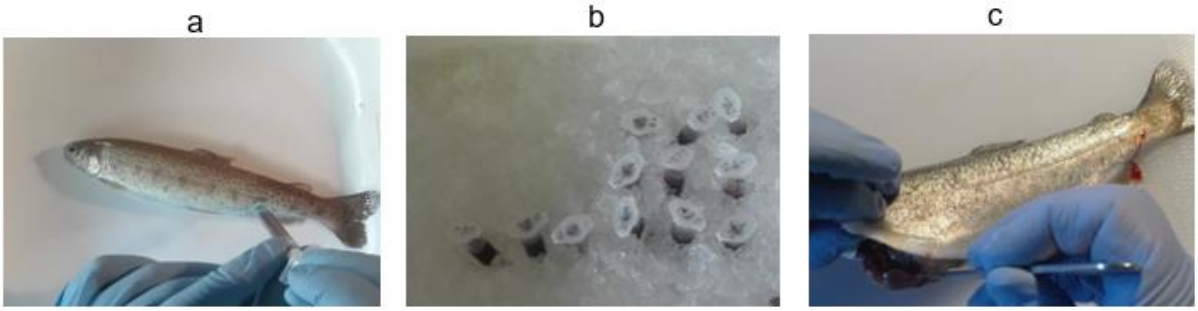

Figure 8 – Rainbow trout blood extraction (a), blood sample collection (b), and liver extraction (c).

3.2.2.2. Triacylglycerol extraction

Methanol (4,6 mL/g) was added to the pulverized frozen sample tube and vortexed. Then, 15,4 mL/g of methyl-tert-butyl ether (MTBE) was added and the mixture was incubated for 1h at room temperature in a shaker, under agitation with a magnet. Phase separation was induced by adding 4 mL of distilled water and let rest for 10 min at room temperature. Upon incubation, the sample was centrifuged at 1,000 g for 10 min. The upper (organic) phase was collected, transferred to a glass vial (dark glass or covered with aluminium foil) and dried in fume hood (hotte) under nitrogen stream or air-evaporate until dry. The lower (water) phase was collected, and carefully separated from the upper fraction, transferred to a vial, freeze and lyophilized (Matyash *et* al., 2008). Reagents specifications are listed in appendix.

3.2.2.3. Triglycerides 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 resuspended in 600 mL of hexane:$CHCl_3$:MeOH (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). TLC's are revealed using a solution of Petroleum Ether/Diethyl Ether/Acetic Acid (7:1:0.1) and with iodine vapor. 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.

3.3. Triacylglycerol data acquisition

3.3.1. ^{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 19F 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.

For ^{1}H acquisition, a spectral width of 10 kHz was used in conjunction with the acquisition time of 3 s with a pulse delay of 5 s. Line broadening was 0.1Hz. The samples were run at a temperature of 25ºC. Peak quantification in NMR was processed in ACD labs software v12.0, Advanced Chemistry Development, Inc.

3.3.2. ^{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 run at a temperature of 25ºC with 0.37 s of acquisition time and a pulse delay of 0.1 s. Line broadening was 1.0 Hz.

Body waters from all the plasma samples were run right before the correspondent liver sample. 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 ^{2}H 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).

Peak quantification in NMR was processed in ACD labs software v12.0 Advanced Chemistry Development, Inc (Toronto, Canadá).

3.4. Triacylglycerol data analysis

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

ω3 FA:

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

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

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:

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

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

The percentage of MUFA's 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 ^{2}H NMR. Briefly, 10 µL of plasma was added to 1000 µl of calibrated acetone. Then, transfer to the NMR tube 490 µL of the mixture and add 40 µL of hexafluorobenzene. Following sample preparation, a ^{2}H 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. 17 the corrected methyl enrichment (A_{eC}) is given by:

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

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), $\%^{1}HS$ 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:

Desaturation rate 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 ^2H_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 the α protons reports the percentage of fatty acid chains that were elongated versus de novo synthesis. Therefore, the elongation rate is given by:

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

Where Ae is the deuterium enrichment of the terminal methyl group of fatty acids and He is the deuterium enrichment of the α protons of FA.

Triacylglycerol bound glycerol synthesis:

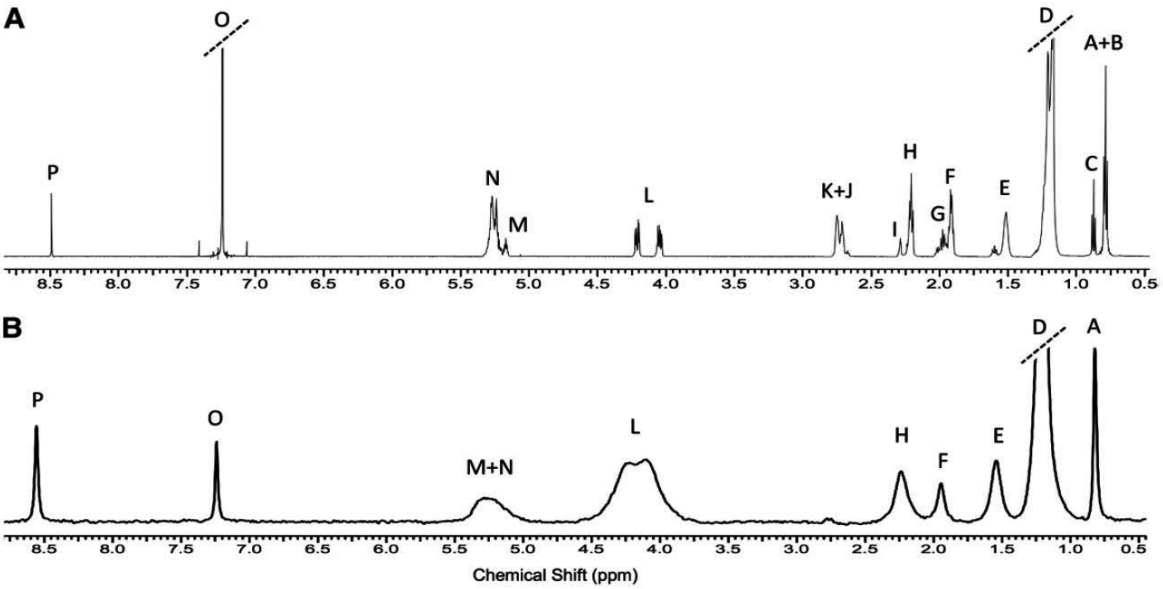

Figure 9 – ^{1}H (A) and ^{2}H (B) chemical shift assignment to the correspondent NMR spectra (Viegas *et* al., 2016). Non ω-3 methyl (A); partial ω-6 methyl (B); ω-3 methyl (C); aliphatic chain methylenes (D); β methylenes (E); monounsaturated allylic hydrogens (F); polyunsaturated allylic hydrogens (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).

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

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

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

3.5. Statistical analysis and calculations

To verify differences, 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 ± error of the mean (SEM). Statistical analysis was conducted with GraphPad Prism v7.0, GraphPad Software (San Diego, California).

4. Results

4.1. Growth trial

The 8-week growth parameters determined are summarized in Table 2. There were no significant differences (P > 0.05) in the growth performance of rainbow trout juveniles between the different supplementation levels of glycerol in diet after eight weeks. The final weight varied between 77.00 g (GLY 5) and 91.46 g (CTRL); weight gain was the lowest and greatest in these treatments. GLY 2.5 and GLY 5 fish had similar HSI values, while CTRL had lower, but not significant values. Similar to HSI, the IBW throughout the 3 conditions did not differ from each other. The FCR was significantly higher in the GLY 5.0. With regard to FE, a decrease in efficiency can be noted in GLY 5.0. During the growth trial, no significant differences were found in survival rates between diets (99,1% overall, throughout the period; data not shown).

Diet	HSI	IBW (g)	FBW (g)	FCR	FE
CTRL	0,95 ± 0,09	20,3 ± 0,1	87,5 ± 3,6	0,98 ± 0,04 [a]	1,02 ± 0,04 [b]
GLY 2.5	1,08 ± 0,19	20,1 ± 0,1	85,3 ± 5,0	0,99 ± 0,04 [a]	1,01 ± 0,04 [b]
GLY 5.0	1,06 ± 0,12	20,2 ± 0,1	79,4 ± 2,4	1,14 ± 0,05 [b]	0,88 ± 0,04 [a]

Table 2 – Initial body weight (IBW), final body weight (FBW), feed conversion ratio (FCR) and feed efficiency (FE) of rainbow trout at 8 weeks of growth. Mean values ± SEM are presented (n = 3). Significant differences between dietary conditions are indicated by different letters (one-way ANOVA followed by Tukey's test; P < 0.05).

4.2. Metabolic trial

4.2.1. Body water

Tank water was kept at 3.9% ^{2}H-enriched throughout the two replicates. The body water ^{2}H-enrichment did not differ 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.

4.2.1. Blood parameters

Glucose and glycerol had no significant differences among treatments. Plasma TAG levels for GLY 5.0 were significantly higher compared with CTRL and GLY 2.5, who had almost identical values (Table 3).

Diet	Glucose (mg/dL)	TAG (mg/dL)	Glycerol (mM)
CTRL	66,00 ± 9,83	194,0 ± 57,8 [a]	0,10 ± 0,04
GLY 2.5	65,67 ± 9,87	194,1 ± 45,2 [a]	0,08 ± 0,02
GLY 5.0	71,33 ± 8,17	246,0 ± 46,0 [b]	0,09 ± 0,02

Table 3 – Glucose, triacylglycerol (TAG) and glycerol of rainbow trout plasma at 8 weeks of growth. 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; $P < 0.05$).

4.2.2. Hepatic triacylglycerol extraction

Two column extraction methods were used to perform TAG purification. The samples were purified by the first extraction method and 3 samples, one of

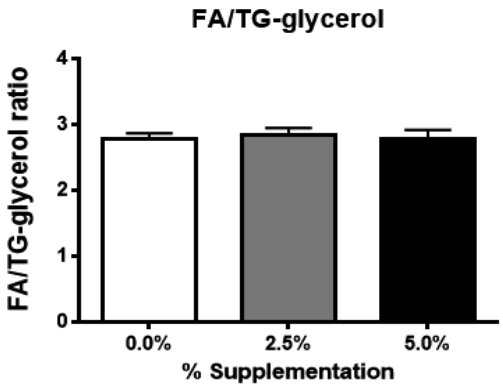

Figure 10 – TAG-bound glycerol expressed as percent of newly synthesized glycerol per day in hepatic TAG. Mean values ± SEM are presented (n = 6).

each treatment conditions, were selected and analysed by ^{1}H NMR. According to the data in Fig. 10B, a great deal of impurities can be spotted in the spectra, making it impossible to quantify certain groups, such as MUFA allylic hydrogens and PUFA allylic hydrogens (Fig. 11 B1). After the second purification, less signal-to-noise ratio, less interference and few overlapping signals could be observed (Fig. 11 A1). Impurities, although few and non-quantification impacting, were consistently present in aliphatic chain methylenes, β methylenes and PU allylic hydrogens. The FA/TAG-glycerol ratio was consistent and was around 3 in all the diets, expected from a successful TAG separation (Fig. 10).

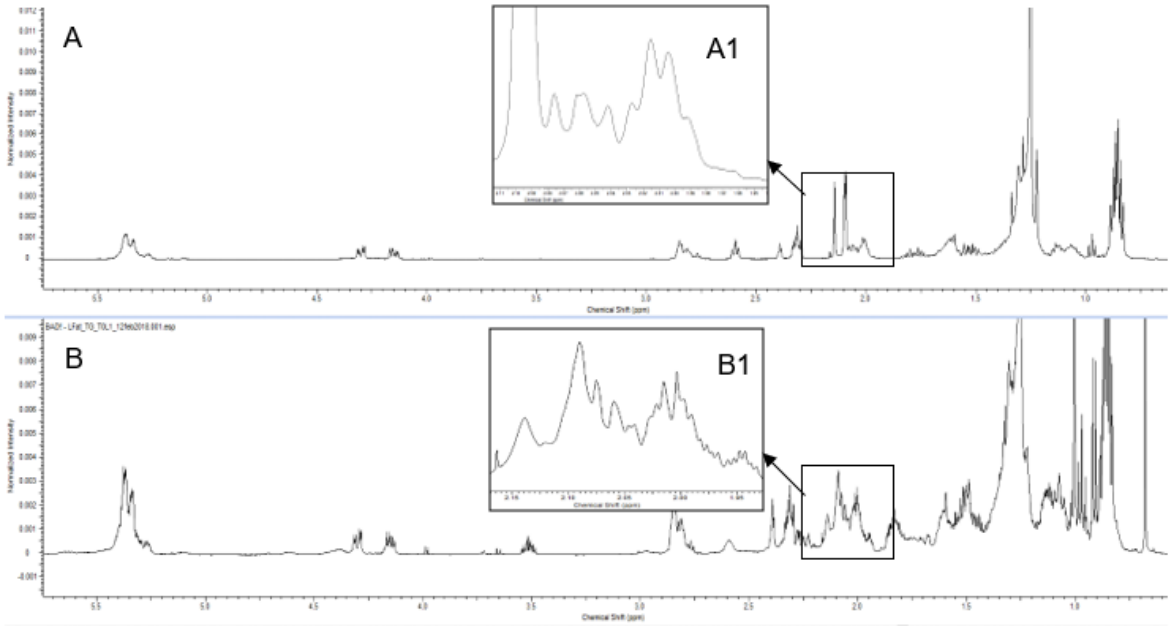

Figure 11 – A, ^{1}H NMR after the both purification methods; B, ^{1}H NMR after the first purification method; A1 and B1 MU allylic hydrogens and PU allylic hydrogens from the respective spectra.

4.2.3. Hepatic triacylglycerol profile

The effect of the glycerol supplemented diet on TAG levels was evaluated. MUFA and PUFA in all treatments did not show variability in their levels. The percentage of SFA, despite not being statistically different, in the

Diet	CTRL	GLY 2.5	GLY 5
ω3	7,32 ± 0,88	7,40 ± 0,43	7,21 ± 0,96
Non ω3	92,68 ± 0,88	92,59 ± 0,43	92,78 ± 0,96
PUFA	36,79 ± 1,75	33,20 ± 1,31	35,35 ± 2,84
MUFA	39,33 ± 3,80	42,50 ± 0,94	45,32 ± 1,53
UFA	76,13 ± 3,99	75,70 ± 1,01	80,67 ± 1,70
SFA	23,87 ± 3,99	24,30 ± 1,01	19,33 ± 1,70
DHA	11,62 ± 0,75	10,39 ± 0,86	12,45 ± 0,72
LO	13,49 ± 0,38 [a]	12,79 ± 0,39 [a]	10,47 ± 0,31 [b]

Table 4 – Liver linoleic acid, ω3 FA, non ω3 FA, PUFA, MUFA, UFA, SFA and DHA percentages among treatments. 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; $P < 0.05$). Mean values that do not share a common letter are significantly different ($p<0.05$).

GLY 5.0 diet has a tendency to decrease in relation to the CTRL and GLY 2.5. The linoleic acid (18:2-ω6) levels were lower in fish fed the GLY 5.0, compared to those fed the CTRL and GLY 2.5 diets. Percentage of DHA and ω3 FA had no significant differences between treatments (Table 4). The liver FA composition had no significant variance among treatments.

4.2.4. Metabolic fluxes

Metabolic fluxes are shown at Fig. 12. The elongation percentage was significantly higher for GLY 5.0 compared with GLY 2.5, but neither group was significantly different from CTRL. High fluctuations were observed on GLY 5 for elongation, desaturation and DNL. Even though desaturation did not show differences among treatments, intriguing results were displayed with a clear upwards in 5% glycerol supplemented diets is present. Concerning the average number of carbons per FA, no significant differences related to either diet

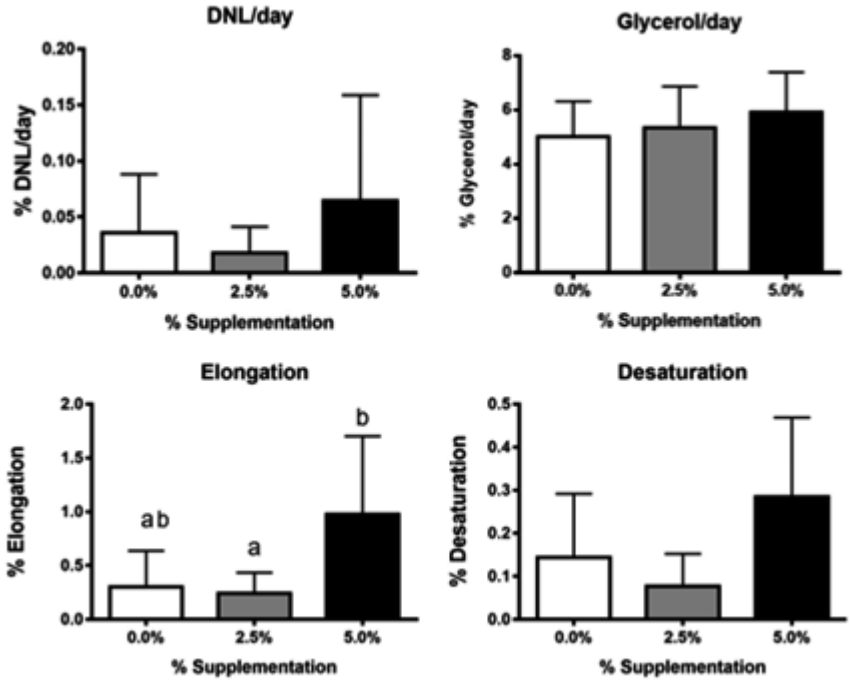

Figure 12 – *De novo* lipogenesis (DNL) per day, Glycerol per day, elongation, desaturation percentages for each treatment. Data are means ± standard deviation (n = 6 individuals/dietary treatment). Statistical differences were determined by one-way ANOVA followed by Tukey's comparison test. Mean values that do not share a common letter are significantly different ($p<0.05$).

keeping in mind that samples that did not show any ^{2}H NMR signal were excluded. Surprisingly, *de novo* glycerol production observed by ^{2}H NMR increased, albeit not statistically significant, proportionally over the increase of supplemented glycerol (Fig. 12).

5. Discussion

Our experimental growth results indicate that rainbow trout can use approximately 5% glycerol in the diet ($p > 0.05$), which is similar to or lower than other animal results. It has already been reported that adding 12% glycerol to low-energy (low-fat) diets did not affect rainbow trout weight gain (Menton *et* al., 1986). Glycerol supplementation studies on other carnivorous fish such as channel catfish (*Ictalurus punctatus*) (Li *et* al., 2010) and Nile tilapia juveniles (Neu *et* al., 2013) found that 10% glycerol and 100 g kg^{-1} glycerol, respectively,

can be used in the diet without affecting growth. Interestingly, juveniles of *Prochilodus lineatus* had fluctuations in their weight gain and from 12% incorporation, the weight gain decreased as the inclusion levels of crude glycerin increased (Balen *et* al., 2017).

Survival rate in all diets tested was greater than 99%. Similar values were described for juveniles of Nile tilapia fed with increasing levels of glycerol (Gonçalves *et* al., 2015; Moesch *et* al., 2016). It is also worth noting that different results for the same species, but in different stages of development, have been described by Neu *et* al. (2012), where the survival of Nile tilapia (*Oreochromis niloticus*) fingerlings was higher in treatment that did not contain glycerol. In light of these results, the response of the animal metabolism to the increased glycerol level in diets may be influenced by differences species and developmental phases. Nevertheless, other factor such as temperature and the tested food source may as well have contributed to this occurrence.

Despite isoenergetic diets with a protein/energy balance that met the needs of the species in this study, FCR was higher on 5% supplementation (GLY 5.0), and thus not being related to studies on carnivorous fish already mentioned at this supplementation level. The isoenergetic state of the feeds was achieved by balancing the glycerol and the cellulose levels, which may have led to the differences recorded.

The liver is the main organ with regard to central metabolism, playing a role in digestion and nutrients absorption and, therefore, monitoring of this organ is essential. HSI is directly correlated with the deposits of lipids and glycogen in the liver. Researchers found a reduction of lipids in the liver with increasing levels of crude glycerol (Li et al., 2010) in catfish, and that crude glycerol was not an effective precursor of lipogenesis or promoted glycogen storage in the liver of rainbow trout (Menton *et* al., 1986). However, in the present study, different levels of glycerol supplementation lead to no alteration on the HSI, which coincide with recent results for juveniles of Amazon catfish (Rôxo *et* al., 2018).

It is relevant to mention that two lipid extraction/purification were performed. This was due to the amount of impurities that were still present after

the first one, making it impossible to quantify crucial TAG groups. Although the desired purity level was achieved, a certain amount of signal was also lost, making certain groups whose levels are already inherently lower, difficult to quantify with accuracy, mainly in deuterium spectra, which is already at tracer level. From all the lipid fluxes addressed in this study, only the elongation was increased on GLY 5.0.

Glycerol is readily incorporated at a cellular level, as demonstrated in glycerol plasma levels. Carnivorous fish have a highly active gluconeogenic activity, relying mostly on protein substrates, to maintain a constant glucose level (Panserat and Kaushik, 2010). Because of this evolutionary trend in regards to a high protein, gluconeogenesis is poorly regulated and it was expected an increase in blood glucose levels, which it was not the case. Surprisingly, blood TAG levels were higher in GLY 5.0 although there was no increase in the percentage of DNL and lipid content in the liver, with exception of linoleic acid. The lack of TAG differences in the liver lead to induce that the TAG present in the blood come from a different source than the liver. The non-existent differences in liver DNL, in a primary evaluative stage, can assure the quality of the filets produced from fish with diets supplemented with glycerol, because the amount of fats exported by the liver remains the same. ATP is a major contributor for the energetic needs in cellular growth and since the final weights between treatments had no differences, it's unlikely that the Krebs cycle is fuelled by the added glycerol. Glycerol might also be converted to glycogen, and it seems to be the most plausible pathway in which it might be incorporated on the liver.

Despite the lack of significant differences in the liver metabolites and substrates, it is possible to perceive the integration of glycerol in this organ and assume that the main channelling of glycerol might not be to other parts of the fish like muscle and perivisceral adipocytes.

6. Conclusion

The rainbow trout can metabolize up to 2,5% of glycerol. Considering that no significant changes in the animal's zootechnical parameters, body composition, hematological and biochemical parameters have been observed up to this level.

Regarding the relevance of this study to aquaculture, it is proven that a supplementation of glycerol up to 2,5% does not promote *de novo* lipogenesis in the liver and it does not change the relative composition of the TAG.

Further studies including another organs of fish and/or featuring a substitution instead of a supplementation can widen the spectrum of knowledge to date.

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Appendix

Acetic acid ≥99.5%	**Sigma**
Chloroform	PanReac
Deuterated Water (99.8%)	CortecNet
Diethyl ether	Sigma-Aldrich
Discovery DSC-NH2 12mL, 2gm - 52641-U	Supelco
Discovery SPE DSC-Si Silica Tube 12mL, 2gm - 52657-U	Supelco
Hexafluorobenzene	Sigma
Hexane, approx. 95%	Fisher Chemical
Methanol ≥99.8%	Sigma-Aldrich
MTBE 99,9%	Sigma
Petroleum ether 60/80	Alfa Aesar
Pyrazine	Alfa Aesar
NMR sample tube 5MM Economy, 7'', 500 MHz	Wilmad LabGlass