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## REVIEW

# Glycerol supplementation in farmed fish species: A review from zootechnical performance to metabolic utilisation

Margarida Coelho<sup>1</sup>  | Mariana Palma<sup>2</sup>  | Ludgero C. Tavares<sup>3</sup>  |  
Stéphane Panserat<sup>4</sup>  | Ivan Viegas<sup>2</sup>  | Leonardo J. Magnoni<sup>5</sup> 

<sup>1</sup>Department of Life Sciences, University of Coimbra, Coimbra, Portugal

<sup>2</sup>University of Coimbra, Centre for Functional Ecology, TERRA Associate Laboratory, Department of Life Sciences, Coimbra, Portugal

<sup>3</sup>CIVG – Vasco da Gama Research Center, University School Vasco da Gama – EUVG, Coimbra, Portugal

<sup>4</sup>INRAE, Université de Pau et des Pays de l'Adour, Exp2S UPPA, Saint-Pée-sur-Nivelle, France

<sup>5</sup>Plant and Food Research Centre, The New Zealand Institute for Plant and Food Research Limited, Nelson, New Zealand

## Correspondence

Leonardo J. Magnoni, Plant and Food Research Centre, The New Zealand Institute for Plant and Food Research Limited, 293 Akersten St. Nelson 7010, New Zealand.  
Email: [leonardo.magnoni@plantandfood.co.nz](mailto:leonardo.magnoni@plantandfood.co.nz)

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## Abstract

Glycerol, an abundant by-product of biodiesel production, has gained attention due to its price and availability for potential commercial applications, and thus utilisation as an animal feed ingredient. This article comprehensively reviews glycerol utilisation in fish and its potential as a dietary ingredient for aquaculture. While dietary inclusion of glycerol may offer cost-effective energy and metabolic intermediates, studies report inconsistent results regarding its effects on nutrient digestibility, zootechnical performance, and product quality. Recent studies however have demonstrated that dietary glycerol supplementation in fish induces metabolic shifts, such as alterations to gluconeogenesis and/or lipogenesis, modifying energy utilisation. Additionally, glycerol has been proposed to reduce protein catabolism, minimising nitrogen excretion and its environmental impact, but its influence on protein retention remains uncertain. Nevertheless, it is important to carefully consider the balance between feed palatability and these potential metabolic alterations when incorporating glycerol in aquafeeds. This review highlights the need for more studies to expand our understanding of glycerol metabolism in fish, since it does not seem to be metabolised by carnivorous species as much as omnivorous. Future research should explore the effects of glycerol supplementation on fish with different feeding habits and in developmental stages, as well as diverse environmental salinities and temperatures. Insight into the impact of impurities and the optimisation of glycerol inclusion in aquafeeds are recommended to support sustainable aquaculture practices and the utilisation of glycerol as a valuable resource.

## KEYWORDS

aquafeeds, energy utilisation, fish metabolism, glycerine, lipid deposition

## 1 | INTRODUCTION

Glycerol (C<sub>3</sub>H<sub>8</sub>O<sub>3</sub>; IUPAC name: propane-1,2,3-triol; alternative names: glycerin or glycerine; CAS: 56-81-5; HMDB0000131; ChEBI:

Margarida Coelho and Mariana Palma are joint first authors.

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17754; MW: 92.0938 g/mol) is a simple sugar alcohol of low molecular weight that is water-soluble, colourless, odourless, viscous, hygroscopic, mostly non-toxic, and sweet-tasting compound. It is involved in several metabolic processes, and it constitutes the backbone of the vast majority of vegetal oils and animal fats.<sup>1</sup>

Until recently, glycerol production was dominated by petrochemical synthesis and saponification of fats. However, the acceleration of the biodiesel market in the early 1990s, reinforced by the fuel crisis (increasing prices of crude oil along with its evident environmental footprint), boosted the production and use of biodiesel from oils and fats as an alternative to fossil fuels.<sup>2</sup> This process involves a transesterification reaction, which yields crude glycerol as a by-product, representing nearly 10% in weight<sup>3–6</sup> and is nowadays responsible for the majority of the available glycerol.<sup>7</sup> Global biodiesel production is projected to increase from 36 billion litres in 2017 to 66.9 billion litres by 2032.<sup>8</sup> As consequence it became crucial to find and develop new commercial applications that could take advantage of this product and improve the sustainability of the biodiesel industry<sup>3</sup> in the context of circular economy. Depending on its production process, treatment and purification level, glycerol can be classified as crude, purified/refined, and pure/commercially synthesised.<sup>3,9</sup> This spectrum of products reflects on its nomenclature where glycerol is the term used for the pure compound whereas glycerin (or glycerine) refers to commercial grades, irrespective of their purity. Glycerol purity from biodiesel production will depend greatly on source of feedstock (edible oils or purified used cooking oils vs. waste-based non-edible feedstocks) and is estimated to be between 60 and 80%, way below technical grade requirements.<sup>10</sup> The remaining glycerol is supplied mostly by the soap or fatty acid industries, available already at high purity since instead of transesterification reactions, require hydrolysis and saponification, which yield purer glycerol contents. Price will invariably reflect this grading, to the point that crude glycerol (80%, vegetable-based) can range from 240, 295, or 395 €/tonne, in United States, China, and Europe, respectively; to refined glycerol (99.5%, technical grade) ranging from 660, 505, or 520 €/tonne, in United States, China, and Europe, respectively.<sup>7</sup> Grading and purity, more than the increase in volume of production per se could be the main drivers for glycerol utilisation as ingredient for animal feed.

In this review, we will refer to this compound generally as glycerol along with the grade of purity whenever available in the cited literature. Crude glycerol possesses low commercial value because of a higher presence of impurities, which may include variable quantities of methanol, fatty acids, methyl ethers, and acid or alkali catalysts used in the production of biodiesel.<sup>11</sup> However, crude glycerol can be refined at an added cost to eliminate them through filtration, chemical additions, or fractional vacuum distillation, yielding diverse commercial grades for different applications.<sup>12</sup> Currently, large-scale biodiesel producers refine crude glycerol to be used in the food, feed, pharmaceutical, and cosmetic industries, but pure glycerol is still used in the production of pharmaceuticals, cosmetics, food additives, beverages, textiles, and lubricants, among others.<sup>3</sup> After its approval by the European Food Safety Authority and the U.S. Food and Drugs Administration as a food additive safe for animal and human consumption,

glycerol has been explored mainly as a food supplement for terrestrial farmed animals. Crude glycerol has been proposed to replace other feed ingredients with similar energetic and metabolic value, mainly carbohydrates (e.g., corn), in several groups of livestock due to its wide availability and price.

Dietary inclusion of glycerol may provide metabolic intermediates for biosynthetic processes, such as glycerol-3-phosphate. These biosynthetic mechanisms have long been addressed in mammalian models<sup>13</sup> and since then an extensive body of literature has been published aiming to unravel glycerol transport,<sup>14–16</sup> alterations in plasma parameters,<sup>17</sup> and its role in metabolic hepatic pathways during fasting,<sup>18,19</sup> exercise,<sup>20</sup> cancer,<sup>15,21</sup> and diet-related diseases.<sup>22,23</sup> These pathways and mechanisms are highly conserved among vertebrates<sup>24</sup> and are, as foundation for the “Glycerol Metabolism” section below, considered valid for fish species. The potential of glycerol as ingredient and hence direct energy source has been evaluated in several production animals such as swine,<sup>25</sup> cattle,<sup>26–28</sup> goats,<sup>29</sup> poultry,<sup>30,31</sup> and fish.<sup>32</sup> Silva et al. have reviewed the use of dietary glycerol in livestock including pigs, laying hens, and broilers.<sup>33</sup> More recently, the use of dietary glycerol in mammals, particularly in ruminants, has been reviewed by Kholif et al.<sup>34</sup> These reviews highlight the apparent disparity in results, since in some cases glycerol improved nutrient digestibility, whereas, in the studies with ruminants, a reduction on the digestion of dietary fibre was observed. There were also variable reports of the effects of dietary glycerol on animal performance and the quality of milk, eggs, and carcasses. However, the inconsistencies observed in these studies may be attributable to different glycerol sources as well as to the diversity in technical grades of glycerol available in the market.<sup>34,35</sup> This appears to be an important factor to take into consideration as dietary inclusion of glycerol may affect the digestibility of animals, particularly of cultured fish. In a scenario where feed formulations result from a complex mixture of ingredients to reach a balanced nutritional composition, differences between studies resulting from different basal formulation cannot be completely disregarded as certain lipid sources may include higher levels of glycerol to start with (e.g., soybean oil). Differences within studies are relatively easier to interpret since glycerol levels normally differ only on the supplementation to the basal (control) diet. Given the increasing application, or at least potential application of glycerol as ingredient for aquafeeds at a larger scale, this review aims to present the current knowledge on the zootechnical performance of fish species fed with glycerol-supplemented diets. Despite valuable contributions and insight, studies with fish larvae and/or post-larvae were not included in the present review.<sup>36–39</sup> In the following section, this review tackles how glycerol is incorporated into aquafeeds (its properties, intake and digestibility), its impact on growth performance, nitrogen excretion and somatic indices. In order to further understand its caloric and nutritional value, this review also discusses the digestion, involvement, utilisation, and consequently the metabolic effects of dietary glycerol supplementation in carnivorous and omnivorous fish separately. Taken together, this document aims to establish future paths for research for this ingredient in fish aquaculture.

## 2 | GLYCEROL IN AQUAFEDS: PROPERTIES AND INTAKE

One crucial consideration in aquafeeds involves maintaining feed consistency, cohesion, and appeal through the incorporation of glycerol in an aqueous medium. Due to its viscous properties, dietary glycerol can improve the flexibility, palatability, and binding properties of extruded diets. This may also be related to its hygroscopic properties and humectant action, which allows the absorption and/or maintenance of moisture from the atmosphere thus improving water retention in feeds.<sup>40</sup> Simultaneously, its emollient properties may improve the viscosity and consistency of the feed, while its capacity to resist freezing can be explored to stabilise the product during shipping and storage, as well as to increase shelf-life, particularly in colder environments. Increased moisture has shown to positively impact feed intake, contributing to a faster intestinal transit and evacuation,<sup>41</sup> but whether glycerol incorporation can provoke or enhance such effects is yet to be evaluated. On the other hand, accelerated microbial spoilage and/or oxidation of aquafeeds including glycerol should be investigated when stored at suboptimal conditions such as elevated humidity and temperature. The stability of glycerol on aquafeeds is yet to be addressed; however, in a biopharmaceutical context, it is considered chemically inert and for this reason often used as an excipient.<sup>42</sup> In this scenario, active impurities resulting from glycerol degradation were only detected after repeated use and improper storage (at 40°C for 6 weeks),<sup>43</sup> which should be unrealistic for aquafeed. The use of unrefined glycerol and the interaction of its impurities with other ingredients should be considered a more relevant matter for aquaculture than the oxidation of glycerol itself. The improved qualities resulting from glycerol incorporation in feeds are relevant for the adaptability of some fish species to farming. For example, glycerol addition between 0.5 and 1% is valuable to manufacture extruded aquafeeds for tuna, as these fish have a preference for softer/moist over regular extruded feeds.<sup>40</sup> In particular, Sotelo-Rodríguez et al.<sup>40</sup> have shown an evident effect of glycerol addition on expansion, density in water, and penetration of the extruded aquafeeds produced for tuna, although the impact of these physical changes on feed intake was not evaluated. Whether they have the potential to increase the palatability and subsequent feed intake of aquafeeds is a relevant area that remains to be investigated in fish.

While our understanding of the biochemistry, physiology, and molecular mechanisms behind the sensorial detection of nutrients by fish continues to increase, it remains uncertain whether glycerol can be directly sensed by fish. Taste receptors have been characterised in fish, revealing common mechanisms across vertebrates, although there appears to be differences between ligand specificities, shifting from amino acids in teleost fish to sugars in mammals.<sup>44</sup> Either through its physical or chemical nuances, separately or in conjugation, several studies have reported effects on feed intake under glycerol-supplemented diets in fish (Table 1). A recent study in juvenile European seabass (*Dicentrarchus labrax*) has shown higher feed intake when fed a diet supplemented with refined glycerol at 2.5%, although that effect was not evident at 5%.<sup>45</sup> Feed intake has been shown to

increase in juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum), when fed a diet supplemented with refined glycerol at 5%, although the effect was not evident at 2.5%.<sup>46</sup> An increase in feed intake has been shown as well in juvenile Nile tilapia (*Oreochromis niloticus*) fed a diet supplemented with crude glycerol at 16%.<sup>47</sup> Even if inconsequential for the final weight of fish, an earlier study noted a decreased feeding response of rainbow trout fed diets supplemented with free glycerol, although feed intake values or details on the source of glycerol supplemented in the feed were not provided.<sup>48</sup> Even though, the study associated informal observation on feeding behaviour with a potential satiation effect of hyperglycaemia due to the conversion of glycerol to glucose. A negative effect of glycerol supplementation on feed intake was observed in channel catfish (*Ictalurus punctatus*) when crude glycerol was added to their diets by more than 15%.<sup>49</sup> These differences in the effect of dietary glycerol in feed intake may be specific to certain species but may also be due to the different methods used to feed and how feed consumption was evaluated across the different studies. In addition, it is possible that compounds, other than glycerol, when included as a crude ingredient in the diets could have a negative impact on their palatability. This is an area of research that needs to be expanded in the near future.

## 3 | GLYCEROL ABSORPTION AND DIGESTIBILITY

Aquaporins (AQP) are water channel proteins that facilitate the transport of water between cell membranes, and therefore provide an osmotic response of fish to the environment. However, some AQP, designated as aquaglyceroporins, can also transport glycerol, promoting the transfer from the digestive tract into the blood circulation (Figure 1).

These include AQP-3, -7, -9, and -10, which are permeable not only to glycerol but also to other small solutes such as urea, being involved in salinity acclimation and osmoregulation in fish.<sup>50–52</sup> The competition of water and glycerol for hydrogen bonds with specific residues is essential for glycerol's mechanism of passage through aquaglyceroporins.<sup>53</sup> The distribution pattern of aquaglyceroporins along the oesophagus, stomach, and intestine/midgut reflects the ability of fish to recover water from the digestive tract.<sup>52,54–56</sup> In addition, the presence of AQP-3 has been confirmed in the kidney of European seabass, where large volumes of water can be reabsorbed when this species is maintained in seawater.<sup>51</sup> Once in the bloodstream, the liver can import glycerol through AQP-9, a major hepatic aquaglyceroporin isoform. Moreover, the expression level of AQP changes for hydro-mineral regulation according to fluctuations in salinity, which may also influence glycerol uptake.<sup>51,57,58</sup> Also, due to the presence of AQPs in multiple organs such as gills, skin, and kidneys, the transport of glycerol could be differentially affected by the internal *milieu* and/or external conditions. These transporters also have important osmoregulatory functions because besides glycerol they also transport water and urea. Therefore, the importance of AQPs transporting glycerol may depend on salinity when comparing fish in freshwater or marine

**TABLE 1** Zootechnical parameters based on weight, feed intake and somatic indices in different fish species fed diets containing glycerol. Tendencies of studied parameters when compared to control (0% glycerol diet) corresponding to increase, decrease, and maintenance are represented by symbols ↗, ↘, and ↔, respectively.

Fish species (glycerol replacement)	Time (days)	IBW (g)	FBW (g)	FI	WG	HSI	LSI	Ref
<i>Carnivorous (marine)</i>								
European seabass ( <i>D. labrax</i> ) (Cellulose/refined glycerol)	90	20.9 ± 0.3	0%: 66.3 ± 2.2 <sup>ab</sup> 2.5%: 75.1 ± 2.0 <sup>a</sup> 5%: 64.5 ± 2.5 <sup>b</sup>	↗2.5%	↔	↗	↗	45
European seabass ( <i>D. labrax</i> ) (Cellulose/refined glycerol)	42	51.4 ± 2.6	0%: 87.3 ± 16.3 2.5%: 75.4 ± 9.7 5%: 87.6 ± 16.3	-	↔	↗	↔	59,60
Gilthead seabream ( <i>S. aurata</i> ) (Dehulled pea/crude glycerol)	90	321 ± 45	0%: 623 ± 66 5%: 617 ± 61	-	↔	↔	↔	89
<i>Carnivorous (freshwater)</i>								
Rainbow trout ( <i>O. mykiss</i> ) (Wheat middlings/refined glycerol)	112 <sup>Exp1</sup> 84 <sup>Exp2,3</sup>	4.8 ± 0.1 <sup>Exp1</sup> 2.1 ± 0.1 <sup>Exp2</sup> 3.1 ± 0.2 <sup>Exp3</sup>	0% <sup>Exp1</sup> : 91.0 <sup>3</sup> 0% <sup>Exp1</sup> : 78.1 <sup>b</sup> 1% <sup>Exp1</sup> : 77.7 <sup>b</sup> 2% <sup>Exp1</sup> : 74.3 <sup>b</sup> 0% <sup>Exp2</sup> : 25.3 <sup>a</sup> 1% <sup>Exp2</sup> : 24.5 <sup>a</sup> 0% <sup>Exp2</sup> : 25.8 <sup>a</sup> 1% <sup>Exp2</sup> : 25.0 <sup>a</sup> 0% <sup>Exp3</sup> : 44.4 <sup>a</sup> 0% <sup>Exp3</sup> : 41.4 <sup>b</sup> 6% <sup>Exp3</sup> : 37.8 <sup>b</sup> 12% <sup>Exp3</sup> : 38.5 <sup>b</sup>	-	↔	-	-	48
Rainbow trout ( <i>O. mykiss</i> ) (Cellulose/refined glycerol)	60	20.2 ± 0.1	0%: 87.5 ± 2.1 2.5%: 85.3 ± 2.9 5%: 79.4 ± 1.4	↗5%	↘	↗	↔	46,59,60,84
Amazon Catfish ( <i>P. punctifer</i> × <i>L. marmoratus</i> ) (Corn bran/crude glycerol)	60	6.5 ± 0.9	0%: 27.7 ± 7.0 5%: 26.7 ± 5.1 7.5%: 35.6 ± 3.3 10%: 28.1 ± 7.1 12.5%: 28.7 ± 5.3	-	↔	↔	-	70
<i>Omnivorous (Freshwater)</i>								
Silver Catfish ( <i>R. quelen</i> ) (Corn/crude glycerol)	75	1.5 ± 0.03	0%: 39.4 ± 1.2 1.5%: 37.6 ± 1.3 3%: 37.7 ± 6.6 4.5%: 40.4 ± 0.8 6%: 44.0 ± 3.8 7.5%: 37.0 ± 0.9	-	↔	↔	-	90
Silver Catfish ( <i>R. quelen</i> ) (Corn/crude glycerol)	206	188.9 ± 0.6	0%: 451.7 ± 35.8 4%: 501.3 ± 28.3 8%: 488.9 ± 9.2 12%: 520 ± 95 16%: 541 ± 89 20%: 509 ± 67	-	↔	↗	-	91

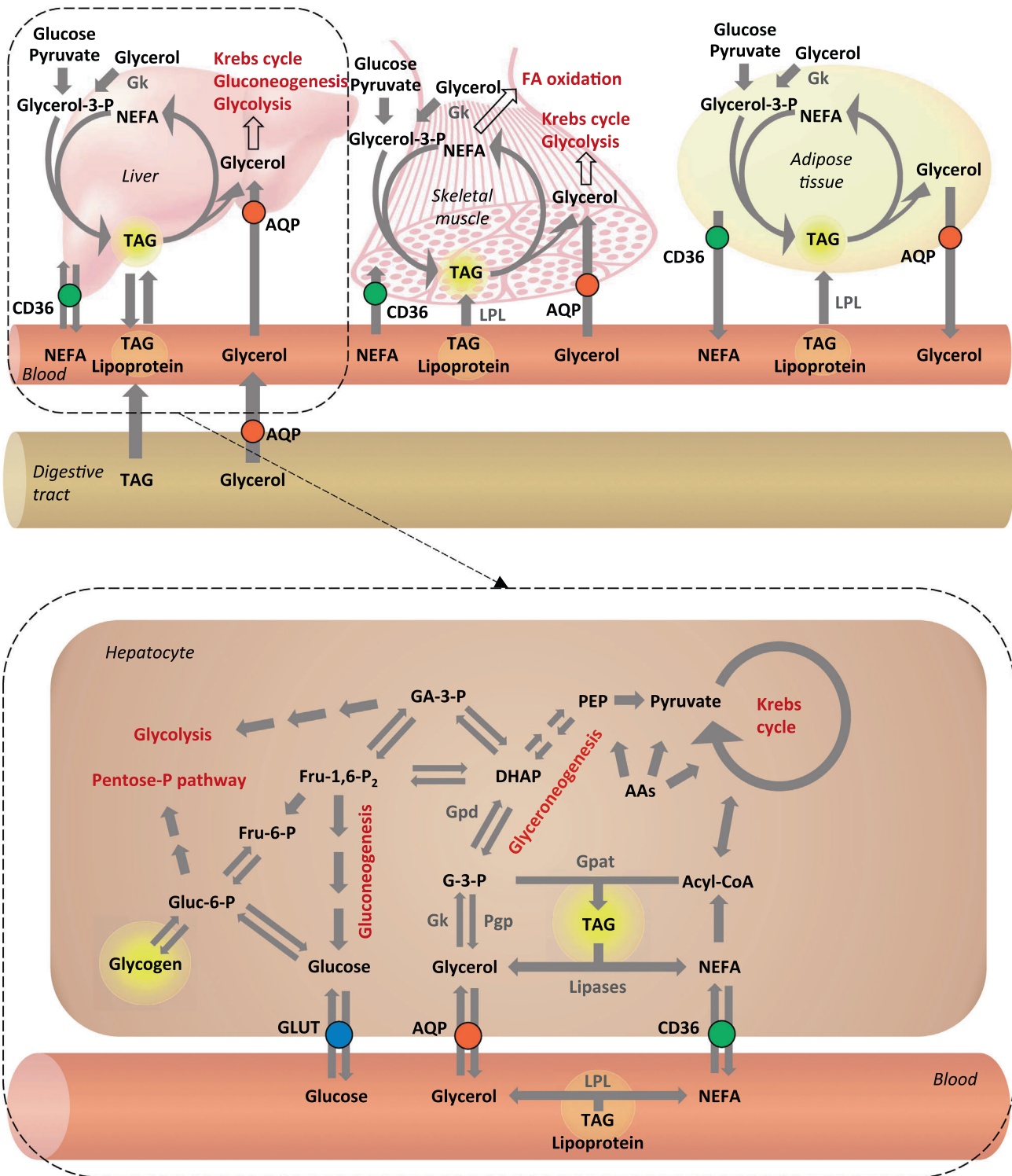
TABLE 1 (Continued)

Fish species (glycerol replacement)	Time (days)	IBW (g)	FBW (g)	FI	WG	HSI	LSI	Ref
Tambaqui ( <i>C. macropomum</i> ) (Corn bran/crude glycerol)	60	17.1 ± 6.1	0%: 54.0 7.5%: 55.3 10%: 51.2 12.5%: 50.5 15%: 55.1	-	↔	↔	-	109
Tambaqui ( <i>C. macropomum</i> ) (Corn bran/crude glycerol)	90	15.3 ± 1.6	0%: 56.8 ± 0.4 <sup>b</sup> 5%: 64.0 ± 0.4 <sup>a</sup> 15%: 64.4 ± 0.4 <sup>a</sup> 22.5%: 53.4 ± 0.6 <sup>c</sup> 30%: 52.7 ± 0.6 <sup>d</sup>	-	↗ ≤ 15% ↘ ≥ 22.5%	↗ ≥ 22.5%	↗ 22.5%	71
Channel catfish ( <i>I. punctatus</i> ) (Corn/crude glycerol)	63	6.8 ± 0.1	0%: 66.3 5%: 63.2 10%: 63.9 15%: 58.6 20%: 46.0	↘ ≥ 15%	↘	↔	-	49
Curimbatá ( <i>P. lineatus</i> ) (Corn/crude glycerol)	64	18.7 ± 2.7	0%: 29.0 ± 0.4 <sup>ab</sup> 4%: 29.8 ± 0.7 <sup>a</sup> 8%: 28.4 ± 1.1 <sup>bc</sup> 12%: 28.8 ± 0.5 <sup>ab</sup> 16%: 27.3 ± 0.5 <sup>cd</sup> 20%: 26.7 ± 1.0 <sup>d</sup>	-	↘ ≥ 16%	↔	-	72
Nile tilapia ( <i>O. niloticus</i> ) (Corn/crude glycerol)	90	7.7 ± 0.1	0%: 4%: 8%: 12%: 16%	↗ 16%	↔	↔	↔	47
Nile tilapia ( <i>O. niloticus</i> ) (Corn/crude glycerol)	30	11.5 ± 2.2	0%: 29.9 4%: 28.3 8%: 28.2 12%: 29.1 16%: 31.1 20%: 31.9	-	↔	↗	-	73
Nile tilapia ( <i>O. niloticus</i> ) (Corn/crude glycerol)	305	25.7 ± 0.5	0%: 196 ± 5 4%: 181 ± 19 8%: 190 ± 9 12%: 179 ± 11 16%: 184 ± 11	-	↔	↗	↔	75
Nile tilapia ( <i>O. niloticus</i> ) (Corn/crude glycerol)	79	29.2 ± 8.4	0%: 146 ± 37 2.5%: 142 ± 35 5%: 140 ± 33 7.5%: 145 ± 34 10%: 143 ± 28	-	↔	↔	↔	76

Note: Relevant differences compared to the control (0% glycerol diet) are indicated with different letters. Relevant differences between different doses of glycerol. Table reports data from referred studies in the last column (Ref), formulas of reported parameters may vary among them.

Abbreviations: FBW, final body weight; FI, feed intake; HSI, hepatosomatic index; IBW, initial body weight; LSI, liposomatic index; Time, duration of the experiment; WG, weight gain.





**FIGURE 1** Glycerol digestion and metabolism in the liver, muscle, and adipose tissue. AAs, amino acids; AQP, aquaporin; DHAP, dihydroxyacetone phosphate; Fru-6-P, fructose-6-phosphate; G-3-P, glycerol-3-phosphate; GA-3-P, glyceraldehyde-3-phosphate; Gk, glycerol kinase; Gluc-6-P, glucose-6-phosphate; GLUT, glucose transporter; Gpat, glycerol-3-phosphate acyltransferase; Gpd, glycerol-3-phosphate dehydrogenase; LPL, lipoprotein lipase; NEFA, non-esterified fatty acids; PEP, phosphoenolpyruvate; Pgp, glycerol-3-phosphate phosphatase; TAG, triacylglycerol. Some intermediates were omitted for clarity.

environments as observed in plasma glycerol levels which were increased in glycerol-supplemented diets for European seabass but not rainbow trout.<sup>59</sup>

The successful cultivation of fish is closely linked to their feeding habits, influencing the specific type of feed needed for optimal growth and development. The diets of carnivorous fish have typically high

digestible energy values of 21 MJ/kg (Table 2). Rainbow trout revealed high apparent digestibility coefficients (ADCs) for refined glycerol of over 99.7% when glycerol was included up to 5% in the diet.<sup>46</sup> Whether this translates into effective growth performance may a posteriori be hindered by the metabolic capacity to utilise circulating glycerol directly as an energy source.<sup>59,60</sup> In omnivorous fish, diets present digestible energy values lower than the diets of carnivorous fish of approximately 13 MJ/kg (Table 2), displaying the gross energy value for crude glycerol of 14.7 MJ/kg, which is equivalent to corn when included as an ingredient in these fish.<sup>47,61</sup> When searching the literature for the effects of glycerol-supplemented feeds in aquaculture, it is noticeable a great volume of studies on Nile tilapia compared to any other species. This abundance of research can be attributed to the fact that Brazil holds the position of the second-highest biodiesel producer globally, trailing only the United States, with an estimated production of 6.37 billion L in 2022, hence the abundance of glycerol as a by-product of biodiesel production.<sup>62,63</sup> In the process of evaluating the use of surplus glycerol it consequently makes sense to do it on Brazil's most relevant species, which are mainly freshwater like Nile tilapia, but also native fish species like tambaqui (*Colossoma macropomum*).<sup>64</sup> In juvenile Nile tilapia, the ADC value reported for crude glycerol was between 79%<sup>47,61</sup> and 89%.<sup>61</sup> However, feeding diets containing different sources of glycerol resulted in lower ADC values for adult Nile tilapia.<sup>65</sup> Interestingly, a diet containing a refined mixture of crude glycerol from animal fat and vegetable oil resulted in higher ADC for this ingredient (81%) than when fed diets including crude (58%) or refined (47%) glycerol from vegetable oil. Therefore, refined glycerol appears to be digested better than crude glycerol, probably due to the absence of other compounds that are present in crude products, although that appears to vary between the sources for this ingredient. Pacu (*Piaractus mesopotamicus*) and silver catfish (locally known as jundiá; *Rhamdia quelen*) are also relevant species to Brazilian aquaculture production in the subtropical region and, due to their feeding regimes are interesting models to evaluate the effects of dietary glycerol supplementation. Both are omnivorous species, although pacu appears to have a wider dietary choice in its natural habitat as it regularly consumes vegetation and fruits.<sup>66</sup> For pacu and silver catfish feed diets with the incorporation of crude glycerol, ADC values were also high, at 97 and 89%, respectively.<sup>67</sup> At least for these species, glycerol supplementation promoted higher digestibility for this ingredient and higher digestible energy when compared with other ingredients such as maize and wheat, revealing the potential to be used as an alternative energetic ingredient. These studies also indicate that differences in the digestible energy of dietary glycerol may influence the capacity of some species to metabolise this ingredient efficiently. Improvements in the way dietary crude glycerol is included in the feed should be explored in the future, similar to that already being done for dietary carbohydrates,<sup>68</sup> targeting nutritional programming in critical early developmental stages to tailor specific functions, such as digestibility and/or pathways of metabolic utilisation through epigenetic regulation of gene expression, tackling of nutrient-sensitive signalling pathways, altered cell number, cell type or structural changes in organs, impaired mitochondrial function, and adaptive clonal selection.<sup>69</sup>

## 4 | DIETARY GLYCEROL, GROWTH PERFORMANCE, AND FEED CONVERSION

The study of different glycerol supplementation levels revealed that there is a limit to how much glycerol can be added to the diet without decreasing the specific growth rate (SGR) or increasing the feed conversion ratio (FCR) of some fish species (Table 2). In juvenile European seabass, SGR was higher in fish fed a diet supplemented with refined glycerol at 2.5% than 5% but remained similar to fish fed a not supplemented diet.<sup>45</sup> However, FCR remained unchanged in all the dietary groups, possibly because the boost in weight gain detected with glycerol supplementation was matched to a similar increase in feed intake.<sup>45</sup> In rainbow trout juveniles the dietary inclusion of pure (free) glycerol up to 12% did not alter SGR nor FCR.<sup>48</sup> Another study of rainbow trout juveniles with supplementation of up to 5% of refined glycerol did not produce alteration to SGR; however, fish displayed increased FCR and decreased protein efficiency ratio (PER), without evident effects at 2.5%.<sup>46</sup> Nevertheless, the increased FCR and decreased PER in that study were likely related to an increased feed intake when refined glycerol was supplemented, along with a lower capacity to efficiently use this compound as a source of energy. Moreover, in Amazon catfish (locally known as pintado; ♀*Pseudoplatystoma punctifer* ♂*Leiarius marmoratus*), higher SGR was observed between 5 and 12.5% crude glycerol supplementation.<sup>70</sup> Regarding omnivorous species, crude glycerol supplementation levels at 15% in tambaqui did not cause alteration in the FCR, although provided higher SGR values, and on the other hand, growth decreased when glycerol was supplemented at higher levels.<sup>71</sup> In channel catfish, crude glycerol supplementation levels equal to or greater than 15% had negative outcomes, increasing the FCR and even impairing growth, as the weight gain of these fish was reduced compared to the fish fed a non-supplemented diet (Table 1).<sup>49</sup> Similarly in curimatá (*Prochilodus lineatus*), diets supplemented with equal or greater levels of 16% crude glycerol lead to higher FCR and lower SGR values.<sup>72</sup> In juvenile Nile tilapia fed a diet supplemented with crude glycerol at 16% showed an increase in the FCR and a decrease in the SGR values,<sup>47</sup> while another study reported the opposing effects in fish of similar body weight by decreasing the FCR value at glycerol levels equal or higher than 16%.<sup>73</sup> Interestingly, studies in Nile tilapia feeding larger juveniles and adults did not show changes in SGR or FCR values,<sup>74–77</sup> suggesting that dietary glycerol might have greater effects when included at earlier developmental stages. Most studies showed that glycerol could be included in fish diets up to 10% without altering SGR but with some variable effects on FCR. The adequate amount of glycerol in terms of feed conversion and growth for most fish species appears to be approximately 5%, although more studies will be necessary to establish optimal supplementation levels for each species and/or stage. However, the current data available indicate that glycerol inclusion levels greater than 15% may pose some limitations to its digestion and metabolic use.



**TABLE 2** Zootechnical parameters based on feeding and growth performance in different fish species fed diets containing glycerol. Tendencies of studied parameters when compared to control (0% glycerol diet) corresponding to increase, decrease, and maintenance are represented by symbols ↗, ↘, and ↔, respectively.

Fish species (glycerol replacement)	Min-Max (Levels)	CP/DE (%/MJ/kg)	Time (days)	SGR	FCR	PER	Surv. (%)	Ref
<i>Carnivorous (marine)</i>								
European seabass ( <i>D. labrax</i> ) (Cellulose/refined glycerol)	2.5–5 (2)	49/21 (%DM)	90	↔	↔	-	94–100	45
European seabass ( <i>D. labrax</i> ) (Cellulose/refined glycerol)	2.5–5 (2)	49/21 (%DM)	42	-	-	-	-	59,60
Gilthead seabream ( <i>S. aurata</i> ) (Dehulled pea/crude glycerol)	5 (1)	48/21 <sup>GE</sup> (%DM)	90	-	-	-	-	89
<i>Carnivorous (freshwater)</i>								
Rainbow trout ( <i>O. mykiss</i> ) (Wheat middlings/refined glycerol)	1–12 (4)	41/–	112 <sup>Exp1</sup> 84 <sup>Exp2,3</sup>	-	↔	↔	98–100	48
Rainbow trout ( <i>O. mykiss</i> ) (Cellulose/refined glycerol)	2.5–5 (2)	49/21 (%DM)	60	↔	↗5%	↘	99–100	46,59,60,84
Amazon catfish ( <i>P. punctifer</i> × <i>L. marmoratus</i> ) (Corn bran/crude glycerol)	5–12.5 (4)	42/19 <sup>GE</sup>	60	↗	↔	-	83–97	70
<i>Omnivorous (freshwater)</i>								
Silver Catfish ( <i>R. quelen</i> ) (Corn/crude glycerol)	1.5–7.5 (5)	39/13 (%DM)	75	↔	↔	↔	78–89	90
Silver Catfish ( <i>R. quelen</i> ) (Corn/crude glycerol)	4–2 (5)	38/18 <sup>GE</sup> (%DM)	206	↔	↔	↗	100	91
Tambaqui ( <i>C. macropomum</i> ) (Corn bran/crude glycerol)	7.5–15 (4)	26/13	60	↔	↔	-	100	109
Tambaqui ( <i>C. macropomum</i> ) (Corn bran/crude glycerol)	7.5–30 (4)	28/13	90	↗15% ↘ ≥ 22.5%	↔	↗ ≤ 15% ↘ ≥ 22.5%	85–96	71
Channel catfish ( <i>I. punctatus</i> ) (Corn/crude glycerol)	5–20 (4)	33/– (%DM)	63	-	↗ ≥ 15%	-	99–100	49
Curimatá ( <i>P. lineatus</i> ) (Corn/crude glycerol)	4–20 (5)	26/16 <sup>GE</sup>	64	↘ ≥ 16%	↗ ≥ 16%	-	100	72
Nile tilapia ( <i>O. niloticus</i> ) (Corn/crude glycerol)	4–16 (4)	33/13 (%DM)	90	↔	↗16%	↘16%	90–98	47
Nile tilapia ( <i>O. niloticus</i> ) (Corn/crude glycerol)	4–20 (5)	34/12	30	↔	↘	-	94–96	73
Nile tilapia ( <i>O. niloticus</i> ) (Corn/crude glycerol)	4–16 (5)	35/14	305	-	↔	-	100	75
Nile tilapia ( <i>O. niloticus</i> ) (Corn/crude glycerol)	2.5–10 (4)	28/13	79	-	↔	-	85–97	76

TABLE 2 (Continued)

Fish species (glycerol replacement)	Min-Max (Levels)	CP/DE (%/MJ/kg)	Time (days)	SGR	FCR	PER	Surv. (%)	Ref
Nile tilapia ( <i>O. niloticus</i> ) (Corn starch/refined glycerol)	5–15 (3)	36/19 <sup>GE</sup>	60		↔	-	91–92	74
Nile tilapia ( <i>O. niloticus</i> ) (Corn/crude glycerol)	6–24 (4)	31/13	50		↔	-	88–93	77
Tilapia ( <i>O. mossambicus</i> ) (Wheat rice bran/crude glycerol)	5–15 (2)	38/20 <sup>GE</sup> (%DM)	40		.	-	100	98

Note: Relevant differences between different doses of glycerol. Table reports data from referred studies in the last column (Ref), formulas of reported parameters may vary among them. Abbreviations: CP, crude protein; DE, digestible energy; DM, dry matter; FCR, feed conversion ratio; GE, gross energy; SGR, specific growth rate; PER, protein efficiency ratio; SGR, survival; time, duration of the experiment.

## 5 | DIETARY GLYCEROL AND NITROGEN EXCRETION

Glycerol is a sugar alcohol and therefore shares metabolic pathways with carbohydrates across all vertebrates, which is why most glycerol-supplemented diets are reducing concomitantly carbohydrate inclusion in feeding trials. The use of dietary carbohydrates in fish has been extensively reviewed by Kamalam et al.,<sup>78</sup> detailing how carbohydrate metabolism is a source of energy and carbon in cultured fish. Interestingly, a link between dietary carbohydrate intake and hepatic de novo lipogenesis has been demonstrated in several fish species<sup>79–83</sup> which may result in body fat deposition, involving its regulation by several mechanisms that appear to be shared in part with dietary glycerol.<sup>84</sup> In species with the same feeding habits, it is expected that they have a similar capacity to use dietary glycerol as has been described for dietary digestible starches. As in other vertebrates, fish do not have a dietary requirement for carbohydrates, as this group can efficiently synthesise glucose from glycerol and non-carbohydrate precursors such as lactate, pyruvate, and amino acids.<sup>85</sup> However, there is a chance that optimal inclusion of dietary carbohydrates, and perhaps glycerol, may increase protein and lipid retention by preventing the catabolism of these nutrients to obtain energy and therefore reducing the pollutant nitrogen discharge from fish farms. Although limited information is available on the utilisation of dietary glycerol, results indicate that at least in rainbow trout this compound does not spare the use of amino acids that can be channelled towards protein accretion, resulting in no apparent changes in growth.<sup>46</sup> In this regard, it has been proposed a lack of control of amino acid catabolism in fish displaying high dietary protein requirements,<sup>86</sup> as these constituents of proteins may be used as well in gluconeogenesis and lipogenesis, which results in nitrogen excreted mainly in the form of ammonia towards the environment.<sup>87</sup>

The total ammonia nitrogen excretion rate increased with refined glycerol supplementation levels in rainbow trout leading authors to conclude that glycerol supplementation (2.5 and 5%) does not promote beneficial effects on rainbow trout performance or in the reduction of the excreted nitrogen waste products. Moreover, in European seabass, another carnivorous species, juveniles showed no changes in the nitrogen efficiency or total ammonia nitrogen excretion rate,<sup>45</sup> suggesting that glycerol is not being effectively used as an energy source to the detriment of proteins. On the other hand, in another study with European seabass juveniles, but with higher initial body weight, glycerol supplementation decreased protein catabolism.<sup>88</sup> The effects of glycerol supplementation on nitrogen excretion and the metabolite profile and bacterial community composition of gut digesta showed that ammonia excretion was not altered by dietary glycerol inclusion, but the 5% glycerol diet promoted an increase in amino acids and a decrease of ergogenic creatine in the digesta metabolome profile, suggesting a decrease of the amino acids' catabolism. These results demonstrate that glycerol-supplemented diets can influence fish gut microbiota, which may, in turn, have an impact on protein retention, and micronutrient digestibility. Regarding the gut microbiome analysis, it was observed an increase in the abundance of

some specific genera and bacterial diversity; however, the gut bacterial communities do not seem to be negatively affected by dietary glycerol, and bigger diversity could reduce the occurrence of bacterial pathogens.<sup>88</sup> Nevertheless, in neither study has glycerol supplementation up to 5% been able to reduce nitrogen excretion. Because carnivorous fishes have low intestinal glucose uptake rates and slow blood glucose clearance, they are not considered efficient users of dietary carbohydrates,<sup>78</sup> and therefore carbohydrate replacement by glycerol may not have such an impact as it would for fish with other feeding habits, but studies on this matter are still limited.

## 6 | DIETARY GLYCEROL AND SOMATIC INDICES

Dietary inclusion of glycerol may provide metabolic intermediates for biosynthetic processes besides replenishing tissue energy storage, sharing some benefits as those proposed for dietary inclusion of carbohydrates, such as supplying metabolic precursors for biological synthesis to sustain growth at a low cost per unit gain. However, this growth should not compromise the metabolic capacity of the liver nor increase the carcass lipid content, which may be reflected in higher hepatosomatic (HSI) and liposomatic indices (LSI) respectively (Table 1). It could be speculated that by forming the structural backbone of triacylglycerol (TAG), dietary glycerol could promote fat accumulation, however clear evidence in this sense has not been gathered. If indeed, fat accumulation occurs in fish subjected to glycerol-supplemented diets it seems relevant to identify where this happens. If in the liver, this would imply alterations in lipogenic fluxes and consequent interference with metabolic mobilisation of nutrients. If in the muscle, this could be detrimental to the quality of the final product (mouthfeel and taste) and ultimately reflect on the price to the consumer. Finally, if deposited as mesenteric fat, this would be impactful by decreasing edible yields and generating waste during the processing of the fish for the market. One could argue that such effects may only manifest under excessive dosage and/or after prolonged utilisation; however, no studies have been specifically designed for long-term assessment of the effects of glycerol utilisation. Ultimately, understanding how excess nutrients are partitioned in the presence of dietary glycerol may be indicative of the metabolic regulation around the utilisation of glycerol.

In carnivorous fish, HSI was found unaltered in studies with gilt-head seabream (*Sparus aurata*),<sup>89</sup> Amazon catfish,<sup>70</sup> and rainbow trout<sup>48</sup> fed glycerol-supplemented diets. However, recent studies in both European seabass and rainbow trout have shown that increasing levels of dietary glycerol supplementation led to higher HSI.<sup>45,59</sup> In omnivorous species, there were also some discrepancies reported by several studies regarding the HSI and dietary glycerol supplementation levels. Mauerwerk et al. reported no changes in the HSI of silver catfish fingerlings,<sup>90</sup> although an increase in HSI was shown at the fattening stage for this species<sup>91</sup> when higher levels of supplementation were used. Similarly, HSI was increased when glycerol supplementation was equal to or higher than 22.5% in tambaqui.<sup>71</sup> On the other

hand, dietary glycerol supplementation reaching 20% saw no reflection in HSI in channel catfish or curimatá.<sup>49,72</sup> In Nile tilapia, dietary glycerol levels up to 24% did not affect the HSI.<sup>77</sup> Similarly, a study by Gonçalves et al. in Nile tilapia juveniles did not reveal changes in the HSI after 90 days of feeding diets supplemented with increasing levels of crude glycerol.<sup>47</sup> On the other hand, in Nile tilapia fingerlings fed only for 30 days had their HSI decreased when crude glycerol was supplemented up to 20%,<sup>73</sup> suggesting that a timely dietary adaptation may be required. Other studies on juvenile Nile tilapia using similar dietary glycerol supplementation but higher initial body weights revealed no changes in the HSI.<sup>74,76</sup> In contrast, Mewes et al. reported increasing HSI values in juvenile Nile tilapia fed to up to 16% of crude glycerol.<sup>75</sup>

Regarding the LSI, which corresponds to a ratio between the amount of adipose tissue stored as mesenteric fat and the body weight of the fish, studies in carnivorous species, including European seabass, gilthead seabream, and rainbow trout did not detect changes in this parameter when fed diets supplemented with glycerol up to 5%.<sup>60,89</sup> However, a study feeding juvenile European seabass showed that LSI values were increased when fed diets supplemented with glycerol up to 5% after 90 days<sup>45</sup> instead of 48 days.<sup>60</sup> While in omnivorous, changes in this parameter were evaluated in tambaqui by Bussons et al., showing that glycerol levels equal to or above 22.5% increased the LSI.<sup>71</sup> In Nile tilapia, no changes in LSI were detected when fed diets supplemented with glycerol, including studies in juveniles.<sup>47,75,76</sup> Surprisingly, a decrease in LSI was found in this species with a higher initial body weight when fed glycerol-supplemented diets.<sup>77</sup> This may imply that dietary glycerol utilisation can be variable between stages reflecting a differential lipid deposition. In addition, nutritional programming appears to be highly relevant in cultured fish as implies changes in nutrient utilisation that take place at early stages. Particularly, traits related to lipid metabolism are more heritable than protein traits in rainbow trout,<sup>92</sup> therefore these characteristics could be more easily controlled with selection programs.

## 7 | GLYCEROL METABOLISM

As previously mentioned, in the intestine glycerol can be absorbed by AQP, which are also responsible for glycerol uptake in other tissues<sup>14,55</sup> (Figure 1). Once absorbed, dietary glycerol is phosphorylated to glycerol-3-phosphate. From here, it can be converted to glucose by gluconeogenesis, and reducing equivalents by the pentose phosphate pathway, providing crucial metabolic energy substrates. On the other hand, glycerol-3-phosphate can enter the Krebs cycle through the glycolytic pathway, producing ATP.<sup>13</sup> The third option for glycerol is esterification with free fatty acids to generate TAG.<sup>93</sup> This means that glycerol can be metabolised differently and promote diverse effects depending on the fish species.<sup>48,89,94-99</sup> But since glycerol is at the crossroads of several metabolic pathways, it can be challenging to study its metabolic fate. The role of glycerol in the gluconeogenic pathway has been extensively described in several fish species, resorting to diverse methodologies.<sup>89,95-99</sup> On the other hand, the glycerol

carbon structure can also be used as a backbone in the synthesis of proteins, amino acids, and lipid molecules.<sup>1,98</sup>

It is worth noting that glycerol is not exclusively provided by the diet but can also be obtained endogenously after complete hydrolysis of TAG stored as body lipid reserves by several neutral lipases present in vertebrates.<sup>100,101</sup> After lipolysis, fatty acids and glycerol are released into the circulatory system and uptaken by tissues, metabolic steps that are highly relevant during food deprivation (fasting and starvation), and endurance swimming.<sup>102–104</sup> In addition, glycerol is required for the synthesis of glycerophospholipids, indispensable components of all biological membranes and used to assemble lipoproteins, involved in TAG and cholesterol transport in blood. In particular, glycerophospholipids play a key role in the homeoviscous adaptation of fish, which is the capacity to remodel biological membranes. This mechanism is key to maintaining fish homeostasis, by adjusting membrane fluidity to overcome detrimental effects of environmental temperature on their physiology.<sup>105</sup> Moreover, glycerol can be synthesised by glyceroneogenesis in vertebrates,<sup>24</sup> which is the synthesis of 3-glycerol phosphate by a pathway that shares steps with gluconeogenesis. Glyceroneogenesis is quite relevant in some fish species, as glycerol synthesised in the liver can be secreted in the plasma serving as a cryo- and osmoprotectant. In particular, high levels of glycerol are found in the plasma of rainbow smelt (*Osmerus mordax*) during winter to avoid freezing.<sup>106</sup> This is a species that can inhabit Arctic waters enduring cold temperatures and have displayed plasma glycerol levels that can exceed 300 mM in fish maintained in seawater at  $-1^{\circ}\text{C}$  for 42 days, showing a similar pattern for glycerol accumulation in tissues. The impact of dietary glycerol on temperature and salinity adaptation remains to be studied.

Ultimately, glycerol is a sugar-alcohol, and once available each fish species reveals a distinctive capacity to use its 3-carbon structure. This is the result of a multitude of factors that involve neuronal sensing and regulation of appetite, hormonal interplay, digestive structure (length, enzymatic capacity, absorption), ability to metabolise, partition and utilise nutrients and all of its interactions. Evolutionary processes shape these factors being the dietary regime one of its main drivers. Carnivorous and omnivorous fish species differ substantially in many aspects concerning carbohydrate utilisation as thoroughly reviewed by Kamalam et al.<sup>78</sup> The following section analyses the metabolic effects of dietary glycerol supplementation under this premise. In addition, the potential role of dietary glycerol as a metabolic fuel readily available in high-energy demand conditions such as swimming and gamete production remains to be investigated.

## 8 | METABOLIC EFFECTS OF DIETARY GLYCEROL SUPPLEMENTATION IN CARNIVOROUS FISH

For a better understanding of the role of dietary glycerol supplementation in fish metabolism, different approaches were applied to compare these effects on European seabass and rainbow trout, as important representative species of marine and freshwater

aquaculture (Table 3). These fish were fed diets supplemented with 2.5 and 5% glycerol and metabolite levels were evaluated in liver, muscle, and plasma. In European seabass, whole body composition displayed similar total lipid, protein, and energy contents, suggesting a reduced impact of dietary glycerol on overall body composition.<sup>45</sup> However, higher glycerol levels were found in the plasma of seabass supplemented with glycerol 24 h post-feeding, which suggests a poor glycerol uptake by the different tissues, the possibility of creating an osmotic imbalance, with glycerol competing with water for aquaporin transport.<sup>59</sup> Moreover, the rapid appearance of dietary glycerol in circulating blood in just 6 h after the meal is likely due to the abundance of aquaglyceroporins across the fish's intestinal system. With less carbohydrate transport to the tissues, hepatic glycogen storage decreased concomitantly, but TAG levels were increased in both plasma and liver.<sup>60</sup> Interestingly, most studies using glycerol-supplemented diets in fish do not report plasma glycerol levels. With the advances in automated and/or semi-automated machinery and routines for systematic metabolite profiles in plasma, this should be addressed for fish species. It would greatly assist in the systematic non-lethal monitoring of fish plasma in aquaculture research and industrial quality control analysis, generating robust metabolite profiles.<sup>107</sup> The fatty acid composition was mostly composed of monounsaturated fatty acids at the expense of lower polyunsaturated fatty acids, as metabolic tracer studies revealed higher rates of fatty acid de novo synthesis on seabass liver.<sup>60</sup> Another study in seabass using a metabolic tracer revealed that glycerol supplementation appears to be mostly used in the gluconeogenic direct pathway, while glycerol conversion via pyruvate and Krebs cycle represented a minor fraction, particularly in fasted fish.<sup>99</sup> Moreover, glycerol appears to be mainly used to replenish hepatic glycogen reserves during this fasting stage.<sup>99</sup> On the other hand, rainbow trout displayed an opposite profile than European seabass, with no changes to plasma glycerol levels, nor in liver TAG 24 h post-feeding.<sup>59,60</sup> However, dietary glycerol was linked to replenished hepatic glycogen reserves, even though this incorporation did not spare the use of gluconeogenic amino acids.<sup>59</sup> In that study, glucose levels were not altered in trout fed diets supplemented with refined glycerol up to 5%, but in another study with higher free glycerol supplementation levels, an increase in circulating glucose was observed in trout in the fed (3 h post-feeding) and fasted state (18 h post-feeding).<sup>48</sup> A nuclear magnetic resonance metabolomics approach on both these species revealed that the muscle metabolite profile was more affected than the liver, although in general, the final metabolite profile of rainbow trout was less affected than that of seabass by the glycerol supplementation up to 5%.<sup>108</sup> The muscle of gilthead seabream fed with 5% glycerol has been evaluated through a proteomics approach, but the few changes on protein levels (17 proteins out of 387 studied) were pointed as a potential stress response by these glycerol-fed fish.<sup>89</sup> At the intestinal level, crucial digestive enzymes, including chymotrypsin, lipase, and trypsin, exhibited changes in seabass when supplemented with 5% glycerol.<sup>88</sup> However, a parallel experiment conducted with rainbow trout showed no alterations in these enzymes, except for amylase.<sup>46</sup> The gene coding of key enzymes and proteins involved in hepatic glycerol metabolism,

**TABLE 3** Biochemical parameters for plasma, liver, and muscle in different fish species fed diets containing glycerol. Tendencies of studied parameters when compared to control (0% glycerol diet) corresponding to increase, decrease, and maintenance are represented by symbols ↑, ↓, and ↔, respectively.

Fish species	Sampling (h)	Plasma				Liver				Muscle				Ref
		Glycerol	Glucose	TAG	Chol	Prot	Glycerol	TAG	Prot	Glycerol	TAG	Prot		
<i>Carnivorosus (marine)</i>														
European seabass ( <i>D. labrax</i> )	6, 24	↗	↔	↗	-	-	↘	-	-	↗	-	-	-	59,60
Gilthead seabream ( <i>S. aurata</i> )	0, 6, 48	-	-	-	-	-	-	-	-	-	↗0 h	-	-	89
<i>Carnivorosus (freshwater)</i>														
Rainbow trout ( <i>O. mykiss</i> )	3, 18	-	↗ ≥ 6%3 h ↗ ≥ 12%18 h	-	-	↔	↔	-	-	-	-	-	-	48
Rainbow trout ( <i>O. mykiss</i> )	6, 24	↗6 h	↔	↗5%	-	-	↗	-	-	↔	-	-	-	59,60,84
Amazon catfish ( <i>P. punctifer</i> × <i>L. marmoratus</i> )	24	-	↔	-	-	-	↔	-	-	-	↘12.5%	-	-	70
<i>Omnivorosus (freshwater)</i>														
Silver catfish ( <i>R. quelen</i> )	24	-	↔	↔	↔	↔	-	-	-	-	-	-	-	90
Silver catfish ( <i>R. quelen</i> )	24	-	↗12%	↔	↔	↔	-	-	-	-	-	-	-	91
Tambaqui ( <i>C. macropomum</i> )	48	-	↗ ≥ 7.5% ↘30%	↘ ≥ 7.5% ↗ ≥ 22.5%	↗ ≥ 7.5%	↔	-	-	-	-	-	-	-	71
Channel catfish ( <i>I. punctatus</i> )	3	-	↗5%	-	-	-	-	-	-	↘ ≥ 15%	-	↘ ≥ 10%	↔	49
Curimatá ( <i>P. lineatus</i> )	24	-	↘4%	↔	↔	↔	-	-	-	-	-	-	-	72
Nile Tilapia ( <i>O. niloticus</i> )	24	-	-	-	-	↔	-	-	-	-	-	-	-	47
Nile Tilapia ( <i>O. niloticus</i> )	ND	-	↘	↔	-	-	-	-	-	↔	-	-	↘4%↗ ≥ 8%	75
Nile Tilapia ( <i>O. niloticus</i> )	24	-	↔	↔	↗	↔	-	-	-	-	-	-	-	76
Nile Tilapia ( <i>O. niloticus</i> )	24	-	↔	↔	-	↘15%	↗ ≥ 10%	↔	↔	↗ ≥ 10%	↔	↔	↔	74
Nile Tilapia ( <i>O. niloticus</i> )	1-2	-	↗ ≥ 18%1 h ↘1.5 h; ↔2 h	-	-	-	-	-	-	-	-	-	↔	77

Note: Relevant differences between different doses of glycerol. Percentages refer to the dose of glycerol. Table reports data from referred studies in the last column (Ref), quantification methods and units of reported parameters may vary among them.

Abbreviations: Chol, cholesterol; Prot, proteins; Sampling, sampling time after last meal in hours (h); TAG, triacylglycerol (or unspecified as lipid or fat).

such as the paralogous genes coding for glycerol transport, glycerol kinase, glycerol-3-phosphate phosphatase, and glycerol-3-phosphate dehydrogenase were all up-regulated after a meal in the liver of rainbow trout, demonstrating that glycerol metabolism could be nutritionally regulated.<sup>84</sup> However, juvenile rainbow trout fed diets with glycerol supplementation (2.5 and 5%) for an extended period showed no regulation of the mRNA abundance for the glycerol metabolism-related genes and seemed to be unable to regulate hepatic glycerol metabolism after dietary changes.<sup>84</sup>

## 9 | METABOLIC EFFECTS OF DIETARY GLYCEROL SUPPLEMENTATION IN OMNIVOROUS FISH

Nile tilapia is one of the most widely studied species and is of interest to evaluate the effects of dietary glycerol on omnivorous fish. In general, different percentages of glycerol supplementation led to different metabolic effects, essentially due to their variable rates of retention and catabolism. However, it is presented as a practical ingredient that can be metabolised into lipids, proteins, and/or carbohydrates by tilapia and other species with similar feeding regimes like the Mozambique tilapia (*Oreochromis mossambicus*).<sup>98</sup> Juvenile Nile tilapia-fed diets supplemented with glycerol up to 16% did not show significant changes in several metabolite levels measured in plasma (Table 3).<sup>47,74–76</sup> Although overall cholesterol levels were maintained, HDL was diminished in most of the fish fed glycerol-supplemented diets.<sup>76</sup> Costa et al. have shown that refined glycerol percentages above 10% caused an increase in TAG content in the liver and with 15% a decrease in hepatic glycogen levels.<sup>74</sup> The muscle was also similarly affected when glycerol was supplemented at 10%, with an increase in TAG, but no differences in glycogen level or protein content. Mewes et al. observed changes in muscle protein content with opposite effects depending upon the levels in which crude glycerol was supplemented in the diets.<sup>75</sup> In studies of carcass composition, only lipid content was different in individuals fed glycerol-supplemented diets, with the highest lipid content at 5% and lowest at 10%.<sup>76</sup> These studies suggest that glycerol might promote lipogenesis in Nile tilapia juveniles, but in Nile tilapia fingerlings, increased levels of glycerol promoted an increase in the whole body crude protein and moisture as well as a decrease in mineral and lipid contents.<sup>73</sup> More insight was obtained by a tracer study on Nile tilapia juveniles, which determined that glycerol primarily follows gluconeogenesis rather than lipogenesis since they observed a decrease in lipid labelling in 5 and 15% glycerol. In addition, that study reported an increase in a non-lipid and non-protein fraction in the liver in Mozambique tilapia fed a glycerol-supplemented diet at 5%, which could be dose-dependent, as glycerol retention and its catabolism decreased at 5% but not at 15%.<sup>98</sup> Most of the plasma metabolite levels were unaffected by dietary glycerol in silver catfish, with only an increase in glucose at 12%, but not at 16 or 20%.<sup>91</sup> Interestingly, channel catfish and curimatá seem to be particularly affected by glycerol supplementation specifically at 4–5%, with opposite effects,

because channel catfish presented higher circulatory glucose levels, while curimatá presented lower glucose levels.<sup>49,72</sup> This difference could be due to the different sampling times after the last meal since blood from channel catfish was sampled 3 h after feeding, while blood from curimatá was sampled after 24 h. While Nile tilapia demonstrated changes in TAG content in the liver and muscle but not in the plasma, tambaqui have shown alterations in TAG plasma content with a decrease in TAG with 7.5 and 15% glycerol and an increase at 22.5 and 30% glycerol. Treatments with 10 and 15% glycerol demonstrated in the liver increased activity of the glucose-6-phosphate dehydrogenase enzyme, a key catalyst in the phosphorylation of glycerol to glycerol-3-phosphate.<sup>74</sup> In turn, the blood plasma levels of pyruvic glutamic transaminase were influenced by the glycerol supplementation. Additionally, glycerol supplementation influenced the blood plasma levels of pyruvic glutamic transaminase.<sup>91</sup>

## 10 | OPTIMAL DIETARY GLYCEROL SUPPLEMENTATION LEVELS

Glycerol appears to have tissue- and species-specific optimal supplementation levels, above which unfavourable effects are reported. Under certain conditions, dietary glycerol could be used as a nutritional tool to improve the final quality of fish meat products and increase shelf life due to its potential to increase the glycogen and ATP content in muscle. In particular, the carnivorous gilthead seabream fed 5% glycerol during the finishing phase showed an increase in muscle glycogen, ATP levels, and fillet firmness.<sup>89</sup> The supplementation did not affect growth, fillet proximate composition, fatty acid profile, and organoleptic properties (aroma and colour). The proteomic analysis revealed an increase in the stress-coping response and a low impact on the general muscle metabolism. For gilthead seabream, a glycerol supplementation of 5% at the finishing phase was therefore considered adequate to increase glycogen deposition in muscle without impacting the fish metabolism and final quality. For European seabass, dietary glycerol up to 5% did not affect the fillet quality or microbial development.<sup>45</sup> Moreover, no differences in whole-body protein, lipid, or energy content were found for this species up to 5%.<sup>45</sup> On the other hand, in juvenile rainbow trout, neither at 2.5 nor at 5% was glycerol effectively used as a metabolic energy source.<sup>46</sup> All these studies have shown that carnivorous species can metabolise dietary glycerol despite the limited capacity of this group to use dietary carbohydrates. However, rainbow trout appears to channel dietary glycerol towards gluconeogenic utilisation in a higher proportion than European seabass, reflected in the increasing synthesis of hepatic glycogen. On the other hand, seabass synthesised FA de novo in the liver at much higher rates than trout with greater hepatic lipid deposition when fed diets supplemented with glycerol up to 5%.<sup>59,60</sup> This suggests that the metabolic fate of dietary glycerol could be slightly different within both species, by converting this substrate towards lipids or carbohydrates in different proportions. In hybrid Amazon catfish juveniles, glycerol could be metabolised with no significant changes in zootechnical parameters detected when



supplemented up to 10% in diets.<sup>70</sup> In the omnivorous silver catfish both as fingerlings and during the fattening phase, the authors considered that glycerol could be included up to 7.5%, improving product performance characteristics such as fillet yield.<sup>90,91</sup> Juvenile channel catfish had no differences in feed consumption, weight gain, FCR, and liver lipid levels when consuming diets with up to 10% glycerol, although plasma glucose was elevated in fish fed 5% glycerol.<sup>49</sup> On the other hand, individuals fed the highest glycerol percentages (15 and 20%) had lower weight gain, FCR, and liver lipid content, which led the authors to conclude that channel catfish seem to be able to utilise glycerol up to 10% supplementation without compromising the production performance. Juvenile tambaqui can be fed with up to 15% glycerol without compromising growth performance.<sup>71,109</sup> In juvenile curimatá, Balen et al. suggested a 4% of glycerol supplementation to improve its growth performance.<sup>72</sup> As described in the earlier section, most of the information on the use of dietary glycerol in omnivorous species has been provided by studies in Nile tilapia, including its effects on different life stages and using diverse diet compositions. As would be expected for a species able to use efficiently dietary carbohydrates, these studies suggest that refined glycerol can be effectively used when included up to 10% without increased hepatic lipid deposition. However, glycerol supplementations above 10% were not recommended in Nile tilapia juveniles because glycerol at these values can promote lipogenesis, negatively affecting the fillet characteristics.<sup>74</sup> Gonçalves et al. estimated an optimal supplementation level of 6% for this species at this development stage.<sup>47</sup> While in Nile tilapia fingerlings, a significant decrease in survival led the authors to not recommend at all the use of glycerol during this development phase.<sup>65</sup> Similarly, a study in carp larvae (*Cyprinus carpio*) revealed that although 10% of glycerol improved most zootechnical parameters, it also decreased survival.<sup>37</sup> However, a short report on Nile tilapia post-larvae in the sex reversal phase stated that up to 11% of glycerol could be used without decreasing fish performance or increasing fish mortality.<sup>61</sup> Moreover, whole-body protein deposition is not increased in Nile tilapia fingerlings, contrary to what was observed in juveniles of this species. Santos and Moron suggested that the inclusion up to 15% of crude glycerine could be used to substitute soy oil as it did not promote significant histopathological effects on the liver and kidney of tambaqui (*Colossoma macropomum*) juveniles.<sup>110</sup> These findings reveal the different potentials of glycerol supplementation in the various stages of fish production, particularly during the initial development phases.

## 11 | CONCLUSIONS

The biodiesel industry's growth has led to an excess of glycerol, prompting investigations into its inclusion in aquafeeds for sustainable practices. Despite the wealth of studies exploring glycerol supplementation in aquafeeds, the heterogeneity in species-specific responses, tissue-specific impacts, dosage variations, and glycerol purity/grading introduces considerable complexity. The costs associated with refinement and purification should be a major bottleneck to the extensive

utilisation of glycerol in aquafeeds. Then, once absorbed the appearance and clearance of circulating glycerol from the diets should greatly assist in the interpretation of some results but plasma glycerol levels are rarely reported. In addition, the timing for incorporating glycerol during the development of the fish seems to be important, particularly at earlier stages. One of the main metabolic shifts observed with this incorporation was the increase in lipogenesis in the omnivorous Nile tilapia<sup>74,76</sup> and hepatic lipogenesis in the carnivorous European seabass.<sup>60</sup> As glycerol enters glycolytic and carboxylic acid pathways, not only can it be used as an energy source but can provide intermediates for fatty acid production and be part of the backbone of TAG and phospholipids, so this effect will be more evident with the inclusion of glycerol in the feeds. Long-term studies could further improve our understanding on whether these metabolic shifts are temporary adjustments or permanent rearrangements of nutrient partitioning.

Nevertheless, Nile tilapia stands out as the most extensively researched species in the context of dietary glycerol supplementation, with our current understanding appearing comparatively limited, especially in the case of carnivorous fish. The large volume of studies on Nile tilapia are related to the introduction of this species in the fisheries of tropical and subtropical regions, particularly Brazil, where biodiesel is also produced in large quantities, originating glycerol as a by-product.<sup>111</sup> Further studies on diverse species, especially under varying temperatures and salinities affecting glycerol digestibility, are recommended. Despite potential benefits in feed palatability and physical attributes, additional investigation into glycerol inclusion is essential. Evaluating its usage across developmental stages, diverse dosages, metabolic turnover, and interaction with environmental factors is critical. Understanding the impact of dietary glycerol on vital organs like the liver, the main nutrient-sensing organ responsible for mobilising and parsimoniously using available nutrients; mesenteric fat tissue, a sink for excess energy that impacts the economic gains for producers; and muscle the final product that should fulfil all the nutritional and organoleptic requirements for human consumption, is crucial for assessing its direct utilisation and influence on nutrient utilisation.

## AUTHOR CONTRIBUTIONS

**Margarida Coelho:** Writing – original draft; writing – review and editing; formal analysis. **Mariana Palma:** Writing – original draft; writing – review and editing; formal analysis. **Ludgero C. Tavares:** Writing – review and editing; formal analysis; data curation. **Stéphane Panserat:** Conceptualization; investigation; funding acquisition; writing – review and editing; resources; supervision. **Ivan Viegas:** Writing – original draft; conceptualization; investigation; funding acquisition; project administration; resources; supervision; writing – review and editing. **Leonardo J. Magnoni:** Writing – original draft; conceptualization; investigation; funding acquisition; project administration; resources; supervision; writing – review and editing.

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### CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest, financial, or otherwise, to declare.

### DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analysed in this review.

### ORCID

Margarida Coelho  <https://orcid.org/0000-0002-0392-1118>

Mariana Palma  <https://orcid.org/0000-0002-0564-8442>

Ludgero C. Tavares  <https://orcid.org/0000-0002-2324-1259>

Stéphane Panserat  <https://orcid.org/0000-0002-4479-9868>

Ivan Viegas  <https://orcid.org/0000-0003-2589-2212>

Leonardo J. Magnoni  <https://orcid.org/0000-0001-8449-6071>

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