fishes

MDPI

Article

Effects of Fasting on Intermediary Metabolism Enzymes in the Liver and Muscle of Rainbow Trout

Montserrat Fernández-Muela [1,†], Rubén Bermejo-Poza [1,*,†], Almudena Cabezas [1], Concepción Pérez [2], Elisabet González de Chavarri [1], María Teresa Díaz [1], Fernando Torrent [3], Morris Villarroel [4] and Jesús De la Fuente [1]

1 Animal Production Department, Veterinary Faculty, Complutense University of Madrid (UCM). Avenida Puerta de Hierro s/n, 28040 Madrid, Spain
2 Department of Physiology, Veterinary Faculty, Complutense University of Madrid (UCM), Avenida Puerta de Hierro s/n, 28040 Madrid, Spain
3 Department of Forestry and Fisheries, College of Forestry Engineering, Technical University of Madrid (UPM), Ciudad Universitaria s/n, 28040 Madrid, Spain
4 Department of Animal Science, College of Agricultural Engineering, Technical University of Madrid (UPM), Avenida Puerta de Hierro 2, 28040 Madrid, Spain
* Correspondence: rbermejo@ucm.es; Tel.: +34-913-943-760
† These authors contributed equally to this work.

Abstract: Fish can go through periods of feed deprivation, either due to natural causes influenced by temperature or management techniques in animal production, affecting their energy metabolism differently, depending on the duration of fasting in days and water temperature. This study showed the effect of different days of fasting before slaughter in rainbow trout by analyzing the intermediary metabolic enzymes in the muscle and liver. For this purpose, a non-fasting group was compared with trout that fasted for 5, 10, and 20 days (55, 107, and 200 degree days (°C d), respectively). A first phase of increased activity of enzymes involved in glycolysis was observed, increasing hexokinase enzyme activity in muscle and pyruvate kinase enzyme activity in muscle and liver. As the fasting days progressed, enzymes involved in the gluconeogenic and glycogenolytic pathways in the liver such as lactate dehydrogenase and glycogen phosphorylase increased their activity regarding the no fasted group. After 20 days of fasting (200 °C d), lipogenic and protein related enzyme activity depletion was observed, leading to significant changes in energy metabolism. Therefore, prolonged fasting with high degree days duration may compromise the energy supply for the vital development of rainbow trout.

Keywords: fasting; rainbow trout; liver; muscle; intermediary metabolism; enzyme activity

Citation: Fernández-Muela, M.; Bermejo-Poza, R.; Cabezas, A.; Pérez, C.; González de Chavarri, E.; Díaz, M.T.; Torrent, F.; Villarroel, M.; De la Fuente, J. Effects of Fasting on Intermediary Metabolism Enzymes in the Liver and Muscle of Rainbow Trout. *Fishes* **2023**, *8*, 53. https://doi.org/10.3390/fishes8010053

Academic Editor: Junyan Jin

Received: 8 December 2022
Revised: 7 January 2023
Accepted: 12 January 2023
Published: 14 January 2023

1. Introduction

Fish are poikilothermic animals whose internal temperature depends on the water temperature, thus some species of fish that inhabit or spend any time in cold waters have been able to develop an adaptation mechanism, being able to survive time periods without ingesting food [1], adapting their metabolism to lower energy expenditure to deal with cooler temperatures [2]. In addition to the natural causes that can produce periods of food deprivation, there are management practices in farmed fish that subject them to periods of fasting.

Fish metabolism is closely related to ambient water temperature, so the cumulative sum of degree days may have a greater influence than the duration of the fast itself.

For this reason, the use of degree days is proposed to estimate the optimal fasting period before handling procedures in rainbow trout (*Oncorhynchus mykiss*), especially in warm water areas, where higher temperatures can produce a greater stress response in rainbow trout and affect their growth [3].

In aquaculture, fasting prior to slaughter is a normal practice to try to empty the digestive system, therefore reducing the possibility of microbial contamination for food safety in the final product. In addition to reducing metabolic activity, it could be beneficial for improving fish meat quality, since reserve mobilization, the composition of fish fat, and muscle deposits can be modified [4].

During the first days of fish fasting, metabolic activity is reduced, decreasing physiological functions, so energy consumption and maintenance expenditure are also reduced [2]. As the fasting progresses, the fish need a greater supply of energy and begin to mobilize their body stores, which could lead to the loss of body weight [5,6]. Faced with a greater demand for energy to maintain their vital functions, fish start to metabolize their own tissues, which could compromise the body's energy reserves [7], so the metabolic response is determined by the use of endogenous reserves [8]. Therefore, as pointed out by Pottinger et al., short-term fasting (less than two weeks) must be differentiated from long-term fasting or starvation [9].

Glucose homeostasis is one of the vital functions, so both the liver and the muscles activate a series of biochemical chain reactions to maintain blood glucose levels, the first being the enzyme glycogen phosphorylase, which is responsible for the release of glucose molecules from glycogen and hexokinase, which is responsible for phosphorylating glucose so that it enters the energy pathway [10]. When glycogen is depleted, other stored nutrients begin to be used, activating a variety of intermediate metabolic pathways with phosphorylation enzymes such as β-hydroxyacyl CoA dehydrogenase, responsible for the hydroxyl group into a keto group for the use of fatty acids as an energy source, or enzymes such as aspartate aminotransferase or alanine aminotransferase are involved in the metabolism of amino acids [11], leading to the consumption of lipid and protein sources in the liver and muscle tissues. In rainbow trout, gluconeogenesis takes place primarily in the liver [12] and to cope with fasting periods, intermediate biochemical pathways are activated to maintain glucose homeostasis, necessary to maintain brain activity, and the regulation of respiratory and mineral balance [11,13]. The last active reserve of rainbow trout is muscle protein [5], which differs from mammals, which first use proteins as energy stores before lipids.

In this sense, as previously indicated, the physiological processes related to energy storage and production in fish can be modified by fasting situations. These energy storage and production pathways are regulated by intermediary metabolic enzymes. In this work, the modification of the metabolic pathways of the intermediate metabolism of muscle and liver in rainbow trout during fasting prior to slaughter will be evaluated.

2. Materials and Methods

2.1. Experimental Design

For this study, a total of 432 rainbow trout were used, obtained from a local fish farm (Cifuentes, Guadalajara, Spain) and with an average weight of 225 ± 5.78 g. Trout were located at the fish farm of the School of Forestry Engineering, at the Polytechnic University of Madrid. The fish farm is located on a small slope and structured in terraces or raceways. The water is taken from an underground well and flows through the different terraces, taking advantage of the slope and providing a constant water flow (1.6 L/s, recirculation). The study was carried out under natural environmental conditions in October, exposing the animals to a natural photoperiod (11 L:13 D) and average water temperature and dissolved oxygen of 12.47 ± 1.42 °C and 9.5 ± 0.5 mg O_2/L, respectively, which were recorded daily.

Four raceways were used for the trial, with a volume of 5.16 m^3 and with a constant water flow and oxygen supply. Each raceway was divided into six compartments by means of stainless-steel plates with holes, with each section having the same volume (0.86 m^3), obtaining a total of 24 compartments or cages (Figure 1).

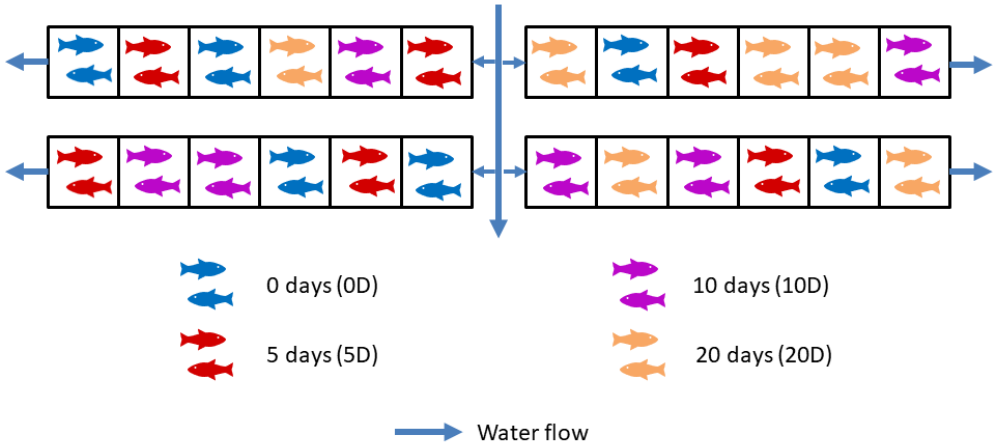

Figure 1. Experimental design. Distribution of trout between the different cages and treatments (pre-slaughter fasting times).

The trout were divided into the cages homogeneously so that there were 18 trout in each cage (stocking density per cage: 4,88 kg/m^3). A week before the test, fish were fed twice a day by hand using a commercial feed (42% crude protein, 23% fat, 4.1% ash, and 2.0% crude fiber, 30 ppm astaxanthin; 1% of their body weight), and in compliance with recommendations for rainbow trout, throwing the food on the surface of the water and observing that they ate and behaved normally.

After the acclimatization week, the experiment was carried out by subjecting the animals to different pre-slaughter fasting periods, with six cages per treatment: no fasting (0 °C d; 0 D), 5 days of fasting (55 °C d; 5 D), 10 days of fasting (107 °C d; 10 D), and 20 days of fasting (200 °C d; 20 D).

Following the pre-slaughter fasting periods, trout were captured by dip nets and stunned by electric shock to the head at 90 W for 2 s, then immediately (<15 s) slaughtered by cutting the spinal cord at the base of the head.

Trout were eviscerated and liver and muscle samples were taken from three trout per cage, placing them in liquid nitrogen and frozen at −80 °C until further analysis.

2.2. Assay Procedures

The activities of the intermediate metabolic enzymes were evaluated in the liver and muscle. These were homogenized in an Ultra-Turrax T25 homogenizer (Janke & Kunkel IKA®-Labortechnik, Staufen, Germany) by ultrasonic disruption in 10 volumes of ice-cold homogenization buffer (50 mM imidazole, 1 mM 2-mercaptoethanol, 50 mM NaF, 4 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 250 mM phenylmethylsulfonyl fluoride (PMSF) sucrose, pH 7.5). The homogenate was centrifuged in a Hermle Z 323 K centrifuge for 30 min at 3220× *g* and 4 °C, and the supernatant was stored at −80 °C for later analysis. The hexokinase (HK), pyruvate kinase (PK), fructose 1,6-bisphosphatase (FBP), lactate dehydrogenase (LDH), glycogen phosphorylase (GPt: total; GPa: activated), glyceraldehyde-3-phosphate dehydrogenase (G3PDH), β-hydroxyacyl CoA dehydrogenase (HOAD), glucose 6-phosphate dehydrogenase (G6PDH), aspartate aminotransferase (GOT), alanine aminotransferase (GPT), and glutamate dehydrogenase (GDH) enzyme activities were evaluated as previously described for Sparus aurata [14–18], after adaptation of the methods described for salmonids [19,20]. Enzyme activities were determined at 25 ± 0.5 °C using a Multiskan GO microplate spectrophotometer (Thermo Scientific). The substrate and protein concentrations necessary to measure the maximum activity of each enzyme were established based on preliminary assays. Reactions began by adding the supernatant to a preset protein concentration, omitting the substrate in the control plates. The protein was assayed in duplicate in the homogenates as described by the Bradford method [21] using bovine serum albumin (Sigma-Aldrich, MO, USA) as the standard. The activity rates of the

enzymes were determined by changes in the absorbance from the reduction of NADH to NADPH, measured at 340 nm (molar extinction coefficient of 6.22 mM^{-1} cm^{-1}) and 37 °C during pre-established times (10–15 min). Enzyme activities were expressed as mU/mg protein.

2.3. Statistical Analysis

The data were analyzed using the Statgraphics v. 19 software (Statgraphics Technologies, Virginia, USA). A prior analysis of normality and homogeneity of variance was performed for all variables using the Shapiro–Wilk and Bartlett test, respectively. When the parameters did not meet the two assumptions, the variables were transformed. The tables presented backward transformed mean values to facilitate the interpretation of the results, although standard errors of the mean (SEM) were presented for the transformed variables. We performed an ANOVA, with pre-slaughter fasting times as the fixed effect. The Bonferroni test was used for the mean comparison ($p < 0.05$) and data were presented in all figures as the mean ± SEM.

3. Results

3.1. Enzymes of the Intermediate Metabolism in Muscle

The effect of the different fasting periods (°C d) on the enzyme activities of the rainbow trout muscle related to glycolytic metabolism are presented in Figure 2. HK and PK enzymes presented a higher activity on fish subjected to 5 days of pre-slaughter fasting than the no fasted and 20 D fish. No significant differences due to fasting times were observed in FBP, GPt, and GPa.

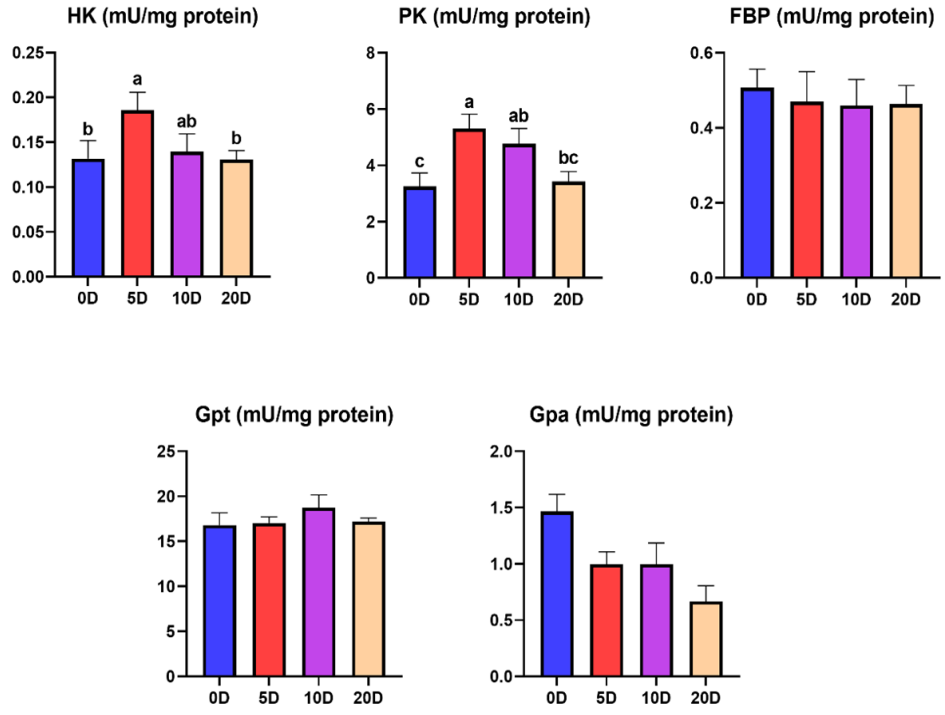

Figure 2. Means ± SEM of enzyme activities (n = 18 per treatment) related to glycolytic and glycogenolysis metabolism in muscle. [a, b, c] Different letters indicate significant differences between pre-slaughter times ($p < 0.05$). HK: hexokinase; PK: pyruvate kinase; FBP: fructose 1.6-bisphosphatase; GPt: total glycogen phosphorylase; GPa: activated glycogen phosphorylase. Transformation data for parameters when performing analysis of variance: Gpa (square root). In the transformed variables, the SEM are presented for the transformed data and means are presented as back-transformed for better interpretation.

The response to different fasting periods of enzyme muscle activity related to lipid metabolism is shown in Figure 3. HOAD enzyme activity was higher in the 10 D and 20 D fish than the other groups. The 20 D fish presented a higher G6PDH enzyme activity than 5 D and 10 D, but no significant differences between treatments were found in the G3PDH and HOAD enzyme activities.

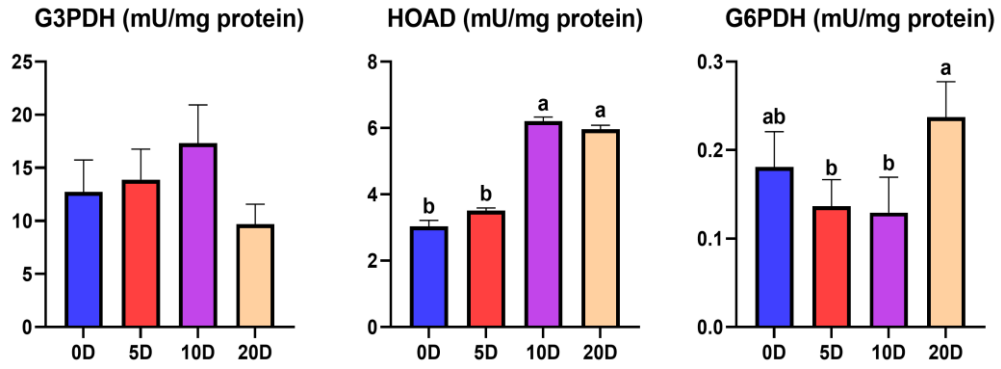

Figure 3. Means ± SEM of the enzyme activities (n = 18 per treatment) related to lipid metabolism in the muscle. [a, b] Different letters indicate significant differences between the pre-slaughter times ($p < 0.05$). G3PDH: glyceraldehyde-3-phosphate dehydrogenase; HOAD: β-hydroxyacyl CoA dehydrogenase; G6PDH: glucose 6-phosphate dehydrogenase. Transformation data for the parameters when performing analysis of variance: G6PDH (square root); HOAD (log10). In transformed variables, the SEM are presented for the transformed data and means are presented as back-transformed for better interpretation.

The GOT enzyme activity in muscle was significantly higher in 5 D fish than 0 D and 10 D. The 5 D and 10 D fish presented a significantly lower enzyme activity of GDH in muscle than 0 D. No significant differences due to fasting times were found in the GPt enzyme activity in muscle (Figure 4).

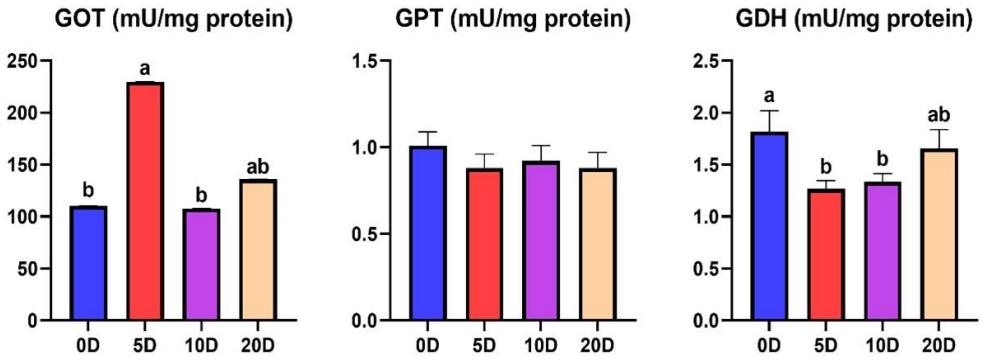

Figure 4. Means ± SEM of the enzyme activities (n = 18 per treatment) related to protein metabolism in muscle. [a, b] Different letters indicate significant differences between pre-slaughter times ($p < 0.05$). GOT: aspartate aminotransferase; GPT: alanine aminotransferase; GDH: glutamate dehydrogenase. Transformation data for parameters when performing analysis of variance: GOT (log10); GDH (log10). In the transformed variables, the SEM are presented for the transformed data and the means are presented as back-transformed for better interpretation.

3.2. Enzymes of the Intermediate Metabolism in Liver

The effect of pre-slaughter fasting times on the intermediate metabolism enzymes related to glycolytic metabolism of the liver of rainbow trout is shown in Figure 5. PK enzyme activity was significantly higher in the 5 D and 10 D fish than the 0 D and 20 D. No fasted fish presented a lower enzyme activity of FBP than the other groups. LDH enzyme

activity was significantly higher in the 0 D and 5 D than the 20 D fish. No significant differences were found in the HK enzyme activity between treatments.

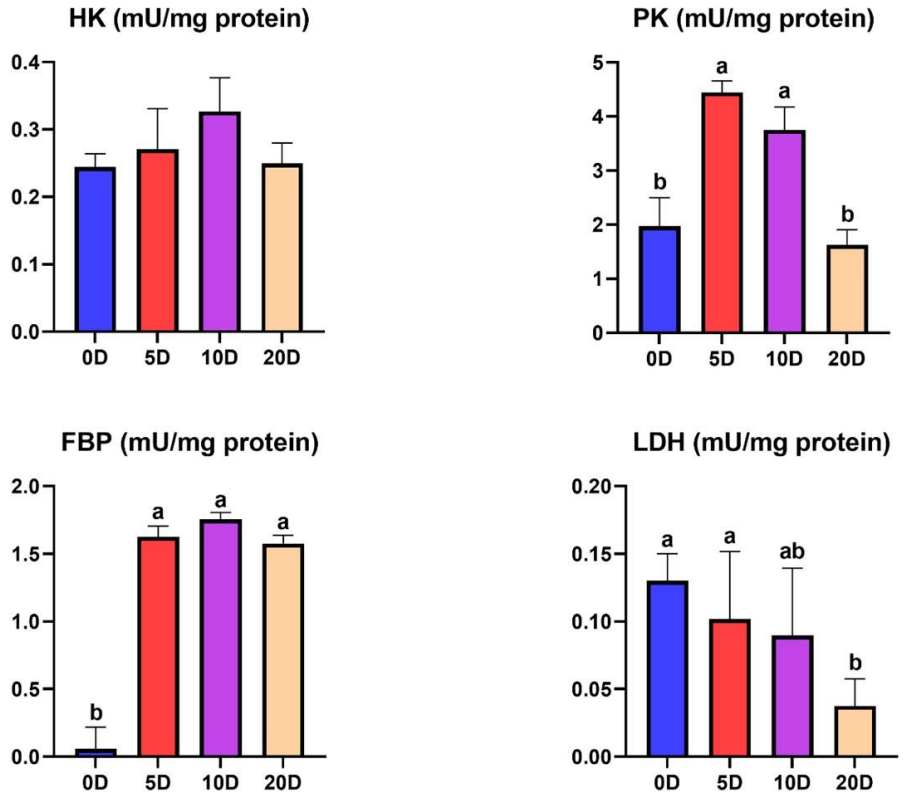

Figure 5. Means ± SEM of the enzyme activities (n = 18 per treatment) related to glycolytic metabolism in the liver. [a, b] Different letters indicate significant differences between the pre-slaughter times ($p < 0.05$). HK: hexokinase; PK: pyruvate kinase; FBP: fructose 1.6-bisphosphatase; LDH: lactate dehydrogenase. Transformation data for the parameters when performing analysis of variance: LDH (square root). In the transformed variables, the SEM are presented for the transformed data and the means are presented as back-transformed for better interpretation.

The results of the enzyme activities involved in the glycogenolysis pathway in the liver are shown in Figure 6. The 20 D fish presented a significantly lower enzyme activity of GPt than 5 D and 10 D. The GPa was significantly lower in the 20 D fish than the rest of the treatments.

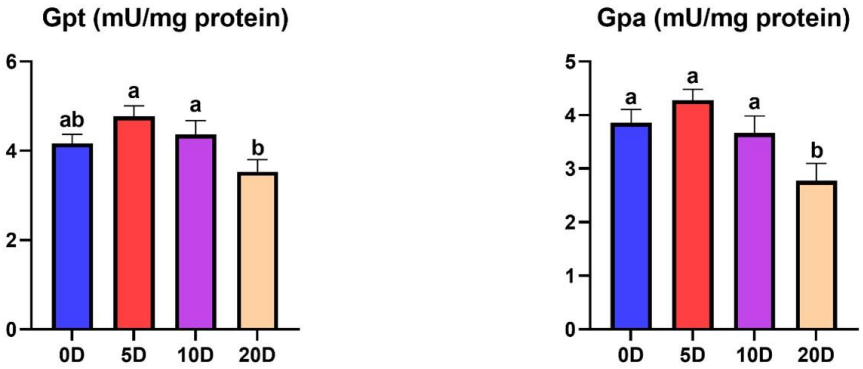

Figure 6. Means ± SEM of the enzyme activities (n = 18 per treatment) related to glycogenolysis metabolism in the liver. [a, b] Different letters indicate significant differences between the pre-slaughter times ($p < 0.05$). GPt: total glycogen phosphorylase; GPa: activated glycogen phosphorylase.

Figure 7 shows the response to different fasting periods of the lipid liver metabolism enzymes. The G3PDH enzyme presented the lowest activity in the 20 D fish and highest in 5 D, while the 0 D and 10 D fish presented intermediate values and was similar between them. The 20 D fish showed a significantly lower enzyme activity of G6PDH than the rest of the groups. No significant differences between fasting times were found in HOAD enzyme activity in the liver.

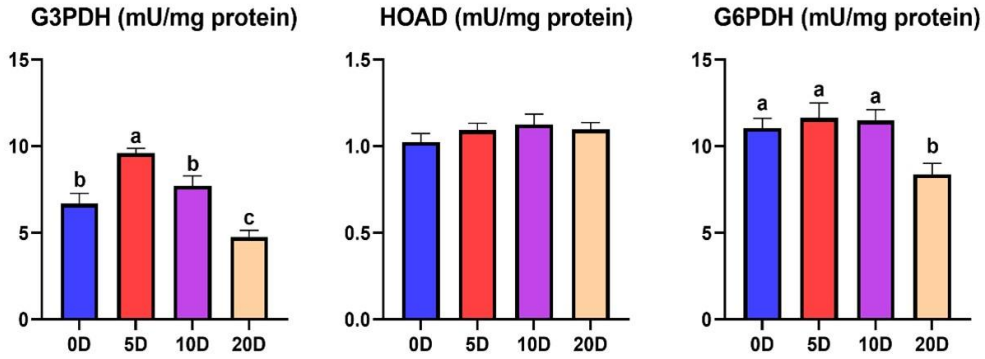

Figure 7. Means ± SEM of enzyme activities (n = 18 per treatment) related to lipid metabolism in the liver. [a, b, c] Different letters indicate significant differences between the pre-slaughter times ($p < 0.05$). G3PDH: glyceraldehyde-3-phosphate dehydrogenase; HOAD: β-hydroxyacyl CoA dehydrogenase; G6PDH: glucose 6-phosphate dehydrogenase.

A significantly higher enzyme activity of GPT in the 0 D and 5 D fish than 20 D was observed. GDH enzyme activity presented the lowest value in the 20 D fish and 10 D fish presented a significantly higher activity than the 0 D fish. No significant differences between fasting times were found in the GOT enzyme activity in the liver (Figure 8).

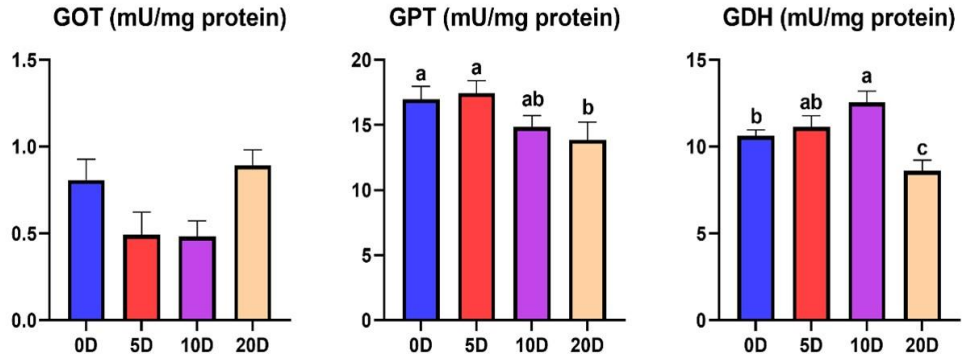

Figure 8. Means ± SEM of the enzyme activities (n = 18 per treatment) related to protein metabolism in the liver. [a, b, c] Different letters indicate significant differences between the pre-slaughter times ($p < 0.05$). GOT: aspartate aminotransferase; GPT: alanine aminotransferase; GDH: glutamate dehydrogenase. Transformation data for parameters when performing analysis of variance: GOT (square root). In the transformed variables, the SEM are presented for the transformed data and means are presented as back-transformed for better interpretation.

4. Discussion

All freshwater fish that live in rivers and lakes have different abilities to adapt to environmental factors such as low temperatures, low oxygen levels, and food shortages during periods of hibernation or winter. The adaptation of fish to periods of food deprivation produces a decrease in metabolic activity and the activation of different pathways aimed at energy production through internal energy sources, activating enzymes that trigger gluconeogenic flow and other metabolic pathways [22,23].

4.1. Enzymes of the Intermediate Metabolism in Muscle

Regarding the intermediate metabolism enzymes of the muscle, the present study showed how the first days of fasting produced a consumption of glucose followed by glycogen and lipids, and as time progresses, fasting leads to protein catabolism. HK enzyme, involved in the glycolytic pathway, showed a peak of activity at 5 days of pre-slaughter fasting (55 °C d), which is supported by other authors who determined that on the first 5 days of fasting, there is greater use of glucose as an energy fuel, which is also observed by the increase in PK, a glycolytic enzyme involved in the pathway of catabolizing glucose. The increase in these enzymes may have kept the glucose stable during the first days of fasting, a fact that coincides with other authors who determined that the glucose level after the fasting period remained stable in *Rhamdia quelen* [8] and rainbow trout (*Oncorhynchus mykiss*) [3,23]. After 10 days of fasting (107 °C d), HK and PK activity decreased, which could indicate that the use of glucose for glycolytic purposes was limited. Therefore, glucose levels after 10 days (107 °C d) and 20 days (200 °C d) of fasting tend to be lower than non-fasted fish [23], but within the basal levels, probably because it is an essential fuel for various tissues [24], possibly at the expense of glycogen stored in the liver.

As the FBP enzyme did not undergo significant alterations between the different fasting groups, its activation would be due to its role as an enzyme involved in gluconeogenesis in muscle to maintain glucose homeostasis. Similar facts have been appreciated by Karatas (2018), who observed that during the first days that fish were feed deprived, glycogen hydrolysis plays an important role in regulating blood glucose homeostasis as it releases glucose from liver glycogen into the blood during fasting [11]. According to previous studies [11], the first metabolic response in fish during a period of food deprivation is the use of liver glycogen, which is a readily available energy store. Harmon et al. (1991) determined that muscle glycogen generally tends to be conserved during fasting, since it is primarily used to support muscle activity [25], which could explain the lack of significant differences in the GPt and GPa enzyme activities in our study. Metón et al. (2003) explained that apart from glycogenolysis, the balance between glycolysis and gluconeogenesis is important for maintaining glucose homeostasis during fasting [26].

Glycerol is used as a substrate for gluconeogenesis, first phosphorylated by the enzyme glycerol kinase to form glycerol-3-phosphate (G3P), and then converted by glycerol-3-phosphate dehydrogenase to dihydroxyacetone phosphate (DHAP), which is an intermediate of the gluconeogenic and glycolytic pathways [27]. The use of lipid fuels as a substrate for the different metabolic pathways saves the lower reserves of carbohydrates (conserved as fuel for specialized organs such as the brain) and minimizes the consumption of muscle protein for fuel [28].

In another study in which trout were subjected to a fasting period, it was observed that in the first 48 h of fasting, liver glycogen was significantly reduced, indicating that the fish first used carbohydrates to supply energy, but after 48 h, showed increased lipid utilization [27]. In our study, HOAD enzyme activity increased after 10 days of fasting (107 °C d) and G6PDH increased significantly after 20 days of fasting (200 °C d), which could indicate the use of lipids as substrates of gluconeogenesis.

Proteins are the main source of energy in the muscle, since in absolute terms, they are the main muscle component [29]. The highest value of the enzyme GDH was found in no fasted fish. After 5 and 10 days of pre-slaughter fasting, (55 °C d), and (107 °C d), GDH activity decreased. This result agrees with Frick et al. (2008), who stated that after 60 days of fasting in lungfish, there was a reduction in GDH activity in the muscle and lung tissues [30]. Furné et al. (2012) maintained that GOT and GDH enzymes remained active during fasting [14], corroborating protein catabolism, as we saw in our study that after 5 D, the GOT activity increased significantly as an active substrate of gluconeogenesis.

4.2. Enzymes of the Intermediate Metabolism in Liver

The increase in the first 5 days of fasting (55 °C d) of enzymes involved in the glycolysis pathway in the liver such as PK indicated a greater use of glucose as energy fuel, remaining high after 10 fasting days (107 °C d). Furné et al. (2012) indicated increased activity of the enzymes involved in glycolysis (hepatic glycolytic enzymes) during the first days of fasting, and a decrease in liver glycogen stores and plasma glucose, and after 5 days of fasting, the activity of the glycolytic enzymes decreased [14]. In our study, after 20 days of fasting (200 °C d), PK activity was lower than the no fasting fish group, corroborated by Dai et al. (2022), who observed that the concentration of several key enzymes and hormones in the process of glycolysis (for example, GCK (glucokinase), HK, PK, or insulin) decreased significantly with fasting [12].

The decrease in glycolysis is linked to an increase in gluconeogenesis, observing reduced glucose levels [31,32]. Inhibition of glycolysis is an adaptive mechanism that is probably related to the decrease in blood glucose concentration and hepatic glycogen consumption [26,33]. Excessive consumption of liver glycogen leads to a low hepatic somatic index. Liver glycogen and muscle glycogen are used as nutrients [12]. Gluconeogenesis takes place primarily in the liver [21,34–36]. Enzyme changes are aimed at enhancing the liver's potential for gluconeogenesis during a period of food deprivation [22].

Facing a period without food, body cells need an energy supply to maintain muscle glucose. The activity of PK and FBP enzymes in the liver increased significantly after five days of fasting (55 °C d), which could be related to energy production and activation of gluconeogenic enzymes in the liver. FBP activity is maximal in trout liver during gluconeogenesis. Our results showed an increase in the activity of FBP after a fasting period that tended to be constant until 20 days of fasting. FBP remained constant after a period of food deprivation in its role of maintaining blood glucose levels, accompanied in longer fasting times with a decrease in liver enzyme activity involved in glycolysis, aimed at maintaining vital functions [6,31,37]. PK phosphorylation during fasting was observed along with other indicators of the maintenance and gluconeogenesis pathway activity as an energy generator such as the increase in lactate and amino acids [13]. Our results show that after 20 days of fasting (200 °C d), LDH dropped significantly, which could indicate an energy compromise. Supporting gluconeogenesis activation, the LDH enzyme underwent an increase in its concentration during the first days of fasting, which seems to indicate the use of lactate with gluconeogenic purposes, and agrees with other authors who determined that gluconeogenesis indicator enzymes from non-carbohydrate substrates such as GPT and the increase in LDH are important in trout gluconeogenesis, and in the muscle, LDH promotes regeneration of NAD+ to continue glycolysis [13].

On the other hand, previous studies have determined that the inhibition of HK, PK, and G6PDH enzyme activity observed in the liver of fish subjected to food deprivation could indicate a reduced rate of glucose uptake and utilization to maintain glycemia [26,37–41]. According to Bermejo-Poza et al. (2017), it is known that the liver plays an important role in maintaining homeostasis during food deprivation, whose color is affected and can be used as an indicator of fasting stress in fish [42]. During longer fasting or starvation, blood glucose levels may be maintained through a metabolic adaptation involving a decrease in liver enzymes involved in glycolysis and the pentose phosphate pathway, and a simultaneous increase in FBP-gluconeogenic 1 and glucose-6-phosphatase [33].

G6PDH is essential to produce NADPH, necessary for fatty acid synthesis. A significant decrease in G6PDH could be related to a decreased lipogenic capacity [22,43], as observed after 20 fasting days (200 °C d) in our study. Nagayama et al. (1972) reported that the activity of hepatic G6PDH decreased when Japanese eel (*Anguilla Japonica*) and rainbow trout (*Salmo gairdneri*) were fasted for 15 days [44]. Glycogen and lipids stored in liver hepatocytes are primary sources of energy during short-term fasting [45]. Favero et al. (2018, 2020) determined that liver glycogen and lipids as well as decreased muscle lipids are associated with decreased hepatosomatic index values during fasting [46,47]. The enzymes GPa and GPt increased after 5 fasting days (55 °C d) to initiate the glycogenolytic

pathway, and together with gluconeogenesis, formed glycogen to distribute to the rest of the body and maintain glycemia. After 20 fasting days (200 °C d), their activity decreased significantly due to scarcity of the substrate, compromising well-being and vital functions.

The G3PDH enzyme, involved in lipid metabolism, increased after 5 days of fasting (55 °C d), which could be explained because it is a substrate for gluconeogenesis, and the significant decrease after 20 days of fasting (200 °C d) could be related to a depletion in the energy reserves. Glycerol is mainly used as a gluconeogenic substrate and glycolytic pathway [28]. A significant decrease in G3PDH and G6PDH may be related to a reduced lipogenic capacity during fasting, as observed after 20 days of fasting (200 °C d), which coincides with Lin et al. (1977), who, after 23 days of fasting, observed a significant decrease in body weight, liver weight, and lipogenic enzyme activities in coho salmon [1].

Under aerobic conditions, energy is produced from the oxidation of amino acids and not from glucose. Amino acid oxidation occurs mainly in the Krebs cycle. The tissues that have more mitochondria are where this oxidation of amino acids occurs, tissues such as the liver, gills, or muscle. The most important transaminases are GPT and GOT. The first step in amino acid catabolism is a transamination reaction, where the amino group is transferred to alpha-ketoglutarate and gives rise to glutamate. Glutamate regenerates alpha-ketoglutarate by releasing ammonium ions by the enzyme GDH. The decrease in GPT activity after fasting for 10 and 20 days (107 °C d) (200 °C d), after reaching its maximum activity at 5 fasting days, could be a protective strategy to prevent the excessive oxidation of body proteins [31]. The GPT enzyme presented the highest activity after 5 days of fasting and GDH after 10 days of fasting (107 °C d), to drop below the activity of the non-fasted group after 20 days (200 °C d). In muscle, there is more GDH activity after long periods of fasting to maintain muscle energy function and obtain substrates to generate energy. The GDH enzyme is found in high concentrations in the liver of fish, having great importance in the oxidation of amino acids. Decreased protein levels may be related to the conversion of energy from protein through gluconeogenesis or the slowing of protein synthesis due to fasting time [11], results that are consistent with this study after 20 fasting days (200 °C d), which resulted in a generalized decrease in the activity of enzymes involved in gluconeogenesis.

5. Conclusions

In the muscle during the first days of fasting, as has been observed, the glycolysis pathway is initiated to maintain stable glucose levels and other metabolic pathways are activated by the consumption of glycogen and lipids. After prolonged fasting or starvation, protein catabolism is observed, unlike mammals that use proteins first when faced with an energy demand, followed by carbohydrate and lipids, so rainbow trout seems to be characterized by first using their carbohydrate and lipid reserves and then protein. After a 5 day fasting period (55 °C d), the activity of enzymes involved in glycolysis in the liver and muscle of rainbow trout increased. After a 10 day fasting period (107 °C d), the activity of glycolytic enzymes began to decrease, with enzymes involved in liver and muscle gluconeogenesis and glycogenolysis being active.

In the liver, gluconeogenesis began, as we can conclude, from the increase in PK after a 5-day fast (55 °C d), increasing enzymes that participate as substrates of this pathway G3PDH, G6PDH, GPT, and glycogenolysis was activated, as noted by the increase in GPa, and GPt. After 20 days of fasting (200 °C d), the energy deposits began to be consumed, observing a decrease in substrates to generate energy and lower enzyme activities of LDH, PK, GPa, GPt, G3PDH, G6PDH, GPT, and GDH. The liver provides the energy needed by the rest of the tissues, which is why it commits the energy needed by the muscle to carry out its functions. Therefore, rainbow trout would tend to decrease the blood glucose and liver glycogen levels after five days of fasting, as explained by the decrease in the activity of the enzymes involved in gluconeogenesis and glycogenolysis as well as the enzymes related to lipogenic capacity and protein metabolism, so there is a greater energy commitment that compromises the well-being and quality of the meat.

Based on our results, in response to fasting, rainbow trout begins to use glucose as an energy reserve and after five days of fasting, gluconeogenic and glycogenolytic pathways are promoted, but with no effect on lipids or protein metabolism, showing that rainbow trout can cope well with up to 55 °C d of fasting. Lipogenic and protein related enzymes increase their activity after 20 days of fasting, changing energy metabolism. Therefore, prolonged fasting and high water temperature can reduce the energy supply and compromise vital functions of rainbow trout, so, based on these results, we can recommend not subjecting rainbow trout to pre-slaughter fasting higher than 55 °C d, and that more studies are needed to determine the optimal pre-slaughter fasting period on this species.

Author Contributions: Conceptualization, M.V. and J.D.l.F.; Methodology, M.V., J.D.l.F., R.B.-P. and E.G.d.C.; Validation, R.B.-P., C.P. and F.T.; Formal analysis, R.B.-P. and J.D.l.F.; Investigation, M.V., R.B.-P. and J.D.l.F.; Resources, M.V. and J.D.l.F.; Data curation, M.F.-M., R.B.-P. and J.D.l.F.; Writing—original draft preparation, M.F.-M., R.B.-P. and J.D.l.F.; Writing—review and editing, M.F.-M., R.B.-P., J.D.l.F., A.C. and M.T.D.; Visualization, M.F.-M., R.B.-P. and J.D.l.F.; Supervision, R.B.-P. and J.D.l.F.; Funding acquisition, M.V. and J.D.l.F. All authors have read and agreed to the published version of the manuscript.

Funding: This project was financed by the Spanish Ministry of Economy and Competitiveness (MINECO), project AGL2013-45557-P.

Institutional Review Board Statement: Ethical review and approval was waived for this study due to the fact that it is a study where non-experimental agricultural practices were carried out and the sampling was carried out after the commercial slaughter of the trout. In addition, this research was part of a project (AGL2013-45557-P) in 2013 that was conducted at the Polytechnic University of Madrid and on that date, we did not need approbation from an Ethical Committee due to the research characteristics of this study.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Lin, H.; Romsos, D.R.; Tack, P.I.; Leveille, G.A. Influence of dietary lipid on lipogenic enzyme activities in coho salmon, Oncorhynchus kisutch (Walbaum). *J. Nutr.* **1977**, *107*, 846–854. [CrossRef] [PubMed]
2. Karatas, T.; Onalan, S.; Yildirim, S. Effects of prolonged fasting on levels of metabolites, oxidative stress, immune-related gene expression, histopathology, and DNA damage in the liver and muscle tissues of rainbow trout (Oncorhynchus mykiss). *Fish Physiol. Biochem.* **2021**, *47*, 1119–1132. [CrossRef] [PubMed]
3. López-Luna, J.; Vásquez, L.; Torrent, F.; Villarroel, M. Short-term fasting and welfare prior to slaughter in rainbow trout, Oncorhynchus mykiss. *Aquaculture* **2013**, *400*, 142–147. [CrossRef]
4. Rasmussen, R.S.; Ostenfeld, T.H.; Ronsholdt, B.; McLean, E. Manipulation of end-product quality of rainbow trout with finishing diets. *Aquacult. Nutr.* **2000**, *6*, 17–24. [CrossRef]
5. Pottinger, T.G.; Rand-Weaver, M.; Sumpter, J.P. Overwinter fasting and re-feeding in rainbow trout: Plasma growth hormone and cortisol levels in relation to energy mobilisation. *Comp. Biochem. Phys. B* **2003**, *136*, 403–417. [CrossRef]
6. Navarro, I.; Gutierrez, J. Chapter 17 Fasting and starvation. In *Biochemistry and Molecular Biology of Fishes*, 1st ed.; Hochachka, P.W., Mommsen, T.P., Eds.; Elsevier Science: Amsterdam, The Netherlands, 1995; Volume 4, pp. 393–434.
7. Vigliano, F.A.; Quiroga, M.I.; Nieto, J.M. Metabolic adaptation to food deprivation and refeeding in fish. *Rev. Ictiol.* **2002**, *10*, 79–108.
8. Barcellos, L.J.G.; Marqueze, A.; Trapp, M.; Quevedo, R.M.; Ferreira, D. The effects of fasting on cortisol, blood glucose and liver and muscle glycogen in adult jundiá Rhamdia quelen. *Aquaculture* **2010**, *300*, 231–236. [CrossRef]
9. Cook, J.T.; McNiven, M.A.; Richardson, G.F.; Sutterlin, A.M. Growth rate, body composition and feed digestibility conversion of growth-enhanced transgenic Atlantic salmon, Salmo salar. *Aquaculture* **2000**, *188*, 15–32. [CrossRef]
10. Moon, T.W. Adaptation, constraint, and the function of the gluconeogenesis pathway. *Can. J. Zool.* **1988**, *66*, 1059–1068. [CrossRef]
11. Karatas, T. Effect of short-term starvation on serum metabolites, antioxidant enzymes and endogenous reserves of rainbow trout, Oncorhynchus mykiss. *Pak. J. Zool.* **2018**, *50*, 1723–1729. [CrossRef]

12. Dai, Y.; Shen, Y.; Guo, J.; Yang, H.; Chen, F.; Zhang, W.; Wu, W.; Xu, X.; Li, J. Glycolysis and gluconeogenesis are involved of glucose metabolism adaptation during fasting and re-feeding in black carp (Mylopharyngodon piceus). *Aquac. Fish.* 2022, *in press*. [CrossRef]
13. Wang, T.; Hung, C.C.; Randall, D.J. The comparative physiology of food deprivation: From feast to famine. *Annu. Rev. Physiol.* **2006**, *68*, 223–251. [CrossRef]
14. Furné, M.; Morales, A.E.; Trenzado, C.E.; García-Gallego, M.; Carmen Hidalgo, M.; Domezain, A.; Sanz Rus, A. The metabolic effects of prolonged starvation and refeeding in sturgeon and rainbow trout. *J. Comp. Physiol. B* **2012**, *182*, 63–76. [CrossRef] [PubMed]
15. Laiz-carrión, R.; Martín Del Río, M.P.; Miguez, J.M.; Mancera, J.M.; Soengas, J.L. Influence of cortisol on osmoregulation and energy metabolism in gilthead seabream Sparus aurata. *J. Exp. Zool. Part A* **2003**, *298*, 105–118. [CrossRef] [PubMed]
16. Sangiao-Alvarellos, S.; Polakof, S.; Arjona, F.J.; Kleszczynska, A.; Del Río, M.P.M.; Míguez, J.M.; Soengas, J.L.; Mancera, J.M. Osmoregulatory and metabolic changes in the gilthead sea bream Sparus auratus after arginine vasotocin (AVT) treatment. *Gen. Comp. Endocrinol.* **2006**, *148*, 348–358. [CrossRef] [PubMed]
17. Polakof, S.; Arjona, F.J.; Sangiao-Alvarellos, S.; Martín del Río, M.P.; Mancera, J.M.; Soengas, J.L. Food deprivation alters osmoregulatory and metabolic responses to salinity acclimation in gilthead sea bream Sparus auratus. *J. Comp. Physiol. B* **2006**, *176*, 441–452. [CrossRef]
18. Vargas-Chacoff, L.; Ruiz-Jarabo, I.; Arjona, F.J.; Laiz-Carrión, R.; Flik, G.; Klaren, P.H.; Mancera, J.M. Energy metabolism of hyperthyroid gilthead sea bream *Sparus aurata* L. *Comp. Biochem. Phys. A* **2016**, *191*, 25–34. [CrossRef]
19. Soengas, J.L.; Strong, E.F.; Fuentes, J.; Veira, J.A.; Andrés, M.D. Food deprivation and refeeding in Atlantic salmon, Salmo salar: Effects on brain and liver carbohydrate and ketone bodies metabolism. *Fish Physiol. Biochem.* **1996**, *15*, 491–511. [CrossRef]
20. Soengas, J.L.; Strong, E.F.; Andres, M.D. Glucose, lactate, and b-hydroxybutyrate utilization by rainbow trout brain: Changes during food deprivation. *Physiol. Zool.* **1998**, *71*, 285–293. [CrossRef]
21. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
22. Suarez, R.K.; Mommsen, T.P. Gluconeogenesis in teleost fishes. *Can. J. Zool.* **1987**, *65*, 1869–1882. [CrossRef]
23. Bermejo-Poza, R.; Fernández-Muela, M.; De la Fuente, J.; Pérez, C.; de Chavarri, E.G.; Díaz, M.T.; Torrent, F.; Villarroel, M. Physio-Metabolic response of rainbow trout during prolonged food deprivation before slaughter. *Fish Physiol. Biochem.* **2019**, *45*, 253–265. [CrossRef]
24. Costas, B.; Aragão, C.; Ruiz-Jarabo, I.; Vargas-Chacoff, L.; Arjona, F.J.; Dinis, M.T.; Mancera, J.M.; Conceição, L.E. Feed deprivation in Senegalese sole (Solea senegalensis Kaup, 1858) juveniles: Effects on blood plasma metabolites and free amino acid levels. *Fish Physiol. Biochem.* **2011**, *37*, 495–504. [CrossRef] [PubMed]
25. Harmon, J.; Eilertson, C.; Sheridan, M.; Plisetskaya, E. Insulin suppression is associated with hypersomatostatinemia and hyperglucagonemia in glucose-injected trout. *Am. J. Physiol.* **1991**, *261*, R609–R613. [CrossRef]
26. Metón, I.; Fernández, F.; Baanante, I.V. Short- and long-term effects of refeeding on key enzyme activities in glycolysis–gluconeogenesis in the liver of gilthead seabream (Sparus aurata). *Aquaculture* **2003**, *225*, 99–107. [CrossRef]
27. Lu, D.L.; Ma, Q.; Wang, J.; Li, L.Y.; Han, S.L.; Limbu, S.M.; Li, D.L.; Chen, L.Q.; Zhang, M.L.; Du, Z.Y. Fasting enhances cold resistance in fish through stimulating lipid catabolism and autophagy. *J. Physiol.* **2019**, *597*, 1585–1603. [CrossRef]
28. De la Roche, M.; Tessier, S.N.; Storey, K.B. Structural and Functional Properties of Glycerol-3-Phosphate Dehydrogenase from a Mammalian Hibernator. *Protein J.* **2012**, *31*, 109–119. [CrossRef] [PubMed]
29. Machado, C.R.; Garofalo, M.A.R.; Roselino, J.E.S.; Kettelhut, I.C.; Migliorini, R.H. Effects of starvation, refeeding, and insulin on energy-linked metabolic processes in catfish (Rhamdia hilarii) adapted to a carbohydrate-rich diet. *Gen. Comp. Endocrinol.* **1988**, *71*, 429–437. [CrossRef]
30. Frick, N.T.; Bystriansky, J.S.; Ip, Y.K.; Chew, S.F.; Ballantyne, J.S. Carbohydrate and amino acid metabolism in fasting and aestivating African lungfish (Protopterus dolloi). *Comp. Biochem. Physiol. B* **2008**, *151*, 85–92. [CrossRef]
31. Pérez-Jiménez, A.; Cardenete, G.; Hidalgo, M.C.; García-Alcázar, A.; Abellán, E.; Morales, A.E. Metabolic adjustments of Dentex dentex to prolonged starvation and refeeding. *Fish Physiol. Biochem.* **2012**, *38*, 1145–1157. [CrossRef]
32. Viegas, I.; Caballero-Solares, A.; Rito, J.; Giralt, M.; Pardal, M.A.; Metón, I.; Jones, J.G.; Baanante, I.V. Expressional regulation of key hepatic enzymes of intermediary metabolism in European seabass (Dicentrarchus labrax) during food deprivation and refeeding. *Comp. Biochem. Physiol. A* **2014**, *174*, 38–44. [CrossRef] [PubMed]
33. Caseras, A.; Metón, I.; Vives, C.; Egea, M.; Fernández, F.; Baanante, A.I. Nutritional regulation of glucose-6-phosphatase gene expression in liver of the gilthead sea bream (Sparus aurata). *Brit. J. Nutr.* **2002**, *88*, 607–614. [CrossRef] [PubMed]
34. Singer, T.D.; Mahadevappa, V.G.; Ballantyne, J.S. Aspects of the energy metabolism of lake sturgeon, Acipenser fulvescens, with special emphasis on lipid and ketone body metabolism. *Can. J. Fish. Aquat. Sci.* **1990**, *47*, 873–881. [CrossRef]
35. Mommsen, T.P.; Moon, T.W. The metabolic potential of hepatocytes and kidney tissue in the little skate, Raja erinacea. *J. Exp. Zool.* **1987**, *244*, 1–8. [CrossRef]
36. Singer, T.D.; Ballantyne, J.S. Absence of extrahepatic lipid oxidation in a freshwater elasmobranch, the dwarf stingray Potamotrygon magdalenae: Evidence from enzyme activities. *J. Exp. Zool.* **1989**, *251*, 355–360. [CrossRef]

37. Kirchner, S.; Seixas, P.; Kaushik, S.; Panserat, S. Effects of low protein intake on extra-hepatic gluconeogenic enzyme expression and peripheral glucose phosphorylation in rainbow trout (Oncorhynchus mykiss). *Comp. Biochem. Physiol. B* **2005**, *140*, 333–340. [CrossRef]
38. Collins, A.L.; Anderson, T.A. The influence of changes in food availability on the activities of key degradative and metabolic enzymes in the liver and epaxial muscle of the golden perch. *J. Fish Biol.* **1997**, *50*, 1158–1165. [CrossRef]
39. Shimeno, S.; Shikata, T.; Hosokawa, H.; Masumoto, T.; Kheyyali, D. Metabolic response to feeding rates in common carp, Cyprinus carpio. *Aquaculture* **1997**, *151*, 371–377. [CrossRef]
40. Pérez-Jiménez, A.; Guedes, M.J.; Morales, A.E.; Oliva-Teles, A. Metabolic responses to short starvation and refeeding in Dicentrarchus labrax. Effect of dietary composition. *Aquaculture* **2007**, *265*, 325–335. [CrossRef]
41. Polakof, S.; Ceinos, R.; Fernández-Duran, B.; Míguez, J.; Soengas, J. Daily changes in parameters of energy metabolism in brain of rainbow trout: Dependence on feeding. *Comp. Biochem. Physiol. A* **2007**, *146*, 265–273. [CrossRef]
42. Bermejo-Poza, R.; De la Fuente, J.; Pérez, C.; González de Chavarri, E.; Diaz, M.T.; Torrent, F.; Villarroel, M. Determination of optimal degree days of fasting before slaughter in rainbow trout (Oncorhynchus mykiss). *Aquaculture* **2017**, *473*, 272–277. [CrossRef]
43. Aster, P.L.; Moon, T.W. Influence of the fasting and diet on lipogenic enzymes in the American eel, Anguilla rostrata (LeSueur). *J. Nutr.* **1981**, *111*, 346–354. [CrossRef] [PubMed]
44. Nagayama, F.; Ohshima, H.; Umezawa, K. Distribution of glucose-6-phosphate metabolizing enzymes in fish. *Bull. Jpn. Sot. Sci. Fish* **1972**, *38*, 589–593. [CrossRef]
45. Figueroa, R.I.; Rodríguez-Sabarís, R.; Aldegunde, M.; Soengas, J.L. Effects of food deprivation on 24 h-changes in brain and liver carbohydrate and ketone body metabolism of rainbow trout. *J. Fish Biol.* **2000**, *57*, 631–646. [CrossRef]
46. Favero, G.C.; Gimbo, R.Y.; Franco Montoya, L.N.; Zanuzzo, F.S.; Urbinati, E.C. Fasting and refeeding lead to more efficient growth in lean pacu (Piaractus mesopotamicus). *Aquac. Res.* **2018**, *49*, 359–366. [CrossRef]
47. Favero, G.; Gimbo, R.Y.; Montoya, L.N.F.; Carneiro, D.J.; Urbinati, E.C. A fasting period during grow-out make juvenile pacu (Piaractus mesopotamicus) leaner but does not impair growth. *Aquaculture* **2020**, *524*, 735242. [CrossRef]