Physio-metabolic response of rainbow trout during prolonged food deprivation before slaughter

Rubén Bermejo-Poza & Montserrat Fernández-Muela & Jesús De la Fuente & Concepción Pérez & Elisabet González de Chavarri & María Teresa Díaz & Fernando Torrent & Morris Villarroel

Abstract Fish normally undergo periods of food deprivation that are longer than non-hibernating mammals. In aquacultured rainbow trout (*Oncorhynchus mykiss*), it is unclear how fasting may affect their physiological adaptative response, especially when they are normally fed daily. In addition, that response may vary with temperature, making it necessary to express fasting duration in terms of degree days. In the current study, trout were fasted for 5, 10, and 20 days (55, 107, and 200 degree days (°C d), respectively). To assess the physiological response of fish to fasting, different

biometric, blood, plasma, and metabolic parameters were measured, as well as liver fatty acid composition. The fish weight, condition factor, and the hepatosomatic index of 5-day fasted trout were not significantly different from those of control fish. Gastric pH increased as fasting progressed while plasma concentrations of glucose, triglycerides, and total proteins decreased significantly after 10 days of fasting, while the percentage of non-esterified fatty acids increased. There were no significant differences in plasma ions (sodium, potassium, and calcium), except for chloride ion which decreased after 5 days of fasting. Liver glycogen decreased after 5 days of fasting while glycogen concentration in muscle did not decrease until 20 days of fasting. Liver color presented a higher chroma after 5 days of fasting, suggesting a mobilization of reserves. Finally, acetylcholinesterase activity in the brain was not affected by food deprivation but increased after 10 days of fasting in liver and muscle, suggesting the mobilization of body reserves, but without severely affecting basal metabolism.

Keywords Food deprivation · Water temperature · Rainbow trout · Stress response

Introduction

In the wild, fish normally cope with long periods without food during migrations and breeding seasons (Hinch et al. 2005; Miller et al. 2009). In aquaculture, fish are usually subjected to short-term fasting during the

production cycle (i.e., a few days, see López-Luna et al. 2013). Additionally, and depending on environmental conditions (especially in winter), it may not be possible for farmers to feed fish for long periods of time, stretching to weeks in extreme cases (Kiessling et al. 1990). Fasting (typically less than 2 weeks) is different from starvation, which can last several weeks (Pottinger et al. 2003). During the early phases without food (7–10 days), fish mobilize readily available metabolite reserves like hepatic glycogen (Soengas et al. 1996), while starvation is more associated with a chronic stress response and is thought to have more dramatic consequences on fish metabolism such as pronounced lipid catabolism, inducing changes in fatty acid profiles, as well as an increase in protein catabolism. This response in last term can lead to important effects of fish weight loss (Barcellos et al. 2010).

For aquaculture producers, it may be useful to identify indicators that differentiate between physiological states of fasting and starvation. Although the cutoff point between the two is not always clear, they produce significantly different metabolic states and can be temperature dependent (Neuheimer and Taggart 2007; Chezík et al. 2013), so endocrine control might be expected to differ markedly (Farbridge and Leatherland 1992).

The lack of food can trigger several physiological responses that together are called the stress response. This response consists of a chain of reactions promoted by a stressor, starting in fish with the neuroendocrine responses, called primary responses, including the rapid release of hormones into the circulation, such as catecholamines and cortisol (Belanger et al. 2001). This is followed by the secondary stress response, which includes various biochemical and physiological changes mediated by hormones liberated in the primary response and leads to hematological, osmo-ionic, and metabolic changes (Oliveira et al. 2011; Simonato et al. 2013). These events will lead to the tertiary response, resulting in a decrease of growth performance or reproductive capacity, as well as the ability to cope with other stressful situations (Wendelaar Bonga 1997).

Most of the theory about starvation physiology is based on experiments using birds and mammals as experimental units. In endotherms, starving animals undergo three different physiological and/or morphological phases which are distinguished by changes in body mass, metabolites, and hormone levels in plasma or enzyme activities related to glucose metabolism (McCue 2010). However, in fish, starvation physiology

is different because ambient temperature has a large effect on their metabolic processes (Bar and Volkoff 2012). Generally, fish grown in colder water are better adapted to starvation than warm water fish, probably since winter is associated with low food availability and reduced activity (Binner et al. 2008). Therefore, we must take into account the water temperature when studying the duration of food deprivation periods in aquaculture and it is recommended to express this period in cumulative degree days ($^{\circ}\text{C d}$), the sum of the average temperatures of the fasting days. This approach is uncommon in aquaculture research, being used only in 5% of all the studies related to temperature and growth between 1980 and 2006 (Neuheimer and Taggart 2007), but the European Food Safety Authority (EFSA 2008) indicates that it is not possible to specify an exact maximum duration of the fast in terms of time only, since its impact on animal welfare depends on water temperature, among other factors.

Feeding habits are also important since carnivorous fish, like rainbow trout, typically feed less frequently than omnivorous and herbivorous fish (Bond 1996; Armstrong and Schindler 2011), and previous studies show that rainbow trout fed once every 2 days during a month had a lower stress response after pre-slaughter fasting (Bermejo-Poza et al. 2015). It is unclear, however, how rainbow trout are affected by different periods of fasting and starvation in terms of degree days and how that may affect their welfare. In this study, we evaluated the physio-metabolic response to prolonged food deprivation in rainbow trout metabolism and compared to short-term fasting.

Material and methods

Experimental design and fish

The trial was carried out at the aquaculture facilities of the School of Forestry Engineering (Madrid, Spain), located on a small slope where the downward water flow is distributed among different raceways. For the experiment, four parallel raceways (volume 5.16 m^3) were filled with freshwater from an underground well, supplying a constant water flow (recirculation) and dissolved oxygen ($8 \text{ mg O}_2/\text{L}$). During the whole experiment, animals were subjected to natural photoperiod (10L:14D). To calculate degree days, water temperature was recorded once every 30 min during the whole trial

using temperature sensors (Hobo-U11, ONSET, Bourne, MA, USA) averaging 10.2 ± 1.1 °C. Each race-way was split into six separate sections with the same water volume (0.86 m^3) using stainless steel separators with grids, allowing the passage of water from one section to another. In this study, 432 rainbow trout, with an initial weight of 225 ± 5.78 g (mean weight $\pm$ SEM), obtained from a farm (Cifuentes, Guadalajara, Spain) were randomly distributed into 24 groups of 18 individuals per compartment (six groups per treatment were in the same raceway). Each group was subjected to either no fasting (0D), to a pre-slaughter fast of 5 days (55 °C d; 5D), 10 days (107 °C d; 10D), or 20 days (200 °C d; 20D). Using this experimental design, we could evaluate each experimental condition six times.

Fish were fed 1% body weight of commercial feed (42% crude protein, 23% fat, 4.1% ash, and 2.0% crude fiber, 30 ppm astaxanthin), in compliance with recommendations for rainbow trout.

Blood and tissue sampling

At the end of each experimental period (0, 5, 10, or 20 days), trout were captured with dip nets and electrically stunned (head only, 90 W, 2 s). Then, they were weighed and fork length measured to calculate the condition factor as indicated in Table 1.

Subsequently, two blood samples of 1 mL were taken from the caudal vein from 12 trout per compartment. The first blood sample was collected in pre-filled syringes with

dry balanced heparin (PICO50 ®, Radiometer Medical Aps, Copenhagen, Denmark) for blood assays. For the evaluation of the remaining parameters (cortisol, glucose, lactate dehydrogenase-LDH activity, triglycerides, non-esterified fatty acids, osmolality, total proteins, albumin, and globulin), a second blood sample was obtained and placed into lithium heparinized tubes that were centrifuged at 1200 g for 15 min to obtain the plasma, which was frozen at -60 °C until further analysis. After blood sampling, fish were killed immediately (< 15 s) by sectioning the spinal cord at the base of the head. Immediately after slaughter, each fish was eviscerated and stomach content was weighed to calculate the empty body weight (fish weight–stomach content) and % relative weight of the stomach contents. Digestive tract (from stomach to anus, including visceral fat), liver and spleen weights were weighed to calculate biometric parameters. Before removing stomach content, gastric pH was measured using a pH-meter (HANNA, mod. HI9125) inserting its probe into the stomach. We sampled the liver of gutted fish, the dorsal musculature (at the level of the dorsal fin), and brain, freezing immediately in liquid nitrogen for later determinations of liver and muscle glycogen concentration, liver fatty acid composition and acetylcholinesterase (AChE) activity of liver, muscle, and brain.

Assay procedures

After collection, blood samples were analyzed immediately (< 5 min) in an ABL90FLEX ® blood gas analyzer

Table 1 Biometric parameters, stomach content, and gastric pH of rainbow trout with different fasting times

	Fasting time				<i>p</i> value
	0D	5D	10D	20D	
Fish weight (g)	270 ± 4.25^a	260 ± 3.68^{ab}	250 ± 4.26^b	229 ± 3.57^c	< 0.001
Fork length (cm)	25.1 ± 0.32	25.8 ± 0.29	25.4 ± 0.38	24.9 ± 0.35	0.31
CF	1.68 ± 0.04^a	1.58 ± 0.03^{ab}	1.53 ± 0.04^b	1.49 ± 0.04^b	0.002
DSI (%)	7.49 ± 0.34^a	6.26 ± 0.20^b	6.30 ± 0.25^b	6.07 ± 0.37^b	< 0.001
Stomach content (%)	12.38 ± 2.06^a	3.53 ± 0.55^b	3.84 ± 1.28^b	2.89 ± 0.52^b	< 0.001
Gastric pH	2.68 ± 0.20^c	2.21 ± 0.10^c	3.52 ± 0.17^b	4.44 ± 0.15^a	< 0.001
HSI (%)	1.33 ± 0.07^a	1.14 ± 0.04^{ab}	1.05 ± 0.04^b	1.02 ± 0.08^b	< 0.001
SSI (%)	0.34 ± 0.02	0.36 ± 0.02	0.44 ± 0.04	0.43 ± 0.03	0.06

Values are mean $\pm$ SEM, $n = 108$ per treatment. ^{a, b, c} Different superscripts within a row indicate significant differences among fasting times ($p < 0.05$). CF (condition factor) = fish weight (g)/fork length (cm)³; DSI (digestive somatic index) = digestive tract (g)/empty body weight (g) $\times 100$; HSI (hepato-somatic index) = liver weight (g)/empty body weight (g) $\times 100$; SSI (spleen somatic index) = spleen weight (g)/empty body weight (g) $\times 100$. 0D, no fasting; 5D, 5 days or 55 degree days (°C d); 10D, 10 days or 107 °C d; 20D, 20 days or 200 °C d

(Radiometer Medical Aps, Copenhagen, Denmark) for determination of pH, ionogram (sodium, potassium, chloride, and ionic calcium), and lactate levels. In plasma obtained of second blood sample, cortisol was measured by enzyme immunoassay using a commercial Cortisol EIA well kit (Radim Ibérica S.A., Barcelona, Spain). Plasma concentrations of glucose and triglycerides were determined using reflectance spectrophotometry (Reflotron® System, Roche Diagnostics, IN, USA) based on methods described by Trinder (1969) and Bucolo and David (1973), respectively. LDH activity determination was based on Furné et al. (2012), measuring the conversion of pyruvate to lactate by monitoring the oxidation of NADH. The concentrations of non-esterified fatty acids (NEFA) were determined by enzymatic-colorimetric method using commercial kits (Randox Diagnostic, London, UK). Total plasma proteins were quantified by refractometry (ATAGo T2-Ne®, Atago, Tokyo, Japan). The albumin concentration was measured by bromocresol green method (Boehringer Mannheim) and globulin concentration as the difference between total protein concentration and albumin. Osmolality was assessed in 50 µL of plasma by measuring freezing point in an osmometer (Fiske one-Ten®, Fiske Co, MA, USA).

Liver and muscle samples were homogenized with perchloric acid to determine glycogen concentration as described by Dreiling et al. (1987). Liver color measurements were taken in fresh liver by triplicate, using a Minolta Spectrophotometer CM-2500c (Minolta, Osaka, Japan). The International Commission on Illumination (CIE) L*a*b* system (CIE 1978) was chosen as the color scale. Using a* and b* parameters, we calculated chroma (C*) and hue (h*) as indicated in Table 2.

Fatty acid methyl esters (FAMES) were formed in duplicate according to Lee et al. (2012). The final organic layers with the FAMES were transferred to a 2-mL vial, then capped and stored at −20 °C until analysis. FAMES were analyzed by gas chromatography with flame ionization detection (Perkin-Elmer Autosystem-1: A, MA, USA). Results were expressed as a percentage of the total FAMES.

Liver, muscle, and brain samples were homogenized with a phosphate buffer pH 8 0.1 M to determine the activity of AChE based on Ellman et al. (1961), with acetylthiocholine iodide 0.075 M and dithiobisnitrobenzoic acid (DTNB) 0.01 M as substrates. Absorbance was measured at 412 nm for 3 min at intervals of 36 s.

Statistical analysis

The data were analyzed using SAS software ver. 9.0 (Statistical Analysis System Institute Inc., Cary, NC, USA). A prior analysis of the normality and homogeneity of variance of all variables was performed using the Shapiro-Wilk test with the UNIVARIATE procedure and Bartlett's test with the ANOVA procedure for residues. When parameters did not meet the two assumptions, the variables were transformed. The tables present the back-transformed mean values for easier interpretation of the results, although the standard errors of the mean (SEM) and significance values are presented for transformed variables. We used the GLM procedure of SAS, with fasting times as fixed effect. The Bonferroni test was used as a post hoc test ($p < 0.05$).

Table 2 Liver/muscle glycogen and liver color parameters (lightness, L*; chroma, C*; and hue, h*) of rainbow trout with different fasting times

	Fasting time				<i>p</i> value
	0D	5D	10D	20D	
Liver glycogen (mg/g)	177.3 ± 10.08 ^a	110.7 ± 10.46 ^b	61.8 ± 7.37 ^c	40.5 ± 3.62 ^c	< 0.001
Muscle glycogen (mg/g)	6.80 ± 0.51 ^a	8.07 ± 2.68 ^a	6.85 ± 0.48 ^a	4.92 ± 0.39 ^b	< 0.001
L*	28.7 ± 0.76	26.6 ± 0.85	27.1 ± 0.84	25.8 ± 0.79	0.08
C*	13.4 ± 0.38 ^b	15.5 ± 0.47 ^a	15.9 ± 0.40 ^a	15.1 ± 0.59 ^{ab}	0.004
h* (°)	46.5 ± 1.83	41.0 ± 1.84	43.2 ± 1.70	44.2 ± 2.66	0.12

Values are mean ± SEM, $n = 36$ per treatment. ^{a, b, c} Different superscripts within a row indicate significant differences among fasting times ($p < 0.05$). L* (lightness); C* (chroma) = $\sqrt{(a^*{}^2 + b^*{}^2)}$; h* (hue) = $\arctan(b^*/a^*) \times 57.29$. 0D, no fasting; 5D, 5 days or 55 degree days (°C d); 10D, 10 days or 107 °C d; 20D, 20 days or 200 °C d

Results

Morphometric measurements and gastric pH

Most biometric parameters and the gastric pH varied significantly with fasting (Table 1). Fish weight and condition factor decreased with fasting, with similar levels between 0D and 5D trout. Fork length did not differ statistically among treatments. Digestive somatic index and stomach content were higher in 0D trout than in the other groups. Conversely, gastric pH was lower in trout of groups 0D and 5D than 10D and 20D. The hepato-somatic index was lower in 10D and 20D trout, with similar levels between 0D and 5D trout. Spleen somatic index showed no significant differences between groups.

Blood and plasma parameters

Blood and plasma parameters data are presented in Fig. 1. Glucose levels decreased significantly with fasting, with similar levels between 0D and 5D trout. The 20D trout had the lowest plasmatic levels of lactate compared to the other groups. Fasting produced a significant decrease in plasma triglycerides compared to levels found in 0D trout. Conversely, NEFA increased with fasting, being the highest in 20D trout. Total plasma proteins and globulin were lower in 10D and 20D trout, showing similar levels in 0D and 5D trout. The albumin/globulin ratio was lower in 0D trout than 20D. Finally, chloride ion concentration was lower in 0D trout than that in the rest of the groups. The remaining parameters (cortisol, LDH, osmolality, pH, albumin, sodium, potassium, and calcium ion) showed no significant differences among fasting times.

Liver/muscle glycogen and liver color

Liver glycogen was highest in 0D trout and decreased significantly with fasting, and 20D trout had the lowest value of muscle glycogen, being similar among the other groups. Liver L* and h* were not significantly different but liver C* increased with fasting, being lowest in 0D trout (Table 2).

Fatty acid composition of liver

The fatty acid composition of liver from each fasting treatment is presented in Table 3. The proportion of total

saturated fatty acids (SFA), C15:0, C16:0, and C17:0 increased with days of fasting, being significantly lower in 0D trout than the rest of the groups. Myristic acid (C14:0) was lower in 10D and 20D trout, with similar levels between 0D and 5D trout. Stearic acid (C18:0) increased in 5D and 10D trout but 20D fish presented similar levels as 0D. Monounsaturated fatty acids (MUFA) presented few significant differences due to fasting, with the lowest proportion of myristoleic acid (C14:1) occurring in 0D trout and highest proportion of oleic acid (C18:1) in 20D trout, being similar between other groups. Apart from linolenic acid (C18:3 n-3), which did not vary significantly with fasting time, the proportion of polyunsaturated fatty acids (PUFA) decreased with fasting.

Acetylcholinesterase activity

The activity of acetylcholinesterase enzyme in liver and muscle increased with fasting, being significantly higher in 10D and 20D trout, while brain acetylcholinesterase activity presented no differences among fasting times (Fig. 2).

Discussion

Morphometric measurements and gastric pH

In the present study, a 10-day fast (107 °C d) caused a significant decrease in fish weight and condition factor compared to controls, which coincides with other studies that report a decrease in body weight in rainbow trout after 1 to 6 weeks without food (Sumpter et al. 1991; Pottinger et al. 2003; Bermejo-Poza et al. 2017). The lack of a difference between 0D and 5D trout sustains the hypothesis that fish can quickly adjust their metabolic rate according to food availability (Lines and Spence 2012). In addition, other authors have observed that short-term fasting increases tolerance to stress, reducing the stress response during handling procedures in aquaculture production (Davis and Gaylord 2011), which could be due to physiological changes during non-feeding periods that allow them to use stored energy reserves for metabolic maintenance (Navarro and Gutierrez 1995). Apart from live weight, condition factor also proved to be a valuable index to measure fasting stress (Bavcevic et al. 2010; Chatzifotis et al. 2011), since it decreased starting after 10 days without food.

Fig. 1 Biochemical parameters in blood and plasma of rainbow trout at different fasting times (mean $\pm$ SEM, $n = 72$ per treatment). Different superscripts indicate significant differences among fasting times ($p < 0.05$). Transformation data for parameters when

performing analysis of variance: lactate (square root); triglycerides ($\log_{10}$). SEM and significance values are presented for transformed data. Means are presented back-transformed for better interpretation

Both stomach content and digestive somatic index (DSI) decreased in 5D trout (55 °C d), which agrees with Lines and Spence (2012) and McMillan and Houlihan (1992) who estimated an average of 1–5 days of fasting to ensure a completely empty stomach. Also, previous studies by our research group confirm that 19–23 °C d of fasting are enough to empty the stomach in rainbow trout (López-Luna et al. 2013; Bermejo-Poza et al. 2017). However, 10 days of fasting (107 °C d) were needed to modify gastric pH, increasing it slightly. That is probably due to decreased stimulation of oxynticopeptic cells in the stomach (Ostos Garrido et al. 1993), which decreases the secretion of hydrochloric acid (Yúfera et al. 2012).

As with fish weight and condition factor, hepatosomatic index (HSI) decreased in 10D and 20D trout. During fasting, the mobilization of body energy reserves decreases liver weight in fish (Navarro and Gutierrez

1995; Gisbert et al. 2011; Torfi Mozanzadeh et al. 2017) but it is not always quantified in terms of degree days. The relative size of the liver is correlated with the nutritional condition of the fish (Caruso et al. 2012), so lower HSI values may indicate a depletion of stored nutrients such as fat and glycogen in this organ, as a consequence of food deprivation (Barcellos et al. 2010; Costas et al. 2011; Davis and Gaylord 2011). However, the effect of fasting on the mobilization of liver reserves may vary with species and experimental conditions. According to the literature, 2–6 days of fasting are needed to decrease the HSI in rainbow trout (Farbridge and Leatherland 1992; McMillan and Houlihan 1992), which is sooner than what was found in the current study, although that effect is also probably temperature dependent, since fish metabolism is higher at higher temperatures (Kumar et al. 2013), and would be better expressed in terms of degree days.

Table 3 Liver fatty acid (FA) profile (expressed as percentage of total FA) of rainbow trout with different fasting times (mean $\pm$ SEM, $n = 36$ per treatment)

	Fasting time				<i>p</i> value
	0D	5D	10D	20D	
SFA	47.1 $\pm$ 3.04 ^b	58.6 $\pm$ 0.85 ^a	60.1 $\pm$ 0.80 ^a	59.1 $\pm$ 1.86 ^a	< 0.001
C12:0	0.06 $\pm$ 0.01	0.06 $\pm$ 0.01	0.08 $\pm$ 0.01	0.07 $\pm$ 0.01	0.28
C14:0	2.00 $\pm$ 0.17 ^a	1.97 $\pm$ 0.05 ^a	1.41 $\pm$ 0.05 ^b	1.48 $\pm$ 0.07 ^b	0.001
C15:0	0.33 $\pm$ 0.03 ^b	0.44 $\pm$ 0.01 ^a	0.44 $\pm$ 0.01 ^a	0.42 $\pm$ 0.01 ^a	0.001
C16:0	32.3 $\pm$ 2.01 ^b	40.1 $\pm$ 0.61 ^a	42.3 $\pm$ 0.50 ^a	43.3 $\pm$ 1.34 ^a	< 0.001
C17:0	0.44 $\pm$ 0.05 ^b	0.60 $\pm$ 0.02 ^a	0.63 $\pm$ 0.01 ^a	0.58 $\pm$ 0.02 ^a	0.001
C18:0	11.9 $\pm$ 1.02 ^b	15.5 $\pm$ 0.47 ^a	15.2 $\pm$ 0.31 ^a	13.3 $\pm$ 0.58 ^b	0.002
MUFA	30.5 $\pm$ 1.43	32.0 $\pm$ 0.98	32.4 $\pm$ 0.87	33.7 $\pm$ 0.95	0.27
C14:1	0.10 $\pm$ 0.02 ^b	0.20 $\pm$ 0.01 ^a	0.19 $\pm$ 0.02 ^a	0.22 $\pm$ 0.03 ^a	0.001
C16:1	1.91 $\pm$ 0.22	1.64 $\pm$ 0.11	1.53 $\pm$ 0.05	1.58 $\pm$ 0.08	0.25
C17:1	0.14 $\pm$ 0.01	0.14 $\pm$ 0.01	0.14 $\pm$ 0.01	0.13 $\pm$ 0.02	0.94
C18:1	25.1 $\pm$ 1.10 ^b	26.7 $\pm$ 0.78 ^b	27.9 $\pm$ 0.90 ^b	29.0 $\pm$ 0.84 ^a	0.032
C20:1 n-9	3.30 $\pm$ 0.32	3.33 $\pm$ 0.20	2.67 $\pm$ 0.19	2.76 $\pm$ 0.11	0.10
PUFA	22.4 $\pm$ 4.30 ^a	9.39 $\pm$ 0.96 ^b	7.52 $\pm$ 0.88 ^b	7.14 $\pm$ 0.98 ^b	0.001
C18:2	4.41 $\pm$ 0.49 ^a	3.05 $\pm$ 0.25 ^b	3.10 $\pm$ 0.23 ^b	2.63 $\pm$ 0.24 ^b	0.004
C18:3 n-3	0.40 $\pm$ 0.07	0.25 $\pm$ 0.04	0.26 $\pm$ 0.06	0.34 $\pm$ 0.04	0.21
C20:2	1.18 $\pm$ 0.15 ^a	0.54 $\pm$ 0.06 ^b	0.41 $\pm$ 0.04 ^b	0.29 $\pm$ 0.03 ^b	< 0.001
C20:3 n-6	0.79 $\pm$ 0.18 ^a	0.29 $\pm$ 0.03 ^b	0.19 $\pm$ 0.02 ^b	0.17 $\pm$ 0.03 ^b	0.001
C20:4 n-6	2.63 $\pm$ 0.55 ^a	1.14 $\pm$ 0.15 ^b	0.81 $\pm$ 0.13 ^b	0.70 $\pm$ 0.11 ^b	0.001
C20:5 n-3	2.26 $\pm$ 0.47 ^a	0.93 $\pm$ 0.12 ^b	0.49 $\pm$ 0.02 ^b	0.69 $\pm$ 0.15 ^b	0.001
C22:5 n-3	0.76 $\pm$ 0.17 ^a	0.19 $\pm$ 0.03 ^b	0.13 $\pm$ 0.02 ^b	0.16 $\pm$ 0.03 ^b	< 0.001
C22:6 n-3	10.0 $\pm$ 2.33 ^a	3.00 $\pm$ 0.42 ^b	2.10 $\pm$ 0.39 ^b	2.20 $\pm$ 0.41 ^b	0.001

Values are mean $\pm$ SEM, $n = 36$ per treatment. ^{a, b, c} Different superscripts within a row indicate significant differences among fasting times ($p < 0.05$). *SFA*, total saturated fatty acids; *MUFA*, total monounsaturated fatty acids; *PUFA*, total polyunsaturated fatty acids. *0D*, no fasting; *5D*, 5 days or 55 degree days ($^{\circ}\text{C d}$); *10D*, 10 days or 107 $^{\circ}\text{C d}$; *20D*, 20 days or 200 $^{\circ}\text{C d}$

Blood and plasma parameters

Under a compromised situation, cortisol secretion modulates many biochemical, physiological, and behavioral responses, such as energy metabolism, ion-osmotic regulation, and growth (Mommensen et al. 1999; Ellis et al.

2012). Thus, we expected that plasma cortisol levels would be affected by food deprivation in the current study, but they were similar among groups. Data about the effect of fasting or starvation on cortisol levels in rainbow trout are inconsistent, with studies reporting an increase in plasma cortisol after periods of food

Fig. 2 Acetylcholinesterase (AChE) activity in brain, liver, and muscle of rainbow trout with different fasting times (mean $\pm$ SEM, $n = 36$ per treatment). Different superscripts indicate significant differences among fasting times ($p < 0.05$)

deprivation (Sumpter et al. 1991), decreased plasma levels (Farbridge and Leatherland 1992), or, as in our case, no effect (Holloway et al. 1994; Reddy et al. 1995). Cortisol contributes to the maintenance of both basal and stress-related homeostasis and varies with temperature, gender, sexual maturity, and genetics (Pottinger 2010), so values tend to be quite variable even among individuals from the same tank/treatment and cannot always be assumed to be related to stress alone (Chrousos and Kino 2009). It should be also considered that cortisol secretion can return to basal values in few days (Ellis et al. 2012), so the lack of changes could be masked after 5 days of fasting.

Regarding the mobilization of body reserves, fasting induces a sequential use of glycogen, lipid, and protein reserves (Collins and Anderson 1995). In the current study, plasma glucose, triglycerides, and total proteins decreased after 10 days of fasting (107 °C d). Periods of food deprivation around 70 °C d can induce hypoglycemia in rainbow trout (Furné et al. 2012). The 10D and 20D trout had lower glucose levels than controls but within basal levels, probably since it is an essential fuel for a number of tissues (Costas et al. 2011), but possibly at the expense of glycogen stored in the liver. An increase in plasma lactate levels is one of the earliest responses associated with “urgent” fuel consumption by anaerobic metabolism in fish under stress (Gatica et al. 2010). Then, these values can be restored by a lactate mobilization for gluconeogenic purposes in liver (Polakof et al. 2006; Liew et al. 2012). At 20 days of fasting (200 °C d), the manifested decrease of plasma lactate might be related to the maintenance of basal glucose levels, which has also been observed in other fasted fish (Soengas et al. 1996; Sangiao-Alvarellos et al. 2005). LDH activity is associated with glucose anaerobic oxidation and used as biomarker for heat stress in fish (Feidantsis et al. 2015). Elevated activity of this enzyme can indicate hepatic, renal, or muscular damage, and this effect can be related to stress stimulus like fasting (Peres et al. 2014). The activity of LDH presented no significant differences between groups, possibly since that requires more degree days of fasting.

Triglycerides are also an important source of energy that fish will use in response to a stressful stimulus such as acclimatizing to different growing densities or fasting (Laiz-Carrión et al. 2012; Millán-Cubillo et al. 2016). As in the case of glucose, plasma triglycerides decreased in 10D trout. Fasting led fish to make notable use of their lipid reserves after 10 days of food deprivation,

possibly since glycogenolysis was not sufficient to normalize glucose levels, and this was reflected in reduced plasma triglycerides (Favero et al. 2018). Lipolysis was confirmed by the high levels of NEFA after 10 days of fasting (Polakof et al. 2006; Mancera et al. 2008).

According to Lauff and Wood (1996), the use of protein as a metabolic fuel during feed deprivation accounts for 24% of the energy needs of rainbow trout. Total plasma protein levels tend to fall after prolonged starvation in several fish species like common carp (*Cyprinus carpio*) and rainbow trout (Shimeno et al. 1997; Polakof et al. 2007), agreeing with our finding of lower total proteins in plasma after 10 days of fasting. However, fasting did not decrease plasma albumin, as found in food-restricted mammals (Straus et al. 1994; Don and George 2004), but the nutritional regulation of albumin is not well understood in fish (Kawanago et al. 2014). On the other hand, our finding of decreased levels of globulin after 10 days of fasting could indicate an impairment of the immune response, where it plays an important role (Nayak et al. 2004).

Regarding plasma ion levels, there were no differences among treatments, with the exception of an increase in plasma chloride after 5 days of fasting (55 °C d), which could be since branchial function was affected. Chloride cells play an essential role in maintaining the ionic balance of the internal environment, absorbing Na^+ and Cl^- , which could be affected by stressors like fasting and reduce their function (Simonato et al. 2013). The fact that food-deprived fish maintained a similar concentration of plasma ions as controls despite reduced energy stores is perhaps not surprising as a loss of ionic balance would almost certainly lead to death (Gonzalez and McDonald 1992; Gingerich et al. 2010).

Liver/muscle glycogen and liver color

As commented above, food deprivation mobilizes nutrients stored in the liver, such as glycogen, especially during the early stages of fasting (Davis and Gaylord 2011). The moment when liver glycogen reserves begin to decrease is variable but is thought to range from 5 to 20 days after food is removed (Sangiao-Alvarellos et al. 2005; Barcellos et al. 2010). Furné et al. (2012) observed that, in rainbow trout, hepatic glycogen is mobilized after 5 days of fasting, which agrees with our results and reflects how glycogen is used to replace the absence of dietary carbohydrate intake (Dias Junior

et al. 2016). However, muscle glycogen did not decrease until 20 days of food deprivation (200 °C d), possibly because it makes a small contribution to total energy expenditure and can be maintained at the expense of hepatic glucose release (Navarro and Gutiérrez 1995).

Since the liver plays an important role in maintaining homeostasis during fasting, its color is affected and can be used as an indicator of fasting stress in fish (Bermejo-Poza et al. 2017). After 5 days of fasting, liver chroma increased in the current study, possibly indicating a decrease in lipid concentration, as seen in other animals such as fasted broiler chickens (Trampel et al. 2005). These results showed that liver color can be a good biomarker for stress response produced by fasting, confirmed with previous results of our group (Bermejo-Poza et al. 2017).

Fatty acid composition of liver

Regarding the fatty acid composition of rainbow trout liver, the results were similar as those of previous studies that reported a higher percentage of saturated and mono-unsaturated fatty acids than polyunsaturated fatty acids (Bayraktar and Bayir 2012; Suárez et al. 2014; Taşbozan et al. 2016). Overall, after 5 days of fasting, we observed an increase of SFA and decrease of PUFA. The percentage of MUFA was not affected by fasting, except for myristoleic acid (C14:1) and oleic acid (C18:1), which increased at 5 and 20 days of fasting respectively, possibly due to the capacity of freshwater fish to desaturate endogenously the saturated fatty acids synthesized to monounsaturated fatty acids (Tocher 2003). Einen et al. (1998) did not find large differences in the fatty acid profile of salmon subjected to fasting periods of up to 86 days although they observed differences in the desaturase enzymes, specifically in the $\Delta 9$ -desaturase. Unfortunately, we did not measure the activity of this enzyme in our study but this could be the reason of the increase of C16:0 in fish subjected to fasting.

After 5 days of fasting, our results indicate that trout used their reserves of PUFA, mainly long chain n-3 fatty acids. Zengin et al. (2013) observed that n-3 fatty acids are the ones that decrease to a greater extent when used as an energy source.

Acetylcholinesterase activity

In liver and muscle, an increase in acetylcholinesterase (AChE) activity after 10 days of fasting (107 °C d) could

be associated with the proteolytic function of this enzyme. During prolonged starvation (in tissues subject to catabolism), AChE (a protease) levels increase and begin to degrade tissue in mice (Suchiang and Sharma 2011) and fish (Kim and Park, 1995). AChE is also important for many physiological functions in fish, such as prey location, predator evasion, and food orientation (Miron et al. 2005). So, increased AChE activity may also be linked with increased food-seeking behavior during fasting. Levels were similar in the liver and muscle of 0D and 5D trout, so it appears that protein catabolism began after 10 days of fasting (when total plasma protein levels also decreased). However, in brain, AChE was not affected, probably since nervous tissue is not normally degraded as a source of energy and most AChE in the nervous system is used to degrade of acetylcholine (Rodrigues et al. 2011).

Conclusions

The results of the present study showed that rainbow trout subjected to 20 days (200 °C d) without food undergo a significant decrease in some biometric parameters and plasma metabolites related to energy metabolism. However, all trout maintained their ion homeostasis in plasma, probably at the expense of metabolic energy (Applebaum et al. 2014; Alix et al. 2017). Fasting for 5 days (55 °C d) had very little effect on the trout, supporting previous studies by our research group showing that rainbow trout can cope well with up to 58 °C d of fasting with little or no effect on metabolic or stress indicators (López-Luna et al. 2013; Bermejo-Poza et al. 2016; Bermejo-Poza et al. 2017). In conclusion, based on the results obtained in this study, a 5-day fasting period (55 °C d) had little impact on biometric parameters and mobilization of body reserves, while 10 and 20 days produced important changes in liver weight and plasma concentration of metabolites related to energy metabolism. However, even after 20 days (200 °C d) of fasting, trout maintained basal levels of plasma ions and brain AChE, which underlines their importance in homeostasis and the preservation of vital functions.

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