# The effect of intermittent feeding on the pre-slaughter fasting response in rainbow trout

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A B S T R A C T

Pre-slaughter fasting, usually measured in days, helps to empty the digestive system but less is known about its effect on fish welfare or flesh quality. We evaluated the combined effects of fasting (measured in degree-days, °C d), preceded by intermittent feeding in the last month of production, on the flesh quality rainbow trout (*Oncorhynchus mykiss*). Trout (n = 240) were fed the same total amount of food either daily (D), once every two days (2D) or once every four days (4D) for one month, and then fasted for 24.3 °C d (2 days) or 102 °C d (9 days). There was a significant interaction between the effects of intermittent feeding and pre-slaughter fasting for most of the parameters measured. Slaughter weight was higher in 2D and 4D trout after two days of fasting, probably since they had more feed in their stomachs. Muscle glycogen and the hepato-somatic index were also higher in 2D trout after 2 days of fasting, suggesting higher energy reserves. There was no interaction between the effects of intermittent feeding and fasting on final muscle pH which was higher after 9 days of fasting, indicating poorer flesh quality. Overall, the results suggest that trout that received a skip-a-day feeding schedule one month before slaughter, adapt better to a two day fast than trout fed daily or once every four days.

## 1. Introduction

Ethical and sustainable aquaculture involves considering fish welfare, but there is little information about the effect of some routine handling procedures on trout welfare, despite its being one of the most produced species in Europe (176.983 metric tons in 2012; APROMAR, 2013). One example of the effects of a routine procedure which we know little about is fasting, which is performed under several different situations including size classification and pre-slaughter handling. Fasting prior to slaughter empties the gut and decreases oxygen demand and waste production (Robb, 2008). It also reduces the amount of feed and feces in the digestive system, so spoilage is delayed and digestive enzyme activity is reduced after *rigor mortis* is resolved. However, fasting may also increase stress levels and if these levels are high enough, it affects the onset and release of *rigor mortis*, which determines the duration of fish freshness (Poli et al., 2005).

The duration of fasting necessary to empty the gut is species and water temperature dependent but may be expected to be from one to five days. Previous authors have suggested that food withdrawal during that period is unlikely to cause significant welfare problems (Lines and Spence, 2012; López-Luna et al., 2013). In commercial practice there are no strict rules controlling the time of pre-slaughter fasting. However, the Farm Animal Welfare Council (FAWC, 1996) suggests 48 h of fasting as a maximum for trout and the Compassion in World Farming (CIWF, 2009) suggests a maximum of 72 h. The European Food Safety Authority (EFSA, 2008) recommends not fasting rainbow trout for over 50 °C d (degree-days). Apart from assessing time limits (in days or hours), it is important to set acceptable ranges in terms of degree-days (López-Luna et al., 2014). In aquaculture, degree-days are used to estimate the amount of time needed for different stages of growth, such as incubating eggs, breeding or fattening (From and Rasmussen, 1992). Some authors have analyzed the effect of short term fasting (up to 65 °C d) on flesh quality (López-Luna et al., 2014) but few studies have considered the effect of feeding in the last month of fattening on pre-slaughter fasting stress and product quality or compared extremes of fasting in terms of degree-days (for example 20 °C days vs. values over 65 °C days).

Much research has been performed on the effect of feeding frequency on fish stress (e.g., Cañon Jones et al., 2012), but less is known about the effects of short intermittent feeding in the last part of fattening on the subsequent response to pre-slaughter fasting. According to Armstrong and Schindler (2011), in the wild, most carnivorous fish eat once every two days. This suggests that feeding once every two days might be acceptable for trout physiologically, like the skip-a-day systems commonly used in poultry (Oyedeji and Atteh, 2005) and tested on other

fish like tilapia (Villarroel et al., 2011). Fish can adapt anatomically and physiologically to intermittent feeding, increasing relative stomach weight and volume, and enabling the fish to eat more when food is available (Mattila et al., 2009). Moreover, during the breeding season, trout can fast for weeks, suggesting that different durations of fasting may depend on previous experience.

In this study we analyzed the effect of intermittent fasting in the last month of fattening combined with two different pre-slaughter fasting times on rainbow trout flesh quality and muscle glycogen reserves.

## 2. Material and methods

### 2.1. Fish and experimental design

The experiment was carried out at a fish farm located in the province of Guadalajara (Spain), on the banks of the Tajuña River. Six parallel raceways (6 m × 30 m) were filled with a constant flow of river water (approximately 0.06 $m^3/s$) that had not passed through other tanks with fish and was not recirculated. We used 240 rainbow trout (*Oncorhynchus mykiss*), 40 per raceway, provided by the fish farm, with an initial average weight of 324 ± 41 g (± s.d.) which were placed in cages (1.5 m × 0.8 m × 0.35 m) in the center of the production tanks.

Each trout was individually identified using Pit-Tags (Pit Tag i-Tag 162, 2 × 12 mm) injected under the dorsal fin a month before the experiment. Fish were subjected to a natural photoperiod and fed by hand a commercial feed (Efico Ys, 587F, Biomar) twice a day during the last month of fattening, from 27 September to 28 October (feed composition: 41% crude protein, 21.5% carbohydrate, 24% crude fat, 6.5% ash and 2.5% crude fiber). Average dissolved oxygen and water temperatures were 10.5 ± 0.5 mg/l and 12.2 ± 1.3 °C, respectively. To calculate degree-days, water temperature was recorded once every 5 min using underwater temperature sensors (Hobo®-U11).

The six groups of trout in the six separate raceways were divided into three treatments (two replicates each), either fed every day (D), once every two days (2D, one day of fasting, skip a day system) or once every four days (4D, three days of fasting). All treatments were given the same amount of food but distributed differently: the trout with the daily feeding schedule received 1.2% of their body weight in food every day, the skip a day groups were fed once every two days (2.4% of their body weight) and, finally, those fed every four days were given 4.8% of their body weight. Because the amount of food provided per day in this group was very high, they received feed to satiety and were fed again one hour later, again to satiety. These feeding intervals were maintained for one month, then before slaughter one group per treatment was fasted two days and the other nine days, corresponding to 24.3 °C days and 102 °C days respectively based on the temperature data.

### 2.2. Slaughtering and analyses

Before slaughter, the water level of the raceway was slowly lowered to half the cage height (which took about 10 min). Trout were captured with dip nets and killed immediately (<15 s) using the "ikijime" method, which involves piercing the brain of the fish using a sharp tip and is suggested to cause less suffering for the fish and the smallest possible changes in flesh quality (Malcolmsen et al., 1995).

After slaughter, fish were placed in a cooler with ice and eviscerated quickly (approximately 10–15 min). Each fish was measured and weighed, and half were eviscerated and the other half were left to measure *rigor mortis*. To measure the concentration of muscle glycogen and the IMP/ATP ratio (R value), we sampled the dorsal musculature at the level of the dorsal fin and liver of gutted fish, freezing immediately in liquid nitrogen for later determination in the laboratory.

#### 2.2.1. Morphometric measurements

Each fish was weighed before the experiment and after slaughter and measured after slaughter. Initial weight and slaughter weight were used to calculate relative growth (expressed as %):

Relative growth
= (Slaughter weight - Initial weight / Initial weight) x 100

Slaughter weight and fork length were used to calculate the body condition or coefficient of condition (CC):

Coefficient of condition (CC)
= Slaughter weight (g) / Fork length$^3$(cm)

Stomach content was weighed to calculate the empty body weight (slaughter weight - stomach content) and was expressed as % relative to the stomach weight. The weights of the digestive system (from stomach to anus, including visceral fat), liver and spleen were weighed too and are expressed as % relative to the empty body weight (digestive somatic index, hepato-somatic index and spleen somatic index, respectively). The carcass yield was calculated as the ratio between slaughter weight and carcass weight and expressed as % too.

#### 2.2.2. Physicochemical flesh quality

Muscle pH was measured at the front end of the dorsal muscle after cutting it away from the head at 0, 2 and 24 h *post-mortem* using a pH-meter (HANNA, mod. HI9125) that adjusts for temperature.

The progression of *rigor mortis* (0 and 24 h *post-mortem*) was measured following Cuttinger's method (Korhonen et al., 1990), placing each trout on a solid flat surface so that the body part behind the posterior end of the dorsal fin was hanging over an edge, unsupported. The *rigor* angle was calculated as $\alpha = \tan^{-1}$ (X/Y), where X is the length (cm) of the horizontal leg of the right-angled triangle and Y is the length (cm) of the vertical leg of the right-angled triangle.

Water holding capacity (WHC) was determined as the amount of water lost by the flesh sample at 24 h *post-mortem* (percentage of water released) using the filter paper press method (Grau and Hamm, 1957). The WHC was calculated as the difference between the total percentage of water of the muscle and the released water.

Finally, color measurements were taken at 0 and 24 h *post-mortem* for muscle using a Minolta Spectrophotometer CM-2500c (Minolta, Osaka, Japan). The CIE 1976L*a*b* system recommended by the International Commission Illumination (CIE, 1978) was chosen as the color scale. Three measurements were taken of the dorsal muscle on the right-hand filet just behind the dorsal fin, as described by Pavlidis et al. (2006).

#### 2.2.3. Muscle glycogen and R-value

The glycogen concentration in muscle was determined using the technique described by Dreiling et al. (1987). Measurements were taken at 0 and 24 h *post-mortem*.

The IMP/ATP ratio (R value) was measured at 0 and 24 h *post-mortem* based on Hönikel and Fischer (1977) and expressed as the ratio of absorbance (nm) at 250/260.

### 2.3. Statistical analyses

The parameters were analyzed using the 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–Wilks test with the UNIVARIATE procedure and Bartlett's test with the ANOVA procedure for residues. The Bonferroni test was used for mean comparison ($p < 0.05$). If the interaction between effects (intermittent feeding, days of fasting and hours *post-mortem*) was significant, planned comparisons among means were performed using Bonferroni test ($p < 0.05$).

For variables related to weight (slaughter weight, relative growth, coefficient of condition, stomach content, digestive somatic index, hepato-somatic index, spleen somatic index and carcass yield) and water holding capacity, we used the GLM procedure of SAS with intermittent feeding (daily, once every two days or once every four days) and days of fasting (two or nine) as fixed effects, including in the model the interaction between the two factors. For slaughter weight, coefficient of condition, stomach content, digestive somatic index, hepato-somatic index, spleen somatic index and carcass yield, initial weight was introduced in the model as covariate.

For the other variables (angle of *rigor mortis*, muscle color, muscle pH, muscle glycogen and IMP/ATP ratio), we performed a repeated measures analysis using the MIXED procedure of SAS. Each trout was considered as the subject, while the intermittent feeding (daily, once every two days or once every four days) and days of fasting (two or nine) were the main factors and hours *post-mortem* was the within-subject factor. For each variable we evaluated which was the best covariance matrix, following the Bayesian information criterion (BIC). Several different matrices were tested (compound symmetry, variance components, autoregressive, heterogeneous autoregressive or unstructured), and we used: variance components for *rigor mortis* angle, color muscle and muscle glycogen and heterogeneous autoregressive for muscle pH and the IMP/ATP ratio.

Finally, we have completed a principal component analysis of principal body measurements and physicochemical flesh quality data using the procedures CORR and PRINCOMP in SAS. The variables for principal components analysis were standardized to a mean of zero and variance of one.

## 3. Results

### 3.1. Morphometric measurements

There was a significant interaction between intermittent feeding schedule and days of fasting for all morphometric measurements except SSI (Table 1). Slaughter weight and relative growth were both higher in 2D trout after two days of fasting and lower after nine days of fasting in 4D trout. This is probably since the stomach content of 2D and 4D trout was also higher than D trout. The CC of 2D and 4D trout was higher after nine days of fasting and similar for D trout at two and nine days of fasting.

Similar to the stomach content results, the DSI was highest in 2D and 4D trout after two days of fasting. The HSI was highest for the 2D group after two days of fasting, but then decreased substantially by nine days of fasting. The SSI was significantly affected by days of fasting but not by intermittent feeding. After nine days of fasting, SSI was higher than after two days in all groups with the greatest increase in 4D trout. Carcass yield was higher after nine days of fasting for 2D and 4D trout.

### 3.2. Physicochemical flesh quality

For *rigor* angle there was a triple interaction between intermittent feeding schedule, days of fasting and hours *post-mortem* (Table 2), but all groups presented similar values at 0 h *post-mortem*, which significantly increased after 24 h. The interaction between intermittent feeding and days of fasting is probably since D and 2D trout had a lower rigor angle at 24 h post-mortem than 4D trout at nine days of fasting.

Table 3 shows the means of flesh pH, muscle glycogen, IMP/ATP ratio and flesh color (L*, a*, b*). The significance of the effects, intermittent feeding, days of fasting, hours *post-mortem* and the interaction between these factors, is shown in Table 4.

For flesh pH there was a triple interaction between all the fixed effects, which was higher after nine days of fasting in all feeding schedule at 0 h *post-mortem*. Also, the pH at 24 h *post-mortem* was higher for nine days than two days fasted trout. Similar to pH, muscle glycogen had an interaction between feeding and days of fasting, since it was lower in 2D trout after nine days of fasting. The IMP/ATP ratio did not vary in terms of feeding or fasting. The WHC was only affected by fasting, being significantly higher after two days of fasting than nine days. The flesh L* did not vary with feeding schedule or fasting but a* only maintained similar values at 24 h (compared to 0 h) in 2D trout after two days of fasting. The b* was not affected by fasting but was higher in D trout at 24 h.

### 3.3. Principal component analysis

Fig. 1 shows a plot of the principal body measurements and physicochemical flesh quality data for the first two principal components (PCs). The data were resolved into six PCs that could explain 72% of the total variance. The first two of these components, namely PC1 and PC2, which accounted for 20.88% and 17.56% of the variance, respectively, showed a meaningful distribution of the samples. Carcass yield is located far from the first PC, opposite the stomach content and DSI, which are negatively correlated ($r = -0.6935$ and $r = -0.8241$, respectively). The IMP/ATP ratio at 0 and 24 h *post-mortem* is near the second PC, which shows the low importance in this PC. The pH at 24 h *post-mortem* is placed 180° from the muscle glycogen indicating a negative correlation among these traits ($r = -0.2404$). Regarding color parameters, a* and b* are located in the same quadrant (positively correlated) but placed 180° from L* parameter, indicating a negative correlation between L* and the other two. Fig. 2 displays the projection of the principal body measurements and physicochemical flesh quality data in the first two PCs. Two separate groups can be observed, 2D trout on the lower left quadrant, where we find stomach content, DSI, HSI and muscle glycogen (0 and 24 h *post-mortem*). On the other hand, trout subjected to a nine-day fasting are located on the upper right quadrant, with carcass yield and pH measurements.

**Table 1**
Mean (± SEM) slaughter weight, relative growth, coefficient of condition (CC), stomach content, digestive somatic index (DSI), hepato-somatic index (HSI), spleen somatic index (SSI), carcass yield and water holding capacity (WHC) of rainbow trout on different intermittent feeding (IF) and days of fasting (D).

| | Daily (D) | | Skip a day (2D) | | Every 4 days (4D) | | Significance (p) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2 (n = 39) | 9 (n = 32) | 2 (n = 32) | 9 (n = 40) | 2 (n = 40) | 9 (n = 34) | IF | D | IF × D |
| Slaughter weight (g) | 353 ± 5.91$^{bc}$ | 352 ± 6.91$^{bc}$ | 398 ± 6.29$^{a}$ | 365 ± 5.49$^{b}$ | 362 ± 5.52$^{b}$ | 330 ± 6.03$^{c}$ | <0.001 | <0.001 | 0.01 |
| Relative growth (%) | 8.8 ± 1.8$^{bc}$ | 7.6 ± 2.1$^{bc}$ | 22.5 ± 2.0$^{a}$ | 12.2 ± 1.7$^{b}$ | 13.5 ± 1.7$^{b}$ | 2.6 ± 1.9$^{c}$ | <0.001 | <0.001 | 0.02 |
| Coefficient of condition | 1.17 ± 0.02$^{c}$ | 1.19 ± 0.02$^{c}$ | 1.24 ± 0.02$^{bc}$ | 1.39 ± 0.02$^{a}$ | 1.19 ± 0.02$^{c}$ | 1.37 ± 0.02$^{ab}$ | 0.009 | <0.001 | 0.08 |
| Stomach content (%) | 30.3 ± 3.6$^{b}$ | 4.3 ± 0.7$^{c}$ | 50.5 ± 3.3$^{a}$ | 14.7 ± 2.7$^{c}$ | 62.3 ± 3.7$^{a}$ | 7.7 ± 1.7$^{c}$ | <0.001 | <0.001 | <0.001 |
| DSI (%) | 11.3 ± 0.4$^{ab}$ | 9.55 ± 0.4$^{bc}$ | 14.4 ± 0.4$^{a}$ | 9.92 ± 0.4$^{bc}$ | 13.3 ± 0.7$^{a}$ | 8.98 ± 0.7$^{c}$ | 0.005 | <0.001 | 0.020 |
| HSI (%) | 1.64 ± 0.06$^{b}$ | 1.48 ± 0.07$^{b}$ | 2.06 ± 0.12$^{a}$ | 1.44 ± 0.06$^{b}$ | 1.43 ± 0.06$^{b}$ | 1.62 ± 0.06$^{b}$ | 0.012 | 0.004 | <0.001 |
| SSI (%) | 0.16 ± 0.02$^{b}$ | 0.17 ± 0.01$^{b}$ | 0.15 ± 0.02$^{b}$ | 0.18 ± 0.03$^{b}$ | 0.11 ± 0.01$^{b}$ | 0.23 ± 0.01$^{a}$ | 0.94 | 0.017 | 0.07 |
| Carcass yield (%) | 83.6 ± 0.7$^{a}$ | 85.3 ± 0.9$^{a}$ | 79.8 ± 0.8$^{b}$ | 84.7 ± 0.7$^{a}$ | 79.1 ± 0.7$^{b}$ | 86.1 ± 0.7$^{a}$ | 0.012 | <0.001 | 0.003 |
| WHC (%) | 68.4 ± 1.87$^{a}$ | 67.02 ± 1.03$^{ab}$ | 67.6 ± 1.64$^{ab}$ | 65.6 ± 1.70$^{ab}$ | 68.9 ± 1.51$^{a}$ | 61.1 ± 1.64$^{b}$ | 0.21 | 0.004 | 0.07 |

$^{a, b, c}$ Different superscripts within a row indicate significant differences among groups (intermittent feeding × days of fasting) ($p < 0.05$).

**Table 2**
Mean (± SEM) *rigor* angle of rainbow trout on different intermittent feeding (IF), days of fasting (D) and hours *post-mortem* (HP).

| | | *Rigor* angle (°) | | Significance (p) | |
|---|---|---|---|---|---|
| | | 0 | 24 | IF | 0.06 |
| Daily (D) | 2 (n = 19) | 32.74 ± 0.88[a] | 86.66 ± 0.88[b, xy] | D | 0.013 |
| | 9 (n = 16) | 33.51 ± 0.96[a] | 89.68 ± 0.96[b, y] | HP | <0.001 |
| Skip a day (2D) | 2 (n = 16) | 35.59 ± 0.96[a] | 86.75 ± 0.96[b, xy] | IF × D | 0.73 |
| | 9 (n = 20) | 34.59 ± 0.86[a] | 90.00 ± 0.86[b, y] | IF × HP | 0.33 |
| Every 4 days (4D) | 2 (n = 20) | 35.37 ± 0.86[a] | 84.21 ± 0.86[b, x] | D × HP | <0.001 |
| | 9 (n = 17) | 31.68 ± 0.93[a] | 89.76 ± 0.93[b, y] | IF × D x HP | 0.021 |

[a, b] Different superscripts within a row indicate significant differences among groups (hours *post-mortem*) ($p < 0.05$).
[x, y] Different superscripts within a column indicate significant differences among groups (intermittent feeding × days of fasting) ($p < 0.05$).

## 4. Discussion

Our results suggest that the type of feeding in the last month before slaughter has significant effects on the response of the fish to pre-slaughter fasting, as demonstrated by the interaction between the two main treatments considered. Trout fed daily suffered a sharp decline in stomach content after two days of fasting, which could be attenuated by feeding them once every two days (skip-a-day system), without a significant decrease in live weight. After nine days of fasting the coefficient of condition (CC), which reflects the nutritional status of fish (Bavcevic et al., 2010; Chatzifotis et al., 2011) decreased, which also tended to increase final flesh pH, compared to two days of fasting.

### 4.1. Morphometric measurements

By day two of fasting (24.3 °C d), the amount of feed in the stomach of 2D and 4D trout was higher than the D trout, and fell to near zero levels by nine days of fasting (102 °C d). This suggests that the higher live weight of 2D trout as compared to D trout may have been related to feed remaining in the stomach, but that does not explain why the live weight of 4D trout was similar to D trout after two days of fasting, since presumably they had four times the amount of feed in their stomachs two days previously. The data on stomach content suggest that the 4D trout ate a similar amount of food per meal as the 2D trout and possibly refused to eat half of the offered food. We did not measure actual food consumption per fish or feed losses in the cages but we noticed that it was difficult for the 4D trout to consume a four day ration. In any case, the 4D option proved to be the worst option.

As stated previously, the DSI decreased after nine days of fasting and the HSI, an indicator of body reserves, also followed the general trend of the stomach content, being lower in fasted fish, probably since trout begin to use body reserves to supply their caloric needs, while minimizing tissue loss. However, the rate of change of HSI may vary with fasting. In rainbow trout, Farbridge and Leatherland (1992) found a decrease with fasting after 48 h, but McMillan and Houlihan (1992) report significantly lower HSI only after six days of fasting. Normal values for HSI in rainbow trout are between 1.5 and 1.8 (Kizak et al., 2013; Rehulka, 2000). In our study, the HSI of 2D trout was higher after two days of fasting than in the rest of the groups and after nine days of fasting it decreased significantly. That could be due to a decrease in glycogen content (Davis and Gaylord, 2011) and/or free amino acids (Costas et al., 2011), the primary sources of energy used in fasting. The higher relative SSI of the 4D trout at nine days of fasting may also imply a higher stress conditions (Sánchez-Muros et al., 2013).

**Table 3**
Mean (± SEM) flesh pH, muscle glycogen, IMP/ATP ratio and flesh color parameters (L*, a* and b*) of rainbow trout on different intermittent feeding (IF), days of fasting (D) and hours *post-mortem* (HP).

| | | Daily (D) | | Skip a day (2D) | | Every 4 days (4D) | |
|---|---|---|---|---|---|---|---|
| | | 2 | 9 | 2 | 9 | 2 | 9 |
| | | (n = 19) | (n = 16) | (n = 16) | (n = 20) | (n = 20) | (n = 17) |
| *Flesh pH* | | | | | | | |
| 0 | | 6.86 ± 0.03[ab, x] | 7.00 ± 0.04[a, x] | 6.79 ± 0.04[b, x] | 6.83 ± 0.03[b] | 6.72 ± 0.03[b, x] | 6.85 ± 0.04[ab, x] |
| 2 | | 6.72 ± 0.04[b, x] | 7.01 ± 0.05[a, x] | 6.75 ± 0.05[b, x] | 6.84 ± 0.04[ab] | 6.58 ± 0.04[b, x] | 6.72 ± 0.04[b, xy] |
| 24 | | 6.31 ± 0.02[b,y] | 6.72 ± 0.03[a, y] | 6.24 ± 0.02[b, y] | 6.73 ± 0.02[a] | 6.31 ± 0.02[b, y] | 6.70 ± 0.02[a, y] |
| *Muscle glycogen (mg/g)* | | | | | | | |
| 0 | | 12.39 ± 1.19[bc] | 17.15 ± 1.29[ab] | 21.10 ± 1.29[a] | 9.52 ± 1.16[c] | 11.51 ± 1.16[c] | 13.45 ± 1.26[bc] |
| 24 | | 9.15 ± 1.19[bc] | 14.65 ± 1.29[ab] | 16.36 ± 1.29[a] | 6.29 ± 1.16[c] | 7.85 ± 1.16[c] | 11.41 ± 1.26[abc] |
| *IMP/ATP ratio* | | | | | | | |
| 0 | | 0.441 ± 0.024[x] | 0.480 ± 0.027 | 0.472 ± 0.027[x] | 0.442 ± 0.024[x] | 0.443 ± 0.024[x] | 0.481 ± 0.026 |
| 24 | | 0.563 ± 0.013[y] | 0.576 ± 0.015 | 0.588 ± 0.015[y] | 0.539 ± 0.013[y] | 0.565 ± 0.013[y] | 0.556 ± 0.014 |
| *Flesh color* | | | | | | | |
| L* | 0 | 48.02 ± 0.92 | 47.41 ± 0.95 | 47.12 ± 0.95 | 45.96 ± 0.85 | 48.57 ± 0.87 | 46.42 ± 0.92[x] |
| | 24 | 48.46 ± 0.92 | 47.26 ± 0.95 | 48.31 ± 0.95 | 48.74 ± 0.85 | 49.28 ± 0.87 | 50.70 ± 0.92[y] |
| a* | 0 | 12.14 ± 0.82 | 12.84 ± 0.85 | 10.63 ± 0.85 | 10.91 ± 0.76 | 9.90 ± 0.78 | 11.00 ± 0.82 |
| | 24 | 11.78 ± 0.82[a] | 10.10 ± 0.85[ab] | 10.64 ± 0.85[ab] | 8.62 ± 0.76[ab] | 8.20 ± 0.78[b] | 9.57 ± 0.82[ab] |
| b* | 0 | 19.12 ± 0.84 | 20.17 ± 0.86 | 16.62 ± 0.86 | 17.90 ± 0.77 | 16.81 ± 0.79 | 17.18 ± 0.84 |
| | 24 | 17.13 ± 0.84 | 16.24 ± 0.86 | 15.12 ± 0.86 | 14.67 ± 0.77 | 13.62 ± 0.79 | 16.00 ± 0.84 |

[a, b, c] Different superscripts within a row indicate significant differences among groups (intermittent feeding x days of fasting) ($p < 0.05$).
[x, y] Different superscripts within a column indicate significant differences among groups (hours *post-mortem*) ($p < 0.05$).

**Table 4**
Summary of p-values of flesh quality parameters on different intermittent feeding (IF), days of fasting (D) and hours *post-mortem* (HP).

| | Significance (p) | | | | | | |
|---|---|---|---|---|---|---|---|
| | IF | D | HP | IF × D | IF × HP | D × HP | IF × D × HP |
| Flesh pH | <0.001 | <0.001 | <0.001 | 0.18 | <0.001 | <0.001 | 0.002 |
| Muscle glycogen | 0.011 | 0.17 | <0.001 | <0.001 | 0.76 | 0.36 | 0.96 |
| IMP/ATP ratio | 0.94 | 0.97 | <0.001 | 0.06 | 0.93 | 0.20 | 0.87 |
| Flesh color | | | | | | | |
| L* | 0.13 | 0.30 | 0.004 | 0.89 | 0.16 | 0.15 | 0.27 |
| a* | 0.001 | 0.93 | 0.003 | 0.14 | 0.91 | 0.12 | 0.42 |
| b* | 0.002 | 0.19 | <0.001 | 0.51 | 0.79 | 0.56 | 0.16 |

Our values for carcass yield was within the range for rainbow trout (79–83%; Kizak et al., 2013) and lower for 2D and 4D fishes after two days of fasting, which could be due to their higher stomach content (and higher DSI), in turn related to pre-slaughter feeding type.

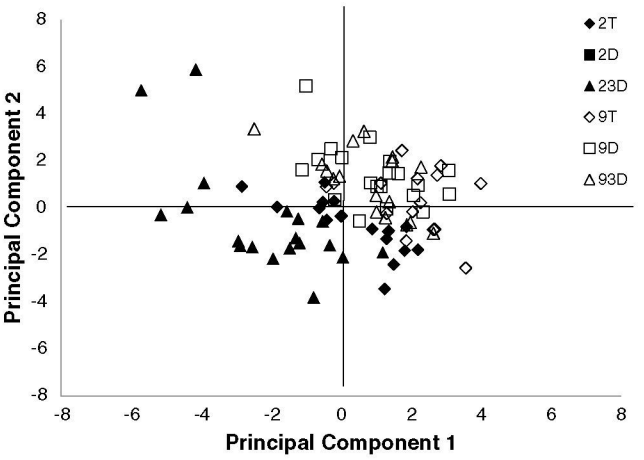


**Fig. 2.** Projection of the body measurements and physicochemical flesh quality data of the six groups studied (intermittent feeding × days of fasting) in the plane defined by the two principal components.

### 4.2. Physicochemical flesh quality

The onset of *rigor mortis* in rainbow trout flesh occurs between 2 and 9 h *post-mortem* and full *rigor* (maximum stiffening) takes place 24 h after slaughter (López-Luna et al., 2014). In our trial *rigor mortis* increased between 0 and 24 h *post-mortem*, so the values obtained at 24 h *post-mortem* may be considered as the maximum *rigor*. In general, fish that underwent nine days of fasting showed a higher *rigor* angle at 24 h *post-mortem* than after two days of fasting. In addition, Love et al. (1974) determined that the establishment of *rigor mortis* is closely related to the pH, noting that the maximum angle at 24 h *post-mortem* corresponds to minimum pH values in fish. Low pH muscle, due to glycolysis, interrupts ATP production through multiple processes, leading to the establishment of *rigor*. The reason that the nine-day fasted trout present higher angles may be that they also presented a higher pH value at 24 h *post-mortem* than the two-day fasted trout, which tends to decrease the rate of establishment of *rigor* (Love and Haq, 2007).

The pH at 24 h *post-mortem* in trout is between 6.5 (Lefevre et al., 2008) and 7 (Robb et al., 2000), so our data were slightly lower than normal (6.24 to 6.73). The flesh pH decreased more in all trout subjected to 24.3 °C d fasting (two days) compared to those with a fasting of 102 °C d (nine days). This could be attributed to the lower glycogen levels in muscle of fish before slaughter after nine days than after two of fasting, thereby producing less lactic acid (Grigorakis et al., 2003). Thus, the pH at 24 h *post-mortem* was higher in all fish fasted for nine days as compared to two days, which we interpret as lower quality flesh (as compared to flesh where pH decreases normally).

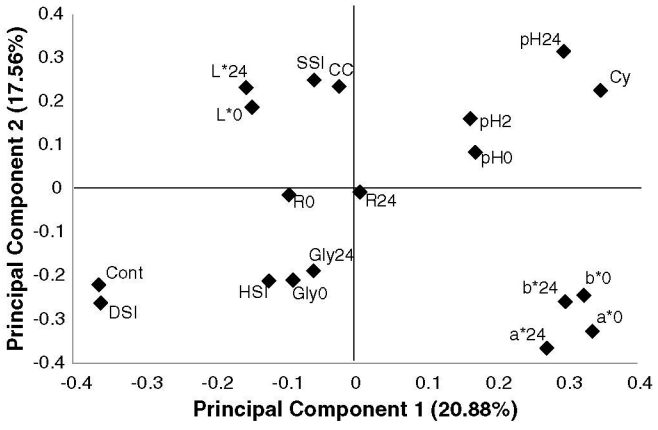


**Fig. 1.** Projection of the body measurements and physicochemical flesh quality data in the plane by the two first principal components. Coefficient of condition (CC); Stomach content (Cont); Digestive somatic index (DSI); Hepato-somatic index (HSI); Spleen somatic index (SSI); Carcass yield (Cy); Muscle glycogen at 0 (Gly0) and 24 h *post-mortem* (Gly24); IMP/ATP ratio at 0 (R0) and 24 h *post-mortem* (R24); L* muscle color value at 0 (L*0) and 24 h *post-mortem* (L*24); a* muscle color value at 0 (a*0) and 24 h *post-mortem* (a*24); b* muscle color value at 0 (b*0) and 24 h *post-mortem* (b*24).

Muscle glycogen decreased after 24 h *post-mortem* in all groups. In 2D trout, muscle glycogen reserves were significantly lower at nine days of fasting than two days, which may be tied with the greatest decrease in stomach content compared to the other treatments. Muscle glycogen only makes a small contribution to the total energy expenditure and is directly related to muscle activity. Some authors suggest that the mobilization of glycogen in the muscle is more related to muscle activity than to fasting itself (Navarro and Gutiérrez, 1995), while others report a reduction in muscle glycogen with only two days of fasting in rainbow trout (Furné et al., 2012) or eight days on brown trout (*Salmo trutta*) (Navarro et al., 1992).

Regarding IMP/ATP, the results suggest that neither intermittent feeding nor fasting had an effect on this indicator of freshness. It should also be pointed out that our mean values (0.460 at 0 h *post-mortem* and 0.564 at 24 h *post-mortem*) were lower than other studies (1.15–1.20 in un-stressed fish, Giuffrida et al., 2007), probably due to the methodology used.

In general, the water holding capacity varied in the same manner as the stomach content, being lowest in 4D trout after nine days (102 °C d), the worst option. Roth et al. (2006) suggested that the physical stress on the muscle fibers or connective tissue proteases releases and accelerates degeneration of the structure in salmon, with the stressed fish having softer flesh. The WHC is also related to muscle pH, so, as the pH is reduced, the net surface charge of muscle protein is reduced, causing it to denature and lose some of its WHC (Toldra, 2003). However, the 4D trout, where the WHC was reduced after nine days of fasting, had a similar pH at nine days of fasting as the other groups, suggesting that other factors in addition to pH could also play a role in the reduction of WHC.

With regard to the color changes on muscle, brightness (L*) was independent of intermittent feeding and days of fasting but dependent of the hours *post-mortem*, showing a small increase after 24 h *post-mortem* resulting in a discoloration of the muscle, as previously seen in salmonids (Robb et al., 2000). Furthermore, the amount of red (a*) and yellow (b*), in addition to decrease with hours *post-mortem*, was

also influenced by the intermittent feeding. Contrary to the lightness, a* and b* had a tendency to decrease their value after 24 h *post-mortem* in all groups and involved a color change towards more green and blue. In our study, increased on L* and decreased on a* and b* may correspond to the normal development of the flesh after slaughter with the consequent decrease in pH, as is described by Warris and Brown (1987) in pig muscle. Similar reductions in the amount of red and yellow had been reported by Álvarez et al. (2008) on other fish (sea bream) and with seven days of storage, so the acidification from anaerobic glycolysis during storage may affect the flesh color. There are studies in fish that determines that the muscle of halibut (*Hippoglossus hippoglossus*) becomes more insoluble with a reduction in pH, thus denaturing (Tomlinson et al., 1965) and resulting in changes in the reflection of light from the surface, hence changing the color perception (Warris, 1996).

### 4.3. Principal component analysis

In general, we observed a strong effect of days of fasting for all variables, which separated fish in two groups (two and nine days of fasting). A negative correlation between carcass yield and stomach content/DSI was also found. Trout subjected to two days of fasting were starved for less time and had higher stomach content and DSI; therefore they finished with a lower carcass yield than trout fasted for nine days. From the production perspective, as our results suggests, a nine-day fasting could be a better choice to maximize carcass yield.

However, nine-day fasted trout had the highest pH at 24 h *post-mortem* and pH was negatively correlated with muscle glycogen, so these fish had lower levels of muscle glycogen before slaughter and produced less lactic acid (Grigorakis et al., 2003), resulting in a higher pH at 24 h *post-mortem* than in trout subjected to only two days of fasting. Because of this, flesh quality of nine-day fasted trout was worse than the other groups.

We also observed a negative correlation between lightness (L*) and the amount of red (a*) and yellow (b*), so those trout with a redder and yellower flesh had a darker color.

## 5. Conclusions

Intermittent feeding before pre-slaughter fasting has effects on slaughter weight, stomach content and muscle glycogen, all of which suggest that trout fed once every two days may adapt better to a two-day or nine-day fast. However, a two-day fast (24.3 °C d), seems better than a nine-day fast (102 °C d) in terms of flesh quality since the latter have a higher final pH.

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