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Reducing the effect of pre-slaughter fasting on the stress response of rainbow trout (Oncorhynchus mykiss)

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Abstract

*Fasting is commonly used in aquaculture to empty the gut before slaughter, but little is known about how feeding frequency before fasting affects the stress response of trout. To find out more, 240 rainbow trout (*Oncorhynchus mykiss*) were separated into three groups with different feeding schedules during the final month of fattening, from 26 September to 28 October 2013 (daily, every two days or every four days) and two durations of pre-slaughter fasting (two days of fasting; 24.3 degree days, to nine days of fasting; 102 degree days). After slaughter, a number of stress-related parameters were measured, such as liver glycogen, skin/gill colour and haematological parameters (cortisol, glucose, lactate, triglycerides, lactate dehydrogenase and creatine phosphokinase). Trout given food every two days on the farm had lower levels of cortisol and higher levels of triglycerides and liver glycogen than the other treatments after two days of fasting; indicating that habituating trout to feed once every two days in the final month of fattening lowered their stress response to two days of fasting before slaughter.*

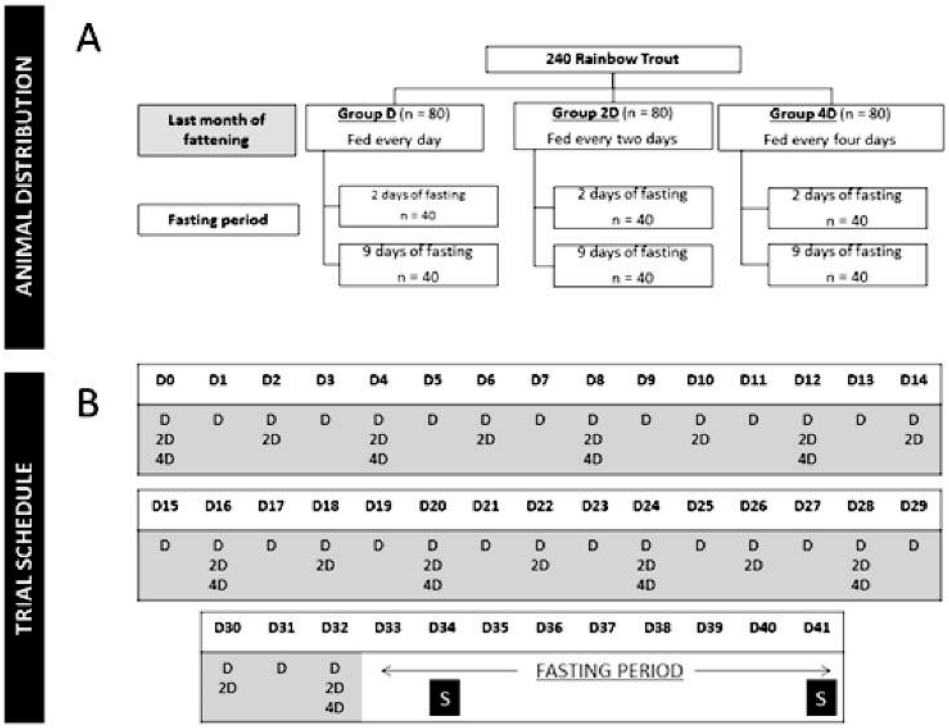
Keywords: *animal welfare, cortisol, fasting, glycogen, rainbow trout, stress response*

Introduction

In aquaculture, the fasting of fish prior to slaughter is commonly practiced to evacuate the gut and reduce oxygen demand and waste production (Lines & Spence 2012). In recent years concerns regarding the maximum permissible duration of the fasting have been raised for different continental (Barcellos *et al* 2010) and marine fish (Morkore *et al* 2008). In trout, the Farm Animal Welfare Council recommends a 48-h limit on fasting (FAWC 1996), arguing that the welfare of farmed fish that have been fed regularly will be affected negatively by a sudden cessation of feeding. However, it is also important to consider the effect of feeding frequency before fasting. Rainbow trout (*Oncorhynchus mykiss*) in the wild, as most carnivorous fish, live a life of feast and famine, where prey is distributed heterogeneously in space and time (Armstrong & Schindler 2011). Fish can store energy when abundant and mobilise it during fasting, although both processes have limitations dependent upon species, life stage, environmental conditions and habituation.

Many recent publications have considered the effect of feeding frequency on fish growth and welfare (eg Cañon Jones *et al* 2012) but fewer have considered the effect of intermittent access to feed and its effect on the reaction to short-term pre-slaughter fasting. In the wild, most carnivorous fish have one meal every two days (Armstrong & Schindler 2011), suggesting that trout could adapt to a skip a day system, commonly used in poultry (Oyedeji & Atteh 2005) and tested on other fish, such as nile tilapia (*Oreochromis niloticus*) (Villarroel *et al* 2011). Although fish may present motivational mechanisms for feeding when nutritional reserves are low (eg Metcalfe & Thorpe 1992), farmed rainbow trout can fast for weeks (Ashley 2007) with no apparent negative effect on stress physiology or behaviour (Pottinger *et al* 2003; Jentoft *et al* 2005). More recently, a number of authors have analysed the effect of short-term fasting (up to three days) on plasma stress indicators in rainbow trout (Hoseini *et al* 2013), including the effect of water temperature (López-Luna *et al* 2013) and its effect on flesh quality (López-Luna *et al* 2014) but few studies have considered the effect of different feeding schedules in the final month of fattening on pre-slaughter fasting stress or compared extremes of fasting in terms of degree days (for example 20ºC days vs values over 65ºC days).

Figure 1



Experimental design. A) Experimental groups and number of fish in each one. Trout fed during the last month of fattening every day (group D), every two days (group 2D) or every four days (group 4D) and different fasting period, with a total of six different groups. B) Trial sequence indicating when each group was fed and the days of sampling (S) at two or nine days of fasting.

Recently, we published results regarding the effect of feeding frequency in the final month of fattening and pre-slaughter fasting on the flesh quality of rainbow trout (Bermejo-Poza *et al* 2015). Here, we describe results from the same trial carried out under commercial conditions but concerning different stress response indicators, such as plasma levels of cortisol, glucose, lactate, triglycerides, lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) as well as liver glycogen, skin/gill colour.

Materials and methods

Fish and experimental design

The experimental design has been described previously in Bermejo-Poza *et al* (2015). Briefly, the trial was carried out at a fish farm in the province of Guadalajara, Spain, on the banks of the Tajuña River (40° 49' 4.605", –2° 44' 49.2072"). Six groups of 40 rainbow trout were placed in cages (1.5 × 0.8 × 0.35 m; length × width × height) in six parallel raceways (30 × 6 m; length × width) receiving a constant flow of river water (approximately 0.06 m s^{-3}), for a total of 240 fish. The headwater for each raceway came directly from the river (had not passed through other tanks of fish) and was not recirculated (one pass). Their initial mean (± SD) weight was 324 (± 41) g.

Each trout was individually identified using 2 × 12-mm Pit-Tags (Pit Tag i-Tag 162, Sweden) injected under the dorsal fin, a month prior to the experiment. During the entire experiment animals were subjected to natural photoperiod and fed a commercial feed during the final month of fattening, from 26 September (Day 0) to 28 October 2013 (Day 32), with the following composition: 41% crude protein, 21.5% carbohydrate, 24% crude fat, 6.5% ash and 2.5% crude fibre. To calculate degree-days, water temperature was recorded once every 5 min during the entire trial using underwater temperature sensors (Hobo®-U11, Bourne, MA, USA).

Two cages of trout were fed every day (D), two once every two days (2D; one day of fasting or skip-a-day system) and two more once every four days (4D; three days of fasting), with a total of six cages for the experiment. All treatments were given the same amount of food in total but D trout received 1.2% of their bodyweight in feed every day, 2D trout were fed 2.4% of their bodyweight once every two days and 4D trout were fed 4.8% of their bodyweight once every four days. Since the amount of food provided per day in this group was very high, they were fed to satiation and fed again to satiation 1 h later. All fish were fed in this manner for a month, then before slaughter three cages (one cage per treatment; n = 40 per treatment) was fasted two days pre-slaughter and the other three were fasted nine days pre-slaughter (one cage per treatment; n = 40 per treatment), corresponding to 24.3°C days (Day 34) and 102°C days (Day 41), respectively, based on the temperature data. This way, all trout started their fasting period the day after they received food for the last time (Figure 1).

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Table 1 Mean (± SEM) haematological parameters of rainbow trout (*Oncorhynchus mykiss*) on different feeding system (FS) and days of fasting (DF).

Haematological parameters	Daily (D)		Skip a day (2D)		Every four days (4D)		Significance (*P*)		
	2	9	2	9	2	9	FS	DF	FS × DF
n	37	31	30	40	39	33			
Cortisol (ng ml^{-1})	21.4 (± 1.7)^{ab}	20.9 (± 1.6)^{ab}	15.0 (± 2.0)^{b}	25.8 (± 1.4)^{a}	21.8 (± 1.3)^{ab}	21.6 (± 1.7)^{ab}	ns	< 0.05	< 0.01
Glucose mg dl^{-1})	131 (± 6)^{ab}	79 (± 5)^{d}	127 (± 7)^{ab}	112 (± 7)^{bc}	142 (± 5)^{a}	88 (± 6)^{cd}	ns	< 0.001	< 0.01
Lactate (mmol l^{-1})	20.5 (± 1.5)^{bc}	14.2 (± 1.3)^{c}	26.9 (± 3.9)^{ab}	36.6 (± 2.9)^{a}	28 (± 2.3)^{ab}	29.1 (± 2.7)^{ab}	< 0.001	ns	< 0.01
Triglycerides (mg dl^{-1})	360 (± 15)^{c}	436 (± 21)^{bc}	871 (± 83)^{a}	354 (± 16)^{c}	522 (± 24)^{b}	293 (± 12)^{c}	< 0.001	< 0.001	< 0.001
LDH (U l^{-1})	571 (± 26)^{b}	551 (± 29)^{b}	587 (± 47)^{b}	567 (± 31)^{b}	878 (± 63)^{a}	430 (± 28)^{b}	ns	< 0.001	< 0.001
CPK (U l^{-1})	541 (± 68)	414 (± 69)	576 (± 85)	443 (± 96)	340 (± 55)	419 (± 64)	ns	ns	ns

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

Slaughtering and analysis

Prior to slaughter, the water level of the raceway was slowly lowered to half the cage height (which took 10 min). The trout were captured with dip nets and killed immediately (< 15 s) using the *ikijime* method, piercing the brain of the fish with a sharp metal tip, as it is suggested to cause the least amount of suffering as well as the smallest possible changes in flesh quality (Malcolmsen *et al* 1995).

Immediately following slaughter, each fish was weighed and blood samples taken from the caudal vein which were divided into two Eppendorf tubes, one with sodium fluoride (NaF) for the determination of glucose and lactate and another with EDTA as an anticoagulant for cortisol, LDH, CPK and triglycerides. Both tubes were centrifuged at 6,000g for 10 min to remove the plasma, and stored immediately at 4°C until analysis.

Determination of concentration of liver glycogen was based on the technique described by Dreiling *et al* (1987), using samples of liver of 0.3 g. The liver was weighed previously in all fish. Finally, colour measurements were taken for skin and gill using a Minolta Spectrophotometer CM-2500c (Minolta, Osaka, Japan). The CIE 1976 L*a*b* system recommended by the International Commission Illumination (CIE 1978) was chosen as the colour scale. Three measurements were taken on the right side of the fish for both skin and gill.

Statistical analysis

The parameters were analysed using the SAS software version 9.0 (Statistical Analysis System Institute Inc, Cary, NC, USA). A Bonferroni test was used to compare means. We used the GLM procedure of SAS with the feeding system (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 the liver glycogen and slaughter weight, liver weight and initial weight were introduced into the model as covariates.

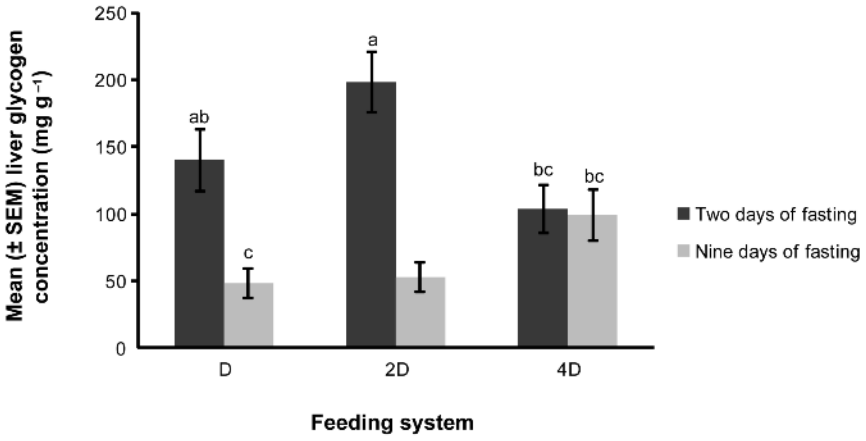
Results

Overall, the haematological parameters and glycogen levels, for the 2D trout were indicative of a lower stress response. As far as production data, slaughter weight was lower after nine days of fasting (102ºC) in 2D (365 [± 5.49] g) and 4D trout (330 [± 6.03] g), while D trout did not vary significantly between two (353 [± 5.91] g) and nine days of fasting (352 [± 6.91] g). The 2D trout also had the highest slaughter weight (381 [± 5.73] g).

Haematological parameters

There was a significant interaction between feeding system and days of fasting for plasma cortisol levels. In feeding systems D and 4D, cortisol levels were similar at two and nine days (24.3 vs 102°C days). On the other hand, 2D trout had lower levels of cortisol at two days of fasting than after nine. Glucose plasma levels also showed a significant interaction between feeding system and days of fasting. In this case, the plasma glucose concentrations in D and 4D trout were lower after nine than after two days of fasting. For lactate, a significant interaction was found between the two factors studied. There were no significant differences in lactate concentrations after two days of fasting between feeding systems. But, after nine days of fasting, the D trout had the lowest values of lactate. Regarding triglycerides, there was a significant interaction between the two fixed factors (FS and DF); after two days of fasting 2D trout showed the highest values, and 4D trout were, in turn, higher than D trout values. These values decreased after nine days of fasting in 2D and 4D trout but not in D trout. There was a significant interaction between feeding system and days of fasting for LDH, where values were similar among groups, except for highest value for 4D trout fasted two days. The CPK values were not significantly different among feeding system or days of fasting (Table 1).

Figure 2



Mean (± SEM) liver glycogen of rainbow trout (*Oncorhynchus mykiss*) on different feeding system (FS) and days of fasting (DF). Different superscripts indicate significant differences among groups (feeding system × days of fasting; $P < 0.05$).

Table 2 Mean (± SEM) skin and gill colour of rainbow trout (*Oncorhynchus mykiss*) on different feeding system (FS) and days of fasting (DF).

Colour	Daily (D)		Skip a day (2D)		Every four days (4D)		Significance (*P*)		
	2	9	2	9	2	9	FS	DF	FS × DF
n	19	16	16	20	20	17			
Skin colour									
L*	30.53 (± 1.56)^{ab}	29.12 (± 1.64)^{abc}	31.22 (± 1.62)^{ab}	23.92 (± 0.75)^{c}	35.17 (± 1.95)^{a}	25.00 (± 0.84)^{bc}	ns	< 0.001	< 0.01
a*	1.51 (± 0.27)^{b}	1.78 (± 0.24)^{ab}	1.56 (± 0.18)^{ab}	0.86 (± 0.12)^{b}	2.94 (± 0.59)^{a}	1.19 (± 0.15)^{b}	< 0.05	< 0.01	< 0.01
b*	6.76 (± 0.59)^{ab}	8.23 (± 0.60)^{a}	7.40 (± 0.71)^{a}	4.88 (± 0.49)^{b}	6.95 (± 0.38)^{ab}	5.84 (± 0.48)^{ab}	< 0.05	ns	< 0.01
Gill colour									
L*	36.76 (± 1.24)^{ab}	34.08 (± 0.83)^{b}	37.77 (± 1.61)^{ab}	34.54 (± 0.72)^{b}	41.15 (± 2.37)^{a}	36.76 (± 0.64)^{ab}	< 0.05	< 0.01	ns
a*	16.26 (± 0.91)^{a}	16.45 (± 0.71)^{a}	14.19 (± 0.94)^{ab}	17.29 (± 0.49)^{a}	11.39 (± 0.96)^{b}	16.66 (± 0.51)^{a}	< 0.01	< 0.001	< 0.01
b*	9.38 (± 0.53)^{ab}	8.80 (± 0.31)^{ab}	8.57 (± 0.47)^{ab}	9.62 (± 0.40)^{a}	7.84 (± 0.47)^{b}	9.91 (± 0.37)^{a}	ns	< 0.05	< 0.01

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

Liver glycogen

Regarding liver glycogen, there was a significant interaction between feeding system and days of fasting. While in 4D trout, liver glycogen remained similar after nine days of fasting, in D and 2D trout there was a significant decrease between two and nine days of fasting. The 2D trout subjected to two days of fasting presented the highest value of liver glycogen (Figure 2).

Skin and gill colour

There was a significant interaction between feeding system and days of fasting for skin L*, a* and b*. The L* was lower in 2D and 4D trout subjected to nine days of fasting than after two days of fasting. Skin a* was similar in D and 2D trout, but in 4D trout was significantly lower at nine days of fasting than after two. Skin b* presented comparable values at both fasting times in D and 4D trout. Regarding gill colour, L* was significantly affected by feeding system and days of fasting, with a slight decrease in all feeding systems after nine days of fasting. Gill a* showed a significant interaction between the two fixed effects (FS and DF). 4D trout after two days of fasting showed lower values than D trout (two and nine days of fasting) and 2D and 4D trout after nine days of fasting. Finally, gill b* also presented a significant interaction between feeding system and days of fasting. 4D trout showed lower values after two days of fastingthan 2D and D trout after nine (Table 2).

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Discussion

Haematological parameters

Plasma cortisol levels were similar after two and nine days of fasting in D and 4D trout. However, in the 2D system, cortisol concentrations were lower after two days than after nine days of fasting. In the literature, there is no clear consensus about the effect of fasting on plasma cortisol concentrations in rainbow trout. Overall, cortisol levels in our study were slightly higher than resting values reported by others (5–20 ng ml^{-1}; Barton & Iwama 1991; Pottinger *et al* 2003). Some studies report an increase in cortisol with fasting (Sumpter *et al* 1991; Varnavsky *et al* 1995; Blom *et al* 2000; Peterson & Small 2004), others find no effect (Reddy *et al* 1995) or even decreased levels with fasting (Farbridge & Leatherland 1992; Small 2005). Our study underlines the importance of considering previous feeding frequencies on the response of fish to fasting, since the 2D trout had lower cortisol levels after two days of fasting than after nine while D and 4D fish showed no changes across two or nine days of fasting. The lack of change in the 4D group could be a result of the prior feeding regime introducing a chronic stress, that has been reported to diminish the cortisol response to an acute stressor (Ings *et al* 2011; Wunderink *et al* 2011), so that the cortisol levels could not increase from day two to day nine of fasting. A similar pattern was found in the D trout, where cortisol levels remained similar at two and nine days of fasting. Here, the reason may be due to the high stress of going from plenty of access to food every day to zero food which could increase social stress and the stress response (Jeffrey *et al* 2014). The lower plasma cortisol levels in 2D trout after two days of fasting suggest a lower stress response, possibly since trout were already habituated to this schedule.

Glucose levels can reach 150 mg 100 ml^{-1} in rainbow trout (Pottinger & Carrick 1999), with basal levels of 70–90 mg 100 ml^{-1} (Jentoft *et al* 2005). Pottinger *et al* (2003) found that it decreased after three days of fasting and it has been demonstrated that five days was sufficient to induce hypoglycaemia (Furné *et al* 2012). As expected, plasma glucose levels decreased after nine days of fasting compared to two days in D and 4D trout. In contrast, glucose concentrations were similar in 2D trout at two and nine days of fasting. This lack of change between glucose levels of 2D trout at nine days of fasting compared to two days could be associated with the elevated concentrations of cortisol observed. In fact, high cortisol concentrations might warrant normoglycaemia through activation of gluconeogenesis (Polakof *et al* 2006). Nevertheless, glucose levels were relatively high after two days of fasting in all treatments, perhaps due to a significant decrease in plasma glucose levels and activation of gluconeogenesis mediated by cortisol (Hoseini *et al* 2013).

Plasma lactate levels were not greatly affected by days of fasting but more by feeding system. Lactate levels should decrease in fasted fish (Blasco *et al* 1992) to enhance the formation of glucose in the liver (Liew *et al* 2012). In the case of rainbow trout, basal levels are around 0.5 mmol l^{-1} and values above 1.3 mmol l^{-1} have been suggested to indicate stress (Ings *et al* 2011). The high levels obtained during our test (14–37 mmol l^{-1}) may be due to other factors besides fasting that were not measured, but probably not a result of the capture since lactate takes 2–4 h to increase in plasma after an episode of acute stress (Olsen *et al* 2005). Other pre-harvest stimuli, such as adjustments of social hierarchies within the groups, may have increased anaerobic muscular activity as well as lactate levels.

Triglycerides should decrease with increased fasting since the fish will mobilise reserves to cope with fasting but those reserves will decrease with time (Costas *et al* 2011; Takahashi *et al* 2011). In our study, triglyceride levels decreased with fasting in all fish except D trout where fish had food every day. Our results suggest that feeding system can affect triglyceride levels in trout after two days of fasting, since D fish had much lower levels in blood by day two of fasting than 2D and 4D trout, indicating that their stores were more depleted, possibly since they did not need to maintain fat reserves when being fed every day. This suggests that the feeding frequency can regulate the storage of triglycerides in trout. The reason that the 4D trout had lower fat reserves compared to 2D trout may be that they used them up during the longer fasting.

The changes in LDH and CPK were similar, with D and 2D trout showing a slight decrease in enzyme activity between two and nine days of fasting. The 4D trout had a higher LDH activity after two days of fasting than after nine days, indicating high muscle activity. High LDH levels are more related to muscle injury, triggered by different types of stress, such as transport (Dobšíková *et al* 2006) or fasting (Vijayan *et al* 2006). Thus, the muscle activity of D and 2D trout was lower, probably reflecting a lower stress response than 4D trout.

Liver glycogen

Fasting mobilises liver nutrients for maintenance and this organ plays an important role as a source of metabolites, especially during the early phases of the fast (Davis & Gaylord 2011). The time taken to reduce these levels with fasting is variable, but can be mobilised within 5–20 days after fasting starts (Barcellos *et al* 2010). Furné *et al* (2012) found that in rainbow trout liver nutrients can be mobilised after five days of fasting. In our study, D and 2D trout had lower concentrations of glycogen in the liver after nine than after two days of fasting. But 4D trout had lower levels after two days of fasting than 2D and 4D trout, and did not mobilise them after nine days of fasting. This could indicate that due to the feeding system previous to the fasting, 4D trout were more used to deprivation of food and mobilised less liver glycogen or that initial liver glycogen levels were lower.

Skin and gill colour

2D and 4D trout fasted for nine days had a darker skin colour (lower L*) than after two days of fasting, which could be related a higher stress level (increased cortisol) due to fasting itself, which in our study occurs in 2D trout. However, in trout fed daily, D trout, there was no significant variation in skin colour due to days of fasting. Höglund *et al* (2000)

showed that social stress produced a darkening of the skin in Arctic char (*Salvelinus alpinus*) because of the dispersing effect of ACTH on chromatophores (Fujii & Oshima 1986) and a similar process has been reported in Atlantic salmon (*Salmo salar*) (O'Connor *et al* 1999) and several other species of salmonid fish like rainbow trout (Abbott *et al* 1985). After two days of fasting there were no differences among feeding systems. On the contrary, after nine days of fasting, b* was lower for 2D trout and a* was lower for 4D trout than after two days, suggesting that feeding system can affect skin colour after nine days fasting, and could lead to a darker skin among other variations in colour.

The colour of the gill is an indicator of health and an important parameter for consumers in evaluating the freshness of fish (Poli *et al* 2005; Álvarez *et al* 2008; Green 2011) and it has been studied mainly related to the storage time (Dowlati *et al* 2013) but there is little information on the effect of fasting on gill colour. An indicator of a fresh fish is a bright red gill colouration (Green 2011). As in the case of skin, trout fasted for nine days showed darker (lower L*) gill colour than after two days. Feeding system and fast duration can affect the colour of the gill since 4D trout had redder and yellowish gills after nine than after two days of fasting. Yellow pigments are formed as result of reaction between protein and oxidised lipid, indicating the quality deterioration of fish (Dowlati *et al* 2013). Again, D trout showed no significant differences in gill colour between durations of fast. This suggests that the 4D feeding system produced a higher stress response in fish and affected the colour of the gill and led to an appearance of less-fresh trout.

Our results indicate that adjusting the feeding regime during the last month of fattening in rainbow trout could help to reduce the stress response during pre-slaughter fasting. However, for most of the data analyses discussed, and as in previous studies (López-Luna *et al* 2013, 2014; Bermejo-Poza *et al* 2015), we used each fish as the experimental unit, and not each cage. This may have implications for the reproducibility of our results, but using many more cages under commercial conditions was not feasible and probably would have created 'unnatural' densities and conditions (for a commercial farm or normal aquaculture setting) with too few fish in a given volume.

Animal welfare implications

Pre-slaughter fasting is a common practice in fish farming and causes a stress response that can be to the detriment of their welfare. The results of this study have two main practical implications for how we consider rainbow trout welfare prior to slaughter. On the one hand, we have shown that different management techniques can be used in the last weeks prior to slaughter to habituate fish to different fasting durations, which reduces their stress response before slaughter. Specifically, trout can get used to fasting for periods of two days in the last month of fattening, which reduces their stress response when fasted for two days before slaughter. On the other hand, we underline that the stress response during fasting is temperature-dependent and that fasting for over 100ºC days appears to have negative effects on trout stress.

Conclusion

Regarding welfare and fasting, trout fasted for nine days had a higher stress response than trout fasted for only two days. Regarding welfare and feeding schedules, the trout fed once every two days had a lower stress response when subjected to two days of fasting prior to slaughter, as reflected by lower cortisol levels and a higher slaughter weight. Feeding trout once every four days has a negative effect on skin and gill colour that could be associated with a higher stress response. Although our results are contingent on our experimental conditions, they suggest that it is possible to reduce the effect of pre-slaughter fasting on the stress response of rainbow trout using different feeding schedules before slaughter. Such information could be used to improve handling, but overall it opens up a new field of study where more research is needed.

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