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Effects of dietary barley on rainbow trout exposed to an acute stress challenge

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ABSTRACT

The present study evaluates the effect of dietary barley, based on its potential stress-relieving properties, on rainbow trout under acute stress challenge (hypoxia and crowding) and their recovery. Diets were formulated containing increasing barley concentrations (0, 4, 8, 16, 32%). Cortisol on plasma and fin, glucose and lactate plasma levels and malondialdehyde (MDA) in muscle were determined under normoxia before the stress test (basal levels), 30 minutes after the acute stress challenge and also during normoxia recovery (6 and 12 hours after the stress). Results showed that at basal levels the inclusion of barley had no influence on cortisol, glucose nor on lactate values. After 30 minutes from the stress challenge, there was a significant increase in cortisol, glucose and lactate concentration in fish of all groups. Plasma cortisol showed the lowest levels in fish fed with diets at a medium (8%) of barley concentration and returned to basal levels 6 hours after the stress stimulus with no differences between diets. Glucose values showed a less clear tendency 30 minutes after the stress challenge with lower levels in the control group, fish fed with 8% and 32% of barley in the diets and returned to basal levels in almost all the groups only 12 hours after the stress challenge. Lactate showed the same trend as with glucose after the stress challenge but it returned to basal levels in 6 hours. Interestingly, there was a significant decrease of lipid oxidation (MDA) in muscle soon after the stress test of fish fed with the highest barley levels. The present results suggest a potential positive effect of dietary barley on trout stress response.

Keywords*: Barley, β-glucans, rainbow trout diets, stress challenge*

1. INTRODUCTION

Fish welfare has become an important, current feature of the aquaculture industry

(Conte 2004, Bertotto *et al*. 2010, Oliva-Teles 2012, Naderi *et al*. 2017). Under intensive aquaculture conditions, fish are subjected to many factors such as stocking densities, handling, weighing, feeding and water quality among others (Conte 2004, Bertotto *et al*. 2010, 2011, Oliva-Teles 2012), which can induce a stress state and negatively affect fish welfare. These factors can also affect the performance and productive parameters, having an important economic impact (Oliva-Teles 2012). A lack of oxygen can regularly occur on fish in their natural environment (Omlin & Weber 2010, Poulsen *et al*. 2011, Pascoli *et al*. 2012, Gesto *et al*. 2015), however, in this situation fish are able to escape from it (Vianen *et al*. 2001), while in aquaculture closed systems fish are forced to put up with it (Poulsen *et al*. 2011). A correct aquaculture management tries to provide the right dissolved oxygen concentration so as to guarantee fish welfare and production efficiency although it is not possible to have a complete control of it. This leads to an occasional oscillation and depletion of oxygen up to values close to hypoxia (Pérez-Jiménez *et al*. 2012) and causes stress. Under hypoxia, fish exhibit behavioural, anatomical and physiological stress responses. Although those responses are protective responses, stress intensity and exposure over time can determine, in the medium and long term, immunosuppression producing a higher susceptibility to diseases and growth depletion (Ming *et al*. 2012). Antibiotics and hormones have been used to control fish diseases, however, these do not contribute to the sustainability of the aquaculture. For this reason, during the last few years, alternative dietary ingredients have gained more attention than conventional methods to mitigate stress response. As far as we are aware, few studies have explored the effects of diet on acute hypoxia. Chagas and Val (2006) described the effect of hypoxia stress on the Amazon fish tambaqui (*Colossoma macropomum*) when they were fed with diets containing increasing L-ascorbic acid concentrations during 10 weeks; McKenzie *et al*.

(2008) reported an increase of tolerance under hypoxia in sole larvae and juveniles fed with diets enriched with essential fatty acids; Pérez-Jiménez *et al*. (2011) also found that sea bream fed with diets with white tea showed similar behaviour as fish fed with control diets after an hypoxia challenge.

The use of β-glucans has been widely studied in aquaculture (Jeney *et al*. 1997, Meena *et al*. 2013, Al-Faragi 2014, Pinedo-Gil *et al*. 2017). β-glucan is one of the most important immunostimulant (Meena *et al*. 2013) and has been widely studied because it plays an important role in immune system (Zeng *et al*. 2016, Miest *et al*. 2016) protecting fish against stress factors (Dawood *et al*. 2015, Zeng *et al*. 2016). Barley is rich in β-glucans and, for this reason, it has been widely used for livestock feeding, but on the other hand it is scarcely used in aquaculture. In this sense, the incorporation in aquafeed of ingredients with specific components that can prepare the animal to overcome eventual adverse situations, such as oxygen depletion, is a challenge for the aquaculture research (Pérez-Jiménez *et al*. 2011).

To study the stress response in fish, different parameters can be used (Bertotto *et al*. 2010, 2011). Plasma cortisol is one of the most commonly used indicators of stress in fish (Barton and Iwama 1991, Weendelar-Bonga 1997, Bertotto *et al*. 2010) but this parameter is not always an ideal marker of stress due to its rapid increase after the exposure to stressors and variability of these values (Bertotto *et al*. 2010, Gesto *et al*. 2015). For this reason, the study of cortisol levels in alternative matrices such as in fin and in muscle can provide a more real and stable measurement of the cortisol level. Along with the increase in plasma cortisol, stress also causes an increase in plasma glucose and lactate levels due to glycogenolysis and gluconeogenesis mediated by the stress hormones (Pankhurst 2011).

At a cellular level, the production of reactive oxygen species (ROS) due to a stressful factor is normal (Pascoli *et al*. 2011, Rahal *et al*. 2014). These ROS can determine oxidative damage to proteins, lipids and nucleic acids (Pascoli *et al.* 2011, Lushchak 2011). During oxidative stress conditions, ROS increase to levels that cells cannot remove causing lipid peroxidation, protein carbonyl formation and cell death. Lipid peroxides are unstable indicators of oxidative stress (Aldini *et al*. 2007, Pascoli *et al*. 2011, Lushchak 2011). Other byproducts of lipid oxidation such as malondialdehyde (MDA) have also been shown to be produced by oxidative stress, that presents a good correlation with stress (Aldini *et al*. 2007, Pascoli *et al*. 2011, Lushchak 2011).

The present study was conducted to evaluate, based on the antioxidant properties of barley, the antioxidant ability as well as the potential stress-relieving properties of dietary administration of barley in rainbow trout (*Oncorhynchus mykiss*).

2. MATERIAL AND METHODS

2.1.Diets

Five isoproteic (40% crude protein) and isolipidic diets (18% crude lipid) were formulated using barley as experimental ingredient (0B: 0% barley; 4B: 4% barley; 8B: 8% barley; 16B: 16% barley; 32B: 32% barley). The composition and proximate analysis of barley diets are described in Pinedo-Gil *et al*. 2017.

The control diet was prepared with the same ingredients as experimental diets but without barley on the formulation. A semi-industrial twin-screw extruder was used (CLEXTRAL BC-45. St. Etienne, France). Raw material was processed at a speed of 100 rpm, at 110 ºC and a pressure of 40-50 atm. The experimental diets were assayed in duplicate. The fish were fed by hand twice a day (8:00 and 15:00), 6 days per week to

apparent satiation level during the whole experimental period. The pellets were distributed slowly to allow all fish to eat.

2.2. Production system

Rainbow trout (*Oncorhynchus mykiss*) were obtained from a local fish farm (Cien Fuentes fishfarm, 19420 Cifuentes, Guadalajara, Spain) and transported alive to the Research Aquaculture Centre of the Agro-Technological Institute of Castilla y León, Segovia, Spain. A total of 400 rainbow trout were used. Fish were randomly allocated in 10 cylindrical fiberglass tanks (500 L) and 40 fish (initial stocking density 19.9 ± 0.1 Kg m^3, with an initial average weight of 247.91 ± 37.92 g) were randomly allocated in each tank (2 tanks for experimental condition). Prior to the feeding trial, all fish were acclimated to the indoor rearing conditions for 1 week and were fed once a day (8:00) to apparent satiation using exclusively the control diet. The study lasted 45 days.

The trial was conducted in a freshwater recirculation system (RAS). During the experimental period, water temperature was maintained at 15.06 ± 0.30 ºC (mean ± SD). The level of dissolved oxygen was 6.20 ± 0.61 mg L^{-1} (61% saturation). All tanks were equipped with aeration and an oxygen probe. Water pH was 7.96 ± 0.22 and ammonia and nitrites concentration in water were 0.93 ± 0.46 and 0.97 ± 0.74 mg L^{-1} respectively. Water flow was 10.35 ± 0.80 L h^{-1}. The photoperiod consisted of 12 hours light and 12 hours dark intervals. All tanks had identical light conditions.

2.3.Stress challenge: acute stress (hypoxia and crowding)

At the end of the experimental period (after 45 days of feeding), fish were exposed to a controlled stress test by decreasing oxygen concentration from 6.20 to 4 mg L^{-1} (acute stress, hypoxia). The concentration of oxygen was decreased by lowering the water in

the tanks to a volume of 50 L and removing the aeration (crowding and hypoxia). Once that water dissolved oxygen reached 4 mg L^{-1} (oxygen-saturated value of 39.7%) (approximately 15-20 minutes), fish were kept in these conditions for 10 minutes, and after this time, tanks were refilled with water and aerated. During the acute stress challenge (hypoxia and crowding), oxygen decreased to levels lower than 2 mg L^{-1} (oxygen-saturated values below 19.8%). Before applying the stress all fish were starved for 2 days.

2.3.1. Sampling

Samples were taken before the stress (basal levels), 30 minutes, 6 hours and 12 hours after the stress. For each sampling time, 6 fish per tank (n=12) were rapidly sacrificed with an overdose of anaesthetic (300 mg L^{-1} MS222, 100% w/w; PHARMAQ®). Fish were bled from the caudal vein using 1 mL syringes (BD Plastipak) and blood was transferred to lithium heparin tubes. Soon after collection, blood was centrifuged at 1200 xg for 10 min at 4 ºC and plasma was maintained at -80 ºC until its analysis. Small portions of muscle (about 1x1x1 cm from the caudal peduncle and without skin) and caudal fin (about 1x1 cm from the upper lobe) were collected and stored at -80 ºC until analysis.

2.3.2. Cortisol analysis

Cortisol was determined in plasma and caudal fin by a specific radioimmunoassay (RIA) as described Bertotto *et al*. (2010) after the extraction in diethyl ether. The sensitivity of the assay was 3.125 pg $well^{-1}$ and was defined as the dose of hormone at 90% binding (*B/B0*).

2.3.3. Glucose and lactate

Glucose and lactate concentrations were determined in plasma by enzymatic colorimetric assays, in particular by GOD-POD (SPINREACT® Ref. 1001191) and LOD-POD (SPINREACT® Ref. 1001330) methods respectively (Kaplan and Pesce 1984). Briefly, aliquots (5 µL) from plasma samples were mixed with 500 µL of reactive and they were incubated for 10 min for glucose determination and 5 min for lactate determination at 37 ºC in dark. The absorbance was determined at 490 nm in a 96-well microplate reader (Bibby Scientific Limited, Jenway 7315, UK). Values were expressed as mg dL^{-1}.

2.3.4. MDA

The amount of lipid oxidation was determined in muscle. 100 mg of tissue was mixed with Tris HCL 0.125 M pH 6.9, centrifuged at 13000 xg at 4 ºC for 15 min. Supernatant was used for the assays.

Lipid oxidation was determined by measuring thiobarbituric acid-reactive substances (TBARS) according to Yoshida *et al*. (2005) as detailed in Pascoli *et al*. (2011). Tetramethoxypropane was used as standard to estimate TBARS formation.

Total proteins in muscle were determined using by the Pierce BCA Protein Assay Kit (bicinchoninic acid assay; Thermo Fisher Scientific) following manufacturer`s instructions. Results were expressed as µg mL^{-1}.

MDA values were expressed as nM of malondialdehyde (MDA) per mg of protein.

2.4.Statistical analyses

Data were analysed by ANOVA using the PROC MIXED (SAS, 2013) with dietary treatment and time after stress challenge as factors of variability and the tanks were

considered as a random effect. The probability of the linear, cubic and quadratic components of variance was calculated by contrast statement to test differences among dietary treatments and sampling time after stress. Differences among means with $p<0.05$ were accepted as representing statistically significant differences.

2.5.Ethical statement

The present study complied with European Union Council Directive 2010/63/UE, and is in accordance with Spanish national legislation (Spanish Royal Decree 53/2013) protecting animals used in experimentation and for other scientific purposes. Moreover the experimental plan has been approved by Animal Ethics Committee of Agro-Technological Institute of Castilla y León (Spain).

Fish in the tanks were checked on a daily basis. At the end of the trials, fish were weighed individually and their health status was assessed by observation, after sedation with MS222 dissolved in water (MS222®; 200 mg L^{-1}). Animals were lastly euthanized by an overdose of MS222 (300 mg L^{-1}) and then dissected.

3. Results

3.1. Cortisol

Basal plasma levels ranged from 2.5 to 5 ng mL^{-1} and these values did not change with the diet ($P>0.05$). Thirty minutes after the stress test a significant increase ($P<0.05$) of plasma cortisol was observed with average values 30 times higher than basal values. The diet showed a significant effect ($P<0.05$), the values reached by fish fed with 8B were significantly lower than the control ones. It was observed that cortisol values in plasma decreased rapidly recovering basal levels after 6 hours and remained the same after 12 hours except for fish fed with 32B diet (Figure 1A).

Results of cortisol in the caudal fin are shown in Figure 1B. Basal values did not show significant differences between diets ($P>0.05$) and significantly increased ($P<0.05$) after the stress test reaching values 6 times higher than basal values. Fish groups fed with 4B, 8B and 32B diets recovered basal values after 6 hours, meanwhile fish fed with control diets and 16B did not recover these values even after 12 hours. Twelve hours after the stress the inclusion of barley had a significant effect on fin cortisol values. Fish fed with 16B diet showed the highest value, which was significantly higher than the fish fed with the control, 4B diet or the diet 8B.

3.2.Plasma glucose and lactate concentrations

Basal glucose plasma levels ranged from 41.70 to 51.06 mg dL^{-1} (without significant differences between groups) and significantly increased ($P<0.05$) 30 minutes after the stress. Fish fed with 4B and 16B diets showed significantly higher glucose values than fish fed with the diet 32B, but there were no significant differences with fish fed with the control and 8B diets. Six hours after the stress, fish fed with the 8B diet were the only group that recovered basal values and showed significantly lower values than the control group. Glucose recovered in most of the groups after 12 hours, except in fish fed with 16B diet (Figure 2A).

Plasma lactate increased in fish of all experimental groups after the acute stress and, after 6 hours, it decreased with a trend similar to that of the plasma cortisol. Basal lactate levels ranged from 18.60 to 23.20 mg dL^{-1} and after 30 minutes from the stress challenge, lactate levels significantly increased ($P<0.05$) to values 3 times higher than basal values. The lowest value was observed in fish fed with the 8B diet (44.89 mg dL^{-1}) and was significantly lower than fish fed with 4B (60.86 mg dL^{-1}) and 16B (58.93 mg dL^{-1}) diets. After 6 hours fish of all groups recovered lactate basal levels, not showing

significant differences (P>0.05) with the control group regardless of the diet and maintained constant values in most of the groups after 12 hours (except with the 32B diet) (Figure 2B).

3.3. MDA

MDA levels are shown in Figure 3. MDA basal values showed significant differences between groups fed with different barley concentrations. Fish fed with the highest barley concentration diet (32B) showed significantly higher MDA values than fish fed with 16B diet but without significant differences with the rest of the diets. Thirty minutes after the stress, fish fed with the highest barley concentration showed significantly lower MDA concentration than control. Between 6 and 12 hours after the stress fish of all groups showed a similar trend. After stress challenge, values observed at the end of the recovery period for fish fed with barley (except for fish fed with 4B diet) were even lower than basal values.

4. Discussion

Results of the present study suggest a potential positive effect on fish under acute stress challenge and their recovery when barley is included in the diet.

Basal plasma and fin cortisol values obtained in the present study, in general are similar to those obtained in similar conditions and in the same specie by Ellis *et al*. (2004) and Bertotto *et al*. (2010). The effect of hypoxia and crowding produced a significant increase in plasma cortisol but returned to basal levels soon after 6 hours as it was observed also in previous studies (Raaij *et al*. 1996). The initial cortisol levels increased significantly after the stress, about 30 times in plasma and 6 times in fin. This shows that the depletion of oxygen levels to values of 4 mg L^{-1} and the exposure to low

oxygen levels and crowding for 10 minutes was a stressful factor for this specie and all matrices were valuable to asses this stress. Cortisol increases in fin may be explained by the lipophilic nature of cortisol, which can diffuse through cell membranes into several tissues such as fins, mucus or muscle (Bertotto *et al*. 2010). The increase in plasma cortisol levels was expected and has been described in several studies after various stress conditions in teleost species (Weendelar-Bonga 1997, Mommsen *et al*. 1999, Pichavant *et al*. 2002, Bertotto *et al*. 2010, Pinedo-Gil *et al*. 2017). The lowest values observed in fish fed with 8B diets may probably be explained by a possible immunomodulatory effect of barley. Barley had a positive effect only when 8% barley was included in diets. The stress-reducing effect of barley could be due to its content of β-glucans (Meena *et al*. 2013), although no mechanisms have been proposed to explain this effect. Dawood *et al*. (2015), Zeng *et al*. (2016) and Miest *et al*. (2016) reported that β-glucan produces an activation of the immune system and protects fish against various stress factors. Cain *et al*. (2003) also observed a decrease in cortisol plasma response when handling stress in tilapia fed a 0.2% β-glucan diet or Jeney *et al*. (1997) observed a significant reduction in cortisol 2 hours and 1 week after a transportation stress in rainbow trout fed with a 0.1% β-glucan diet. Some authors (Aluru and Vijayan 2006, Ings *et al*. 2012, Gesto *et al*. 2013, 2015) observed that rainbow trout needs between 4-8 hours to recover basal cortisol plasma levels. Results of the present study showed that 6 and 12 hours after the stress test, plasma cortisol levels significantly decreased to basal levels, which was in accordance with the studies reported by other authors (Ings *et al*. 2012, Gesto *et al*. 2013, 2015).

Plasma glucose and lactate increased during stress challenge in the present study as reported in fish by several other authors (Raaij *et al*. 1996, Pichavant *et al*. 2002, Pinedo-Gil *et al*. 2017). These results were expected because in stressful conditions, the chromaffin cells release catecholamine hormones, adrenaline and noradrenaline towards

blood circulation that in conjunction with cortisol mobilize and elevate the glucose production in fish through gluconeogenesis and glycogenolysis pathways (Iwama *et al*. 1999). All this makes it possible to cope with the energy demand produced by the stressor for the "fight or flight" reaction and to increase lactate due to the muscle anaerobiosis (Mommsen *et al*. 1999, Chagas and Val 2006, Ming *et al*. 2012, Pérez-Jiménez *et al*. 2012). In the present study, plasma glucose and lactate increased in all experimental groups after the stress challenge and then decreased during the recovery period. Before the stress (basal levels) there were no significant differences in glucose and lactate levels in fish of all the groups. However, 30 minutes after the stress fish fed with the highest barley concentration showed the lowest glucose plasma values with no significant differences with the control group and fish fed with 8B diets. Six and twelve hours after the stress period, glucose and lactate plasma levels recovered to basal values in almost all the groups. These results point at a possible effect of barley in glucose metabolism overall to low concentrations, however, this effect was not observed when the concentration of barley increased in the diet, maybe associated to the presence of compounds which could interfere in high doses in the glucose metabolism. Fish facing up a stressor mobilize energy reserves as an adaptive response. It seems that barley could provide certain resistance characteristics reducing the stress associated with the depletion of oxygen, and modifying the plasma glucose metabolic pathway, although further studies should be conducted to verify this effect and try to explain the compounds present in the barley involved in this mechanism. Another explanation of the barley effect on cortisol, glucose and lactate levels may be related to the cortisol synthesis. Barley can control lipid peroxidation and that may have prevented the production of cholesterol (Ming *et al*. 2012), thereby avoiding the cortisol production (Kitabchi 1967).

Lipid oxidation results showed a less clear effect of the stress challenge on trouts. MDA values resulted higher in fish fed with the highest barley concentration (32B) than those fed with 16B diets before the stress challenge (basal levels). Interestingly, a significant decrease of MDA was observed after the stress challenge in fish fed the highest barley level, with the lowest recorded MDA value when compared with the control group. The use of different tissues and techniques in lipid oxidation evaluation makes difficult to

compare the results of the present study with those of other studies but the low levels of MDA in fish fed with barley diet after the stress test and during their recovery suggest a potential antioxidant effect of this dietary ingredient. Some studies have shown an increase on lipid oxidation under hypoxia (Lushchak *et al*. 2005, Pérez-Jiménez *et al*. 2011); other studies such as the described by Chagas and Val (2006) showed that lipid oxidation is prevented by antioxidants. Interestingly and in accordance with the present results, Leveelahti *et al*. (2014) in a study of three species (the epaulette shark, threespine stickleback and rainbow trout) exposed to hypoxia, reported that in general, fish do not show an increase in redox-active antioxidant defence in response to oxidative stress associated with hypoxia. Rather, the changes in antioxidant defences during hypoxia are very much species- and tissue-specific and are not linked to the levels of hypoxia tolerance. It is well known that the response of MDA is tissue-specific (Lushchak and Bagnyukova 2006) and depends on the type of stress. In the present study, the lowest MDA level observed in fish fed with the highest barley level suggests a protective effect from oxidative stress. Lushchak *et al*. (2005) observed in carp that TBARS levels in liver increased due to the hypoxia, however in brain and liver, lipid peroxides decreased, maybe due to low oxygen availability which could reduce the reactive oxygen species (ROS) levels and thus reducing lipid oxidation.

5. CONCLUSIONS

Based on the results it seems that the inclusion of barley on rainbow trout diets could modulate stress markers, which were determined in plasma, fin and muscle. Cortisol levels significantly decreased when fish were fed at medium (8%). Glucose values significantly decreased when fish were fed at maximum barley concentrations (32%) and lactate at medium barley concentrations (8%) with no significant differences with the control group and fish fed with 8B diets in both markers. It was also observed a

significant decrease on lipid oxidation (MDA) in muscle. All these indicate a better control of acute hypoxia and crowding when fish were fed with barley. Although further studies should be conducted in order to verify these interesting results with other markers and study a dose dependent behaviour.

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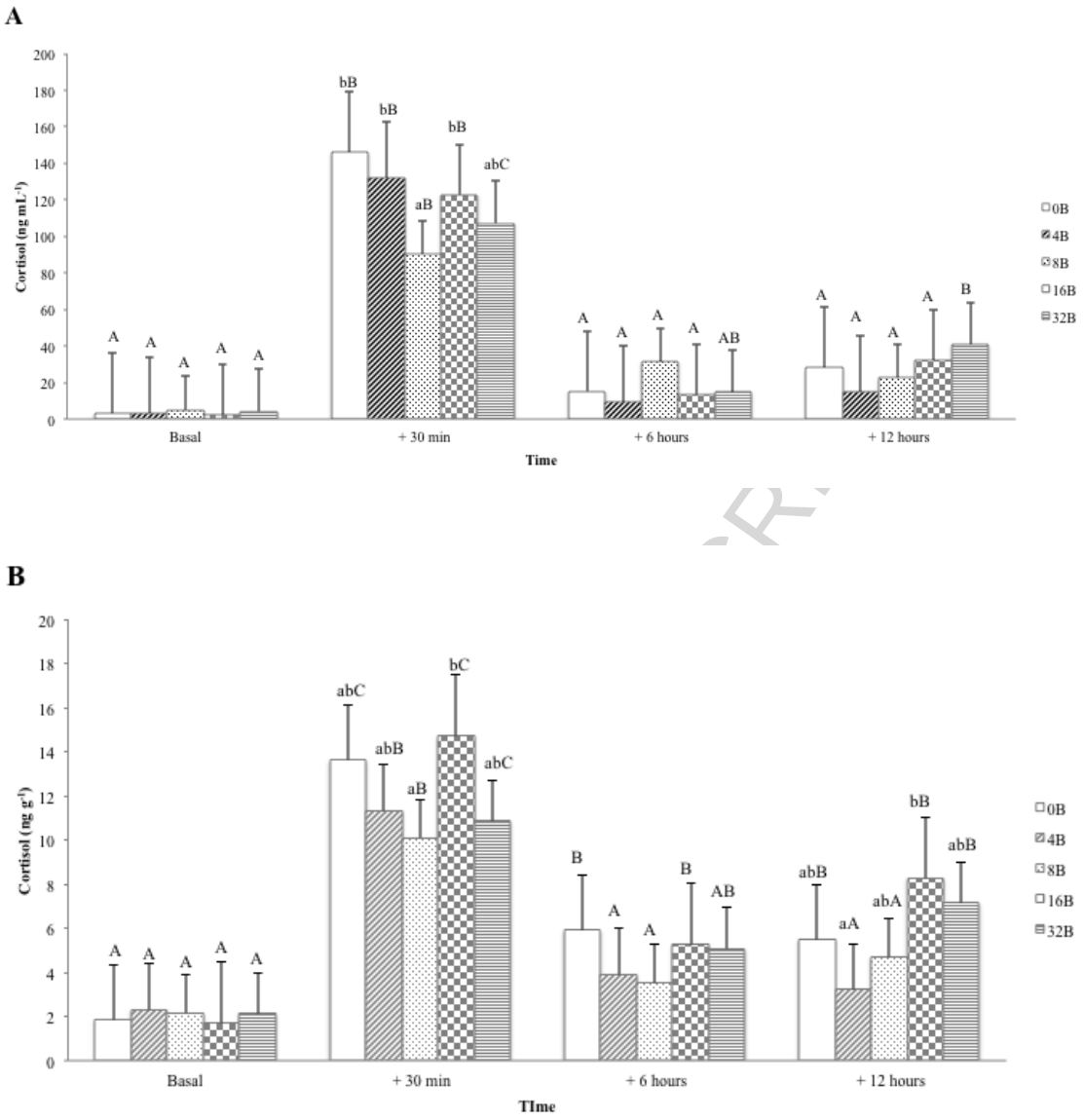

Figure 1. Effect of barley on plasma cortisol (ng mL^{-1}) (A) and fin cortisol (ng g^{-1}) (B) content of rainbow trout under normoxia (basal), 30 minutes, 6 and 12 hours after stress. Data are expressed as least-square means ± SEM, n=6. Capital letters indicate significant differences ($p<0.05$) at different time points and small letters above the bars indicate significant differences ($p<0.05$) between experimental diets in the same time point. Different experimental diets: 0B (0% barley); 4B (4% barley); 8B (8% barley); 16B (16% barley); 32B (32% barley).

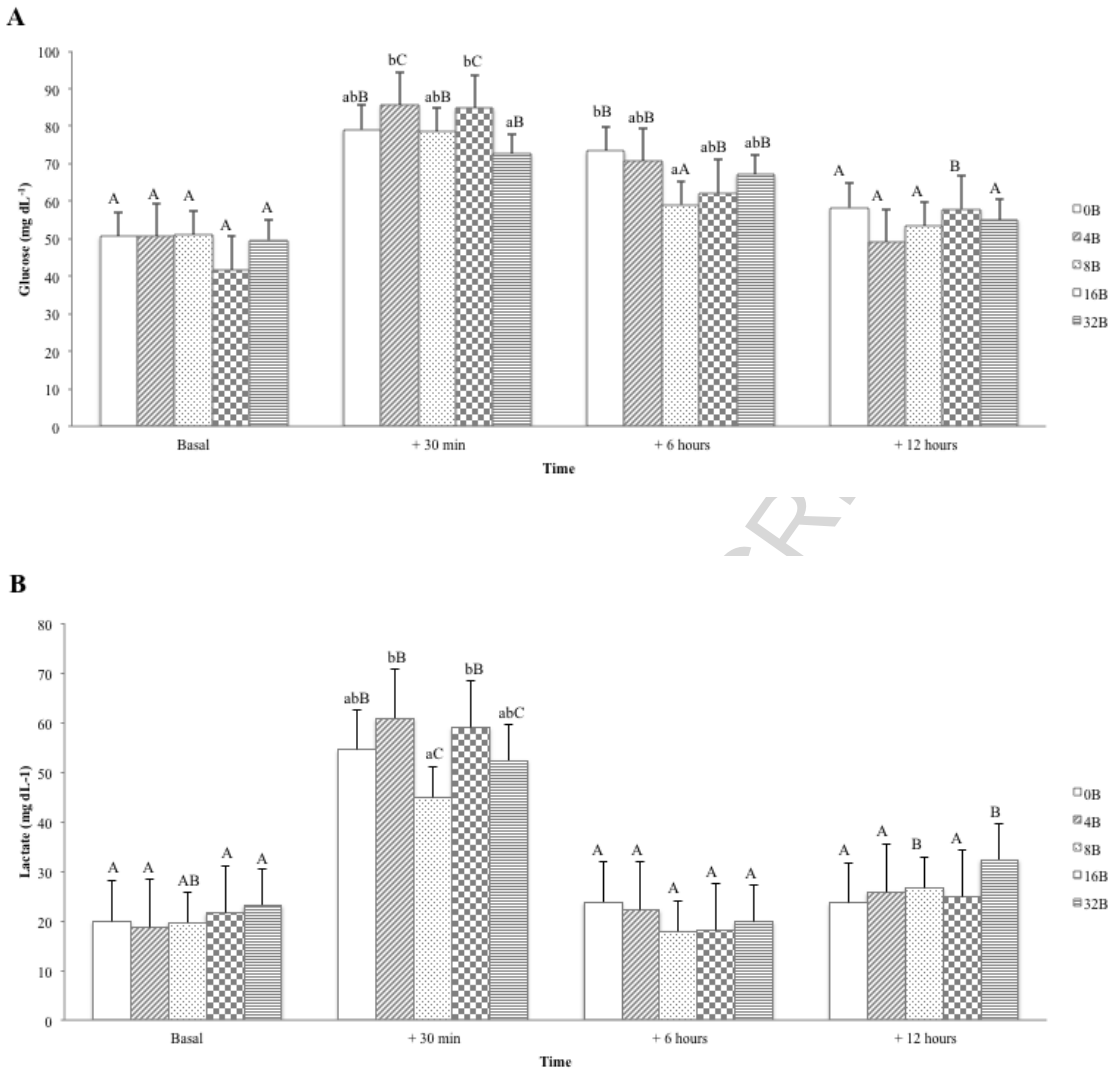

Figure 2. Effect of barley on plasma glucose (mg dL^{-1}) (A) and lactate (mg dL^{-1}) (B) content of rainbow trout under normoxia (basal), 30 minutes, 6 and 12 hours after stress. Data are expressed as least-square means ± SEM, n=6. Capital letters indicate significant differences ($p<0.05$) at different time points and small letters above the bars indicate significant differences ($p<0.05$) between experimental diets in the same time point. Different experimental diets: 0B (0% barley); 4B (4% barley); 8B (8% barley); 16B (16% barley); 32B (32% barley).

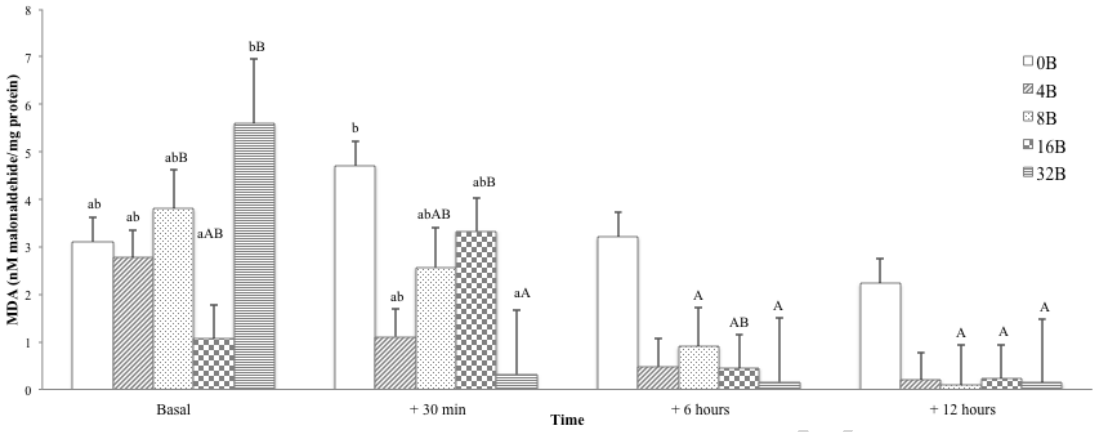

Figure 3. Effect of barley on MDA values (nM malonaldehide/mg protein) of rainbow trout under normoxia (basal), 30 minutes, 6 and 12 hours after stress. Data are expressed as least-square means ± SEM, n=6. Capital letters indicate significant differences ($p<0.05$) at different time points and small letters above the bars indicate significant differences ($p<0.05$) between experimental diets in the same time point. Different experimental diets: 0B (0% barley); 4B (4% barley); 8B (8% barley); 16B (16% barley); 32B (32% barley).