Pinedo-Gil, J.; Martín-Diana, AB.; Bertotto, D.; Sanz-Calvo, MÁ.; Jover Cerda, M.; Tomas-Vidal, A. (2018). Effects of dietary inclusions of red beet and betaine on the acute stress response and muscle lipid peroxidation in rainbow trout. Fish Physiology and Biochemistry. 44(3):939-948. doi:10.1007/s10695-018-0483-3

Springer

Dear Author

Here are the proofs of your article.

- You can submit your corrections **online,** via **e-mail** or by **fax**.
- For **online** submission please insert your corrections in the online correction form. Always indicate the line number to which the correction refers.
- You can also insert your corrections in the proof PDF and **email** the annotated PDF.
- For **fax** submission, please ensure that your corrections are clearly legible. Use a fine black pen and write the correction in the margin, not too close to the edge of the page.
- Remember to note the **journal title**, **article number**, and **your name** when sending your response via e-mail or fax.
- **Check** the metadata sheet to make sure that the header information, especially author names and the corresponding affiliations are correctly shown.
- **Check** the questions that may have arisen during copy editing and insert your answers/corrections.
- **Check** that the text is complete and that all figures, tables and their legends are included. Also check the accuracy of special characters, equations, and electronic supplementary material if applicable. If necessary refer to the *Edited manuscript*.
- The publication of inaccurate data such as dosages and units can have serious consequences. Please take particular care that all such details are correct.
- Please **do not** make changes that involve only matters of style. We have generally introduced forms that follow the journal's style.
- Substantial changes in content, e.g., new results, corrected values, title and authorship are not allowed without the approval of the responsible editor. In such a case, please contact the Editorial Office and return his/her consent together with the proof.
- If we do not receive your corrections **within 48 hours**, we will send you a reminder.
- Your article will be published **Online First** approximately one week after receipt of your corrected proofs. This is the **official first publication** citable with the DOI. **Further changes are, therefore, not possible.**
- The **printed version** will follow in a forthcoming issue.

Please note

After online publication, subscribers (personal/institutional) to this journal will have access to the complete article via the DOI using the URL:

`http://dx.doi.org/10.1007/s10695-018-0483-3`

If you would like to know when your article has been published online, take advantage of our free alert service. For registration and further information, go to: http://www.link.springer.com.

Due to the electronic nature of the procedure, the manuscript and the original figures will only be returned to you on special request. When you return your corrections, please inform us, if you would like to have these documents returned.

Metadata of the article that will be visualized in OnlineFirst

1	Article Title	**Effects of dietary inclusions of red beet and betaine on the acute stress response and muscle lipid peroxidation in rainbow trout**	
2	Article Sub- Title		
3	Article Copyright - Year	**Springer Science+Business Media B.V., part of Springer Nature 2018** **(This will be the copyright line in the final PDF)**	
4	Journal Name	Fish Physiology and Biochemistry	
5	Corresponding Author	Family Name	**Pinedo-Gil**
6		Particle	
7		Given Name	**Julia**
8		Suffix	
9		Organization	Polytechnic University of Valencia
10		Division	Research Group of Aquaculture and Biodiversity, Institute of Animal Science and Technology
11		Address	Camino de Vera, 14, Valencia 46071
12		e-mail	julia.pinedo.gil@gmail.com
13	Corresponding Author	Family Name	**Martín-Diana**
14		Particle	
15		Given Name	**Ana Belén**
16		Suffix	
17		Organization	Subdirection of Research and Technology, Agro-Technological Institute of Castilla y León, Consejería de Agricultura y Ganadería
18		Division	
19		Address	Finca de Zamadueñas, Ctra. Burgos km. 119, Valladolid 47071
20		e-mail	mardiaan@itacyl.es
21	Author	Family Name	**Bertotto**
22		Particle	
23		Given Name	**Daniela**
24		Suffix	
25		Organization	University of Padova
26		Division	Department of Comparative Biomedicine and Food Science

27		Address	Viale dell'Università 16 Agripolis, Legnaro 35020, PD
28		e-mail	
29	Author	Family Name	**Sanz-Calvo**
30		Particle	
31		Given Name	**Miguel Ángel**
32		Suffix	
33		Organization	Subdirection of Research and Technology, Agro-Technological Institute of Castilla y León, Consejería de Agricultura y Ganadería
34		Division	
35		Address	Finca de Zamadueñas, Ctra. Burgos km. 119, Valladolid 47071
36		e-mail	
37	Author	Family Name	**Jover-Cerdá**
38		Particle	
39		Given Name	**Miguel**
40		Suffix	
41		Organization	Polytechnic University of Valencia
42		Division	Research Group of Aquaculture and Biodiversity, Institute of Animal Science and Technology
43		Address	Camino de Vera, 14, Valencia 46071
44		e-mail	
45	Author	Family Name	**Tomás-Vidal**
46		Particle	
47		Given Name	**Ana**
48		Suffix	
49		Organization	Polytechnic University of Valencia
50		Division	Research Group of Aquaculture and Biodiversity, Institute of Animal Science and Technology
51		Address	Camino de Vera, 14, Valencia 46071
52		e-mail	

53	Schedule	Received	5 August 2017
54		Revised	
55		Accepted	14 February 2018

56	Abstract	This study evaluates the effects of red beet (RB) and betaine on rainbow trout submitted to an acute stress challenge. A control diet was compared with four experimental diets in which red beet (14 and 28%) and betaine (0.9 and 1.63%) were incorporated in different concentrations according to a factorial design. Cortisol in

		plasma and fin, glucose and lactate plasma levels, and malondialdehide (MDA) in muscle were all measured before the stress challenge and 30 min and 6 and 12 h after the stress challenge as parameters to determine the diet effects. RB and betaine had no effect on cortisol, glucose, and MDA basal levels. However, lactate basal levels were significantly lower on fish fed with RB and betaine. Thirty minutes after the stress challenge, there was a significant increase in plasma and fin cortisol, glucose and lactate concentrations, although fish fed with diets containing RB and betaine showed significantly higher plasma cortisol values. MDA values of fish fed with 14% RB and 0.9% betaine were significantly higher than MDA values from fish fed with 28% RB and 1.63% betaine. After 6 and 12 h, plasma and fin cortisol and lactate levels recovered in a similar trend. Glucose plasma levels recovered in almost all groups 12 h after the stress. Also, MDA values recovered basal levels after 6 and 12 h. RB and betaine did not enhance the tolerance to the stress challenge compared to the control group, although the presence of these ingredients had no negative effect on any of the stress indicators.
57	Keywords separated by ' - '	Red beet - Betaine - Rainbow trout - Acute stress challenge
58	Foot note information	

Fish Physiol Biochem
https://doi.org/10.1007/s10695-018-0483-3

Effects of dietary inclusions of red beet and betaine on the acute stress response and muscle lipid peroxidation in rainbow trout

Julia Pinedo-Gil · Ana Belén Martín-Diana · Daniela Bertotto · Miguel Ángel Sanz-Calvo · Miguel Jover-Cerdá · Ana Tomás-Vidal

Received: 5 August 2017 / Accepted: 14 February 2018

Abstract This study evaluates the effects of red beet (RB) and betaine on rainbow trout submitted to an acute stress challenge. A control diet was compared with four experimental diets in which red beet (14 and 28%) and betaine (0.9 and 1.63%) were incorporated in different concentrations according to a factorial design. Cortisol in plasma and fin, glucose and lactate plasma levels, and malondialdehide (MDA) in muscle were all measured before the stress challenge and 30 min and 6 and 12 h after the stress challenge as parameters to determine the diet effects. RB and betaine had no effect on cortisol, glucose, and MDA basal levels. However, lactate basal levels were significantly lower on fish fed with RB and betaine. Thirty minutes after the stress challenge, there was a significant increase in plasma and fin cortisol, glucose and lactate concentrations, although fish fed with diets containing RB and betaine showed significantly higher plasma cortisol values. MDA values of fish fed with 14% RB and 0.9% betaine were significantly higher than MDA values from fish fed with 28% RB and 1.63% betaine. After 6 and 12 h, plasma and fin cortisol and lactate levels recovered in a similar trend. Glucose plasma levels recovered in almost all groups 12 h after the stress. Also, MDA values recovered basal levels after 6 and 12 h. RB and betaine did not enhance the tolerance to the stress challenge compared to the control group, although the presence of these ingredients had no negative effect on any of the stress indicators.

Keywords Red beet · Betaine · Rainbow trout · Acute stress challenge

J. Pinedo-Gil (✉) · M. Jover-Cerdá · A. Tomás-Vidal
Research Group of Aquaculture and Biodiversity, Institute of Animal Science and Technology, Polytechnic University of Valencia, Camino de Vera, 14, 46071 Valencia, Spain
e-mail: julia.pinedo.gil@gmail.com

A. B. Martín-Diana (✉) · M. Á. Sanz-Calvo
Subdirection of Research and Technology, Agro-Technological Institute of Castilla y León, Consejería de Agricultura y Ganadería, Finca de Zamadueñas, Ctra. Burgos km. 119, 47071 Valladolid, Spain
e-mail: mardiaan@itacyl.es

D. Bertotto
Department of Comparative Biomedicine and Food Science, University of Padova, Viale dell'Università 16 Agripolis, 35020 Legnaro, PD, Italy

Introduction

Fish welfare is crucial for the farming industry not only for public perception, marketing, and product acceptance, but also in terms of efficiency, quality, and quantity (Øverli et al. 2006; Ashley 2007). The welfare of farmed fish is influenced mainly by physical disturbances such as handling, weighing, crowding, grading, transport, temperature, and dissolved oxygen which cause fish stress (Barton and Iwama 1991; Chagas and Val 2006; Bertotto et al. 2010, 2011; Madaro et al. 2015). The primary stress response in fish involves the release of catecholamines and activation of the hypothalamic-pituitary-interenal (HPI) axis, and the synthesis and release of cortisol. Both catecholamines

and cortisol cause an energy source mobilization, depletion of glycogen stores, and increase of glucose and lactate plasma levels (Ashley 2007; Zolderdo et al. 2016). Therefore, the levels of glucose and lactate in plasma are often used alongside cortisol to assess stress level in animals (Rollo et al. 2006, Ashley 2007).

Due to those stressful factors, a decisive goal in aquaculture is to find different alternatives to manage stress. The development of novel methods to reduce stress responses and/or strengthen immunity is an important area of study, a good example being the use of nutrients and other compounds such as ascorbic acid in gilthead seabream (Ortuño et al. 2003), in rainbow trout (Dabrowski et al. 2004), or in fish tambaqui (Chagas and Val 2006), vitamin E in gilthead seabream (Montero et al. 2001; Ortuño et al. 2003), fatty acids in gilthead seabream (Van Anholt et al. 2004), β-glucans in rainbow trout (Jeney et al. 1997) or in yellow croaker (Zeng et al. 2016), or betaine in *Labeo rohita* (Virtanen 1995; Kumar et al. 2012). Over the last decade, there has been an increasing interest of the use of natural compounds present in fruits, vegetables, and herbs as antioxidants and functional nutrients (Ganessan et al. 2011). Betaine is a natural compound that is widely found in animals, plants, and microorganisms and has been used as a dietary feed supplement in animal nutrition for more than 50 years due to its antioxidant and functional properties that protect against stressful factors (Kujala et al. 2002; Ganessan et al. 2011; Rabeh 2015). However, what about the activity of these functional compounds when they are not isolated but present in the whole raw material of the diet? Does it have the same effect? Few studies have been found about this topic. Red beet (*Beta vulgaris* L.) is a rich source in natural betaine but also in other important nutrients such as magnesium, sodium, vitamin C, and betalains (Pinedo-Gil et al. 2017). To the best of our knowledge, the use of red beet on aquaculture has been limited, possibly, due to their high fiber content that reduces fish digestibility (Hemre et al. 2002; Krogdahl et al. 2004; Tan et al. 2006; Enes et al. 2006; Wu et al. 2007; Cui et al. 2010) or due to a low palatability which would lead to a reduction of fish intake and growth, or due to probability of the presence of some antinutritional component such as tannins, oxalates, or phytates which can also promote growth inhibition (Francis et al. 2001; Pinedo-Gil et al. 2017). However, Pinedo-Gil et al. (2017) showed a positive effect of red beet and betaine on quality parameters of rainbow trout. The main objectives of the study were to test, based on the antioxidant properties of betaine observed in other animals, the antioxidant ability as well as the potential stress-relieving properties of dietary administration of red beet and betaine in rainbow trout diet and the ability of these ingredients to control or reduce the stress.

Material and methods

Production system

The trial was conducted at the Aquaculture Research Centre of Segovia, Spain, in 10 cylindrical fiberglass tanks (500 L) within a freshwater recirculation system (RAS). During the experiment, water temperature remained constant at 15.04 ± 0.27 °C (mean ± SD). The level of dissolved oxygen was 6.49 ± 0.37 mg L^{-1} (64% saturation). All tanks were equipped with aeration and an oxygen probe. Water pH was 7.91 ± 0.14, and ammonia and nitrite concentrations in water were 0.65 ± 0.40 and 0.43 ± 0.30 mg L^{-1}, respectively. Water flow was 10.29 ± 0.84 L h^{-1}. The photoperiod consisted on 12 h of light and 12 h of dark intervals and all tanks had identical light conditions.

Fish, diets, and feeding

A total of 400 rainbow trout from a commercial fish farm (Piscifactoría Cien Fuentes, 19420 Cifuentes, Guadalajara, Spain) were used. Fish were randomly allocated in 10 tanks, 40 fish per tank (initial stocking density 20.0 ± 0.1 kg m^3, with and initial average weight of 250 ± 48.63 g). Prior to the feeding trial, all fish were acclimated to the indoor rearing conditions for 1 week, and fish were fed once a day (8:00) to apparent satiation exclusively using the control diet. The study lasted 45 days.

Five isoproteic and isolipidic diets were formulated using red beet and betaine as experimental ingredients. Two red beet concentrations (14 and 28%) and 2 betaine concentrations (0.9 and 1.63%) were introduced: control (0% red beet, 0% betaine), RB141 (14% red beet, 0.9% betaine), RB142 (14% red beet, 1.63% betaine), RB281 (28% red beet, 0.9% betaine), and RB282 (28% red beet and 1.63% betaine). The composition and proximate analysis of red beet diets are described in Pinedo-Gil et al. (2017). Control diet was prepared using the same ingredients than

experimental diets but without red beet and betaine in the formulation. It was not a commercial diet. The diet extrusion process is described in Pinedo-Gil et al. (2016). The experimental diets were administered in replicate (two groups). 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.

Stress trial

The stress trial was carried out after the feeding experimental period (45 days) by submitting the fish to a decrease of water oxygen concentration from 6.34 to 4 mg L^{-1}. The reduction of oxygen was obtained by lowering water level to a volume of 50 L and removing the aeration. Once the oxygen dissolved in water reached 4 mg L^{-1} (oxygen-saturated value of 39.7%) (approximately 15–20 min), fish were kept in these conditions for 10 min, and after this time, tanks were filled again with water and aerated. During the acute stress challenge (hypoxia and crowding), oxygen decreased to less than 2 mg L^{-1} (oxygen-saturated value below 19.8%). Before applying the stress, all fish were starved for 2 days.

Sampling

Samples were taken before the stress test (basal levels) and 30 min, 6 h, and 12 h after the stress. Fish were sacrificed with 300 mg L^{-1} MS222 (100% *w*/*w*; PHARMAQ®). Once fish were deeply anesthetized, they were blooded from the caudal vein with 1-mL syringers (BD Plastipak) and blood put in heparinized tubes on ice. When all the fish of every tank were bled (1.5–2 mL from each fish), the heads were removed and maintained in ice until all fish were blooded. Soon after collection, blood was centrifuged at 1200×*g* for 10 min at 4 °C and plasma (500–750 μL) and transferred to 1 mL eppendorfs and frozen at − 80 °C until analysis. Small portions of side muscle (about 1 × 1 × 1 cm from the caudal peduncle and without skin) and caudal fin (about 1 × 1 cm from the upper lobe) were collected and conserved at − 80 °C until analysis.

An equal number of fish from each tank was subjected to the same sampling procedure at each time of sampling (6 fish per tank, $n = 6$).

Cortisol analysis

Cortisol was measured in plasma and fin by a specific radioimmunoassay (RIA) as described by Bertotto et al. (2010) after extraction in diethyl ether. Briefly, a 96-well microtiter plate (Optiplate, Perkin Elmer Life Sciences, Waltham, MA, USA) was coated with anti-rabbit γ-globulin serum raised in goat, and the antiserum, diluted 1:1000 in 0.15 mM sodium acetate buffer, pH 9 at 4 °C, was incubated overnight. The plate was washed twice with PBS and incubated again overnight at 4 °C with the specific antiserum solution. It was then carefully washed with PBS, standards, quality controls, unknown extracts, and ^{3}H tracer were added, and the plate was reincubated overnight at 4 °C. Finally, the plate was washed with PBS, added with 200 μL scintillation cocktail (Microscint 20, Perkin Elmer Life Sciences), and counted on a β-counter (Top-Count, Perkin Elmer Life Sciences).

The sensitivity of the assay was 3.125 pg $well^{-1}$ and was defined as the dose of hormone at 90% binding (B/B_0).

The anti-cortisol serum showed the following cross-reactions: cortisol 100%, prednisolone 44.3%, 11-deoxycortisol 13.9%, cortisone 4.95%, corticosterone 3.5%, prednisone 2.7%, 17-hydroxyprogesterone 1.0%, 11-deoxycorticosterone 0.3%, dexamethasone 0.1%, progesterone < 0.01%, 17-hydroxypregnenolone < 0.01%, and pregnenolone < 0.01%.

Glucose and lactate

Glucose and lactate concentrations were determined only in plasma. They were measured by an enzymatic colorimetric assay, in particular by GOD-POD (SPINREACT® Ref. 1001191) and LOD-POD (SPINREACT® Ref. 1001330) method, respectively (Kaplan and Pesce 1984). Briefly, aliquots (5 μL) from plasma samples were mixed with 500 μL of reactive and incubated for 10 min for glucose determination and 5 min for lactate determination at 37 °C 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}.

Lipid peroxidation

Muscle was used to determine the amount of lipid peroxidation. One hundred milligrams of tissue was

homogenized with Tris HCL 0.125 M pH 6.9 (500 µL), centrifuged at 13,000×*g* 4 °C for 15 min, and supernatant used for the assays.

The amount of lipid peroxidation was determined in muscle by measuring thiobarbituric acid-reactive substances (TBARS) according to Yoshida et al. (2005). Thiobarbituric acid reaction was carried out by mixing 0.2-mL sodium dodecyl sulfate solution (8.1% *w*/*v*), 1.5 mL acetic acid buffer (20% *v*/*v*, pH 3.5), 1.5 mL thiobarbituric acid (TBA 1% *v*/*v*), 0.775 mL water, and 0.05 mL ethanol containing butylated hydroxytoluene (0.8 wt% *w*/*v*) with 25 µL of supernatant. The reaction mixture was incubated at 100 °C during 60 min and then cooled in ice followed by vigorous mixing with 1 mL water and 5 mL of n-butyl alcohol and pyridine (15/1, by volume). Afterwards, the mixture was centrifuged at 1400×*g* at 0 °C for 10 min, and the supernatant was measured spectrophotometrically at 535 nm. Tetramethosypropane was used as standard to estimate TBARS formation as nmoles of malondialdehyde (MDA) equivalents per g of tissue.

Statistical analyses

All statistical analyses were carried out using software SAS (SAS version 9, SAS Institute Inc., Cary, North Carolina, USA). Data were analyzed by ANOVA using the PROC MIXED with dietary treatment and time after stress as variable factors and the tank as random effect. The probability of the linear, cubic, and quadratic components of variance was calculated by contrast statement to test differences according to sampling time after stress. The contrast statements were used to test differences between diets containing 14 and 28% of red beet and 0.9 and 1.63% betaine corresponding to the different experimental treatments. Differences among means with $P < 0.05$ were accepted as representing statistically significant differences.

Ethical statement

The rainbow trout *Oncorhynchus mykiss* (Walbaum) study complied with both European Union Council Directive 2010/63/UE, which lays down minimum standards for the protection of animals, and Spanish national legislation (Spanish Royal Decree 53/2013) protecting animals used in experimentation and for other scientific purposes and approved by Animal Ethics Committee of Agro-Technological Institute of Castilla y León (Spain).

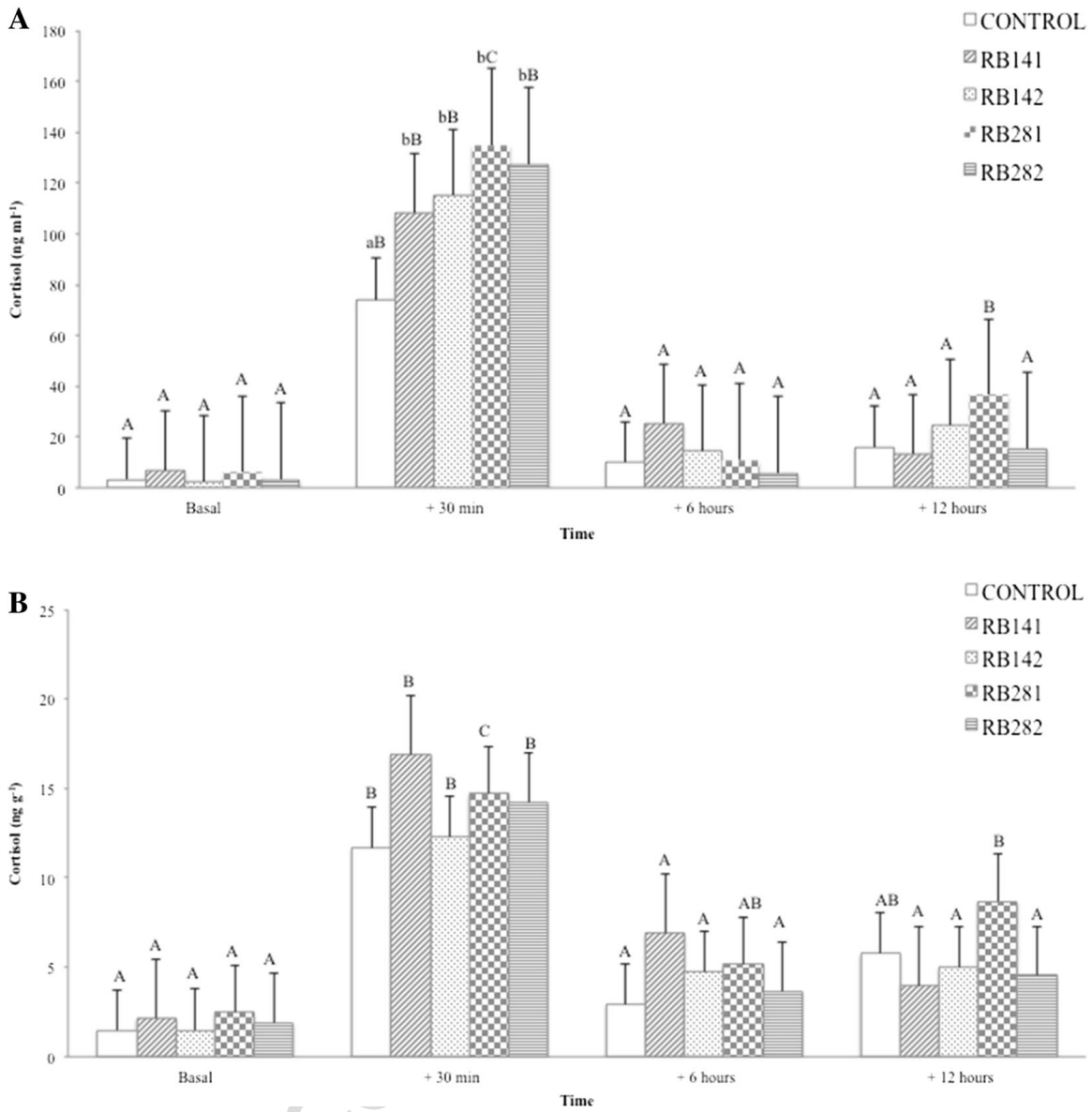
Fish in the tanks were checked on a daily basis. At the end of the trials, fish were weighed individually and their health status was assessed by observation, after sedation with MS222 dissolved in water (MS222®; 200 mg L^{-1}) to minimize animal suffering. Animals were euthanized by excess of MS222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

Results

Cortisol

Before stress (basal levels), plasma cortisol levels were low without significant differences among the experimental diet groups (Fig. 1a). Basal levels range from 3.32 to 6.69 ng mL^{-1}. Thirty minutes after the stress, plasma cortisol levels significantly increased ($P < 0.05$) with values more than 30 times higher than basal values. This significant increase occurred in all groups but fish fed with diets containing red beet and betaine (107.94–134.88 ng mL^{-1}) showed significantly higher values compared with the control group (73.99 ng mL^{-1}). After 6 and 12 h from stress, plasma cortisol levels significantly decreased ($P < 0.05$) in all groups returning to basal values, except in fish fed with diet RB281 (28% red beet and 0.9% betaine) where cortisol plasma levels remained higher (36.51 ng mL^{-1}) than basal values (6.00 ng mL^{-1}; $P < 0.05$) only at 12 h. Some uncontrolled stress probably occurred after 6 h as the basal levels were recovered after 6 h.

The effect of red beet and betaine on fin cortisol levels of rainbow trout after acute stress challenge and subsequent recovery is shown in Fig. 1b. Results showed that different red beet and betaine concentrations had no effect on fin cortisol levels during the stress experimental trial. Fin cortisol levels were low before the stress (basal levels) showing values ranging from 1.48 to 2.44 ng mg^{-1}. As occurred in cortisol plasma levels, 30 min after the stress, fin cortisol concentration significantly increased ($P < 0.05$) in all experimental groups, although the increase was only 7 times higher than basal values. Also, fish groups fed with red beet and betaine recovered overtime without significant differences with the control, although final values were higher than the levels before stress.



Q1 **Fig. 1** Effect of red beet and betaine on plasma cortisol (**a**) and fin cortisol (**b**) content of rainbow trout before the acute stress challenge (basal), 30 min after stress and 6 and 12 h after stress. Data were expressed as least-square means ± SEM, $n = 12$. Different capital letters above the bars indicate significant differences ($p < 0.05$) at different time points of the same group and different small letters above the bars indicate significant differences ($p < 0.05$) between different experimental diets in the same time point. CONTROL (0% red beet, 0% betaine), RB141 (14% red beet, 0.9% betaine), RB142 (14% red beet, 1.63% betaine), RB281 (28% red beet, 0.9% betaine), and RB282 (28% red beet, 1.63% betaine) are the different experimental diets

Plasma glucose and lactate

Results of plasma glucose levels are shown in Fig. 2a. Basal levels range from 45.08 to 57.72 mg dL^{-1} and were not affected by the diet. After acute stress challenge (+ 30 min), there was a significant increase ($P < 0.05$) of glucose reaching values 1.5 times higher than basal values. The recovery overtime (6 and 12 h after the stress) did not follow the same pattern for every experimental group, and fish fed diet RB141 did not recover basal levels after 12 h.

Figure 2b shows that basal plasma lactate levels were significantly affected ($P < 0.05$) by the diet. Control group showed the highest lactate level (39.43 mg dL^{-1}), and the lowest was observed in those fish fed with the diets with 0.9% betaine (diets RB141 and RB281, 23.57 and 21.42 mg dL^{-1}, respectively). Thirty minutes after the acute stress

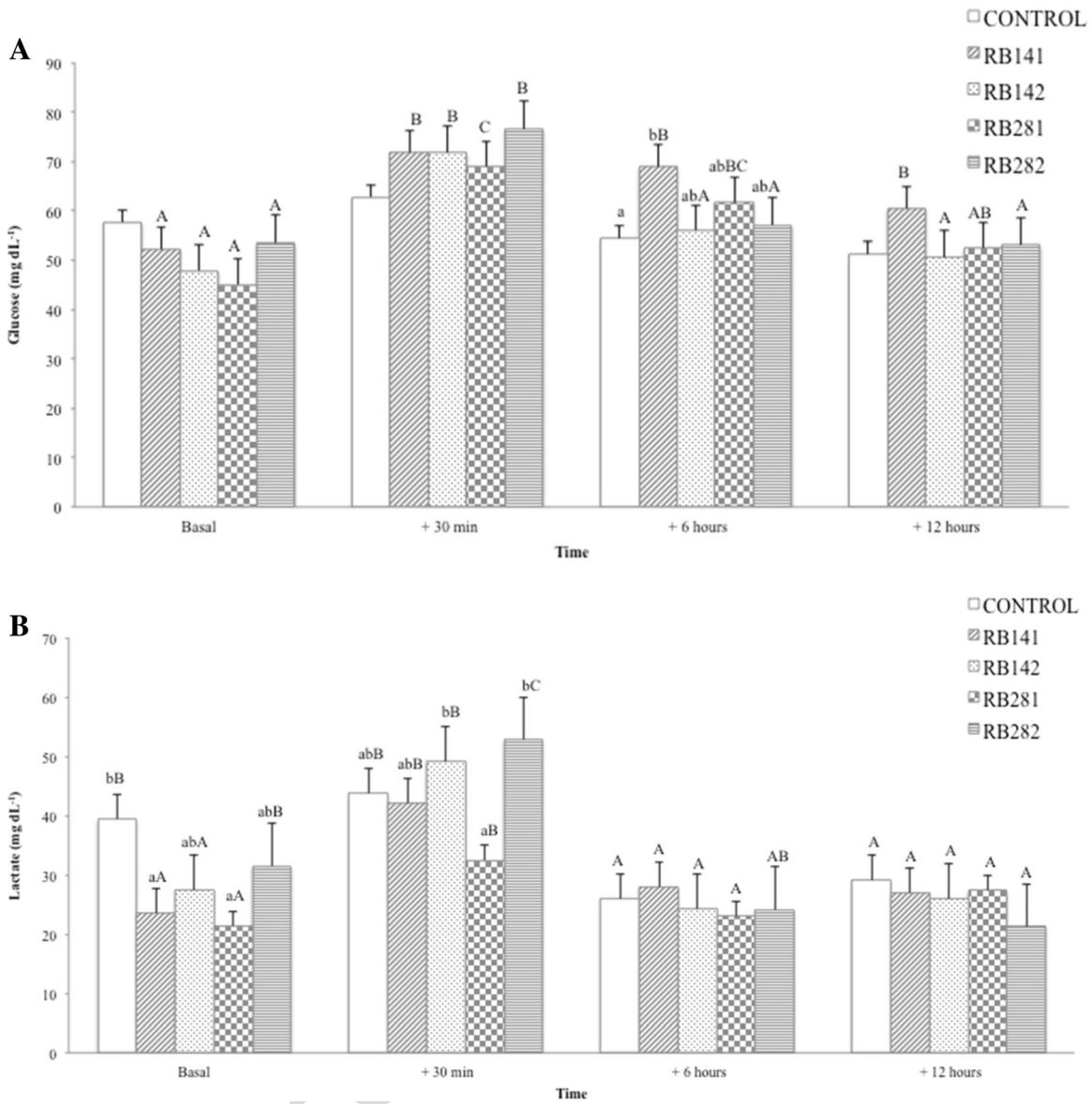


Fig. 2 Effect of red beet and betaine on plasma glucose (**a**) and lactate (**b**) content of rainbow trout before the acute stress challenge (basal), 30 min after stress and 6 and 12 h after stress. Data were expressed as least-square means ± SEM, $n = 12$. Different capital letters above the bars indicate significant differences ($p < 0.05$) at different time points of the same group and different small letters above the bars indicate significant differences ($p < 0.05$) between different experimental diets in the same time point. CONTROL (0% red beet, 0% betaine), RB141 (14% red beet, 0.9% betaine), RB142 (14% red beet, 1.63% betaine), RB281 (28% red beet, 0.9% betaine), and RB282 (28% red beet, 1.63% betaine) are the different experimental diets

challenge, a significant increase ($P < 0.05$) of lactate in all groups was observed, except for the control group. The diet had a significant effect, the least effected being fish fed with diet RB281, while lactate values were significantly lower in fish fed with the control diet than fish fed with diets at higher betaine concentration. After 6 and 12 h of recovery, fish of all groups recovered basal lactate levels.

Lipid peroxidation

Before the stress challenge, no significant differences on MDA values were observed regardless of the diet ($P > 0.05$). Thirty minutes after the stress, fish fed with diet RB141 (14% RB and 0.9% betaine) showed a significant increase on MDA concentration. Although no significant differences were observed with the control group, lower MDA values were observed on fish fed

at higher RB and betaine concentration (RB282, 28% RB and 1.63% betaine). Overtime the recovery period (6 and 12 h after the stress), no significant differences were observed regardless of the diet (Fig. 3).

Data showed that high RB concentrations (28% RB) did not produce an increase on MDA values; meanwhile, fish fed with the control diet and lower RB concentrations (14% RB) showed an impact on MDA values 30 min after the stress but recover basal levels after 12 h (Fig. 3).

Discussion

Results of the present study showed that the incorporation of red beet and betaine on rainbow trout diets had a significant effect on fish under acute stress challenge responses and recovery. Differences between the used indicators and matrixes can be observed although their trend along the stress challenge was similar.

Basal cortisol plasma values (about 4 ng mL^{-1}) were similar to values reported by other authors (Tintos et al. 2006; Bertotto et al. 2010) confirming the state of no stress of the fish before the stress challenge. The cortisol levels increased significantly 30 min after the stress in all groups and regardless of the diet. An increase in plasma cortisol is well reported after various stress conditions in teleost species (Bertotto et al. 2010; Ming et al. 2012; Pérez-Jiménez et al. 2012). Cortisol, together with the catecholamines, is involved in adaptive mechanisms developed by fish to maintain oxygen supply to the tissues under hypoxia situations (Pichavant et al. 2002). However, in the present study, the higher values were recorded in fish fed diets containing red beet in comparison with the control diet suggesting that this ingredient did not enhance the tolerance of rainbow trout to a stress challenge although all groups recover with the same trend. On the other hand, fin cortisol levels showed a similar trend but it was not affected by the diet. Nevertheless, fin cortisol levels were about 10 times lower than plasma cortisol values. These differences could be explained by the different diffusion rates in the various matrices even if the kinetics of the hormone in the various matrices should be better-understood (Bertotto et al. 2010). Plasma and fin cortisol levels returned to control levels regardless of the diet 6 h after the exposure and remained low after 12 h. Similar results were observed by Sadoul et al. (2015) in rainbow trout submitted to stress confinement and by Fast et al. (2008) in Atlantic salmon after a heat-stress with recovery in 5–6 h.

Plasma glucose basal values were not affected by the inclusion of red beet and betaine. However, plasma lactate basal values were significantly affected by the inclusion of red beet and betaine. Significantly lower values were observed in fish fed with red beet and betaine compared to fish fed with the control diet. Plasma glucose and lactate levels increased after the stress since it has been verified that the increase of cortisol and catecholamines released by the perception of a stressor produces the mobilization of energy stores to provide metabolic fuel, usually in the form of glucose and lactate, to overcome a stress challenge (Ings et al. 2012). In fish, it is generally accepted that catecholamines are mostly responsible for the increase of glycogenolysis while cortisol is believed to induce gluconeogenesis, and its role on promoting glycogenolysis, if any, is less clear (Janssens and Waterman 1988; Mommsen et al. 1999). In this study, 30 min after the acute stress challenge, as expected, fish glucose plasma levels significantly increased compared with the basal levels, regardless of the diet. However, lactate basal values significantly increased, and surprisingly, fish fed with diets containing red beet and betaine showed significantly higher values than fish fed with the control diet. This suggests that these ingredients did not enhance the tolerance of rainbow trout to a stress challenge even though all fish recover basal levels in the same way regardless of the diet. At 6 h, the inclusion of red beet and betaine on diets had a significant effect on rainbow trout plasma glucose level recovery. Fish fed with diets containing red beet and betaine presented significantly higher glucose levels than fish fed with the control diet. Only fish fed the highest betaine and red beet concentration (diets RB142, RB281, and RB282) returned to basal values by 12 h, while fish fed with diets with 14% red beet and 0.9% betaine (diets RB141) maintained plasma glucose levels higher than the basal ones even after 12 h. Overall, as it occurred with plasma cortisol values, higher values were recorded in fish fed diets containing red beet in comparison with those fed with the control diet.

The response dynamic of cortisol, glucose, and lactate levels as stress markers was as expected and similar to the results reported by several authors (Aluru and Vijayan 2006; Fast et al. 2008; Ming et al. 2012; Gesto et al. 2013, 2015). However, for all parameters, it could be observed that red beet and betaine did not

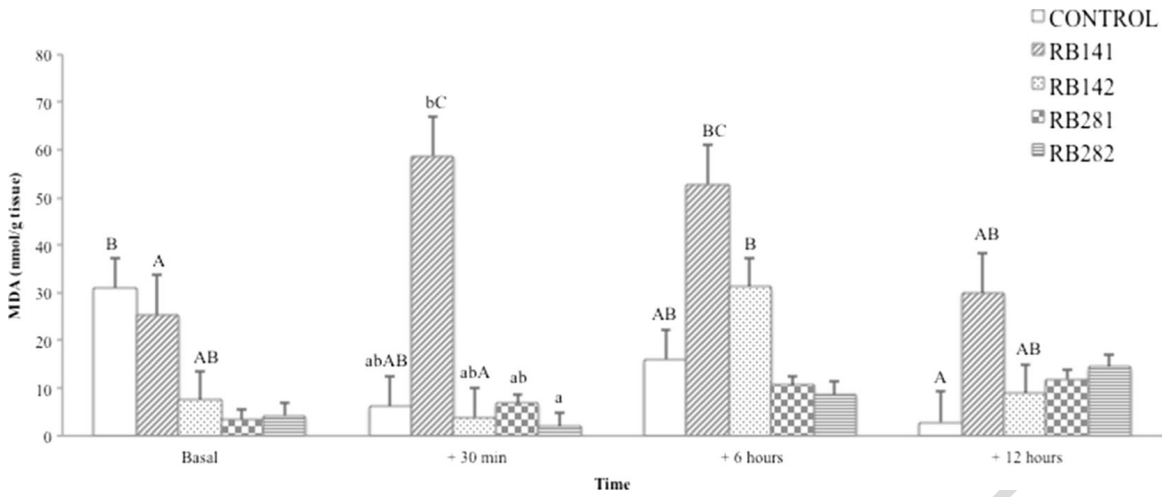


Fig. 3 Effect of red beet and betaine on MDA (nmol per g of tissue) of rainbow trout before the acute stress challenge (basal), 30 min after stress and 6 and 12 h after stress. Data were expressed as least-square means ± SEM, $n = 12$. Different capital letters above the bars indicate significant differences ($p < 0.05$) at different time points of the same group and different small letters above the bars indicate significant differences ($p < 0.05$) between different experimental diets in the same time point. CONTROL (0% red beet, 0% betaine), RB141 (14% red beet, 0.9% betaine), RB142 (14% red beet, 1.63% betaine), RB281 (28% red beet, 0.9% betaine), and RB282 (28% red beet, 1.63% betaine) are the different experimental diets

have an enhancing effect, although the recovery had a similar trend for every stress index.

Regarding lipid peroxidation levels, expressed as MDA, results showed that the inclusion of red beet and betaine did not significantly affect before the stress challenge. After the stress (30 min), red beet and betaine significantly affect MDA values. The stress had a significant effect on the control group and on fish fed with 14% RB, but all groups recovered basal values 12 h after the stress challenge. Oxidative stress is common under stressful conditions (Pérez-Jiménez et al. 2012). This oxidative stress produced free radicals that may attack polyunsaturated fatty acid producing lipid peroxidation (Chagas and Val 2006; Ming et al. 2012) and several studies reported an increase of lipid peroxidation under hypoxia (Lushchak et al. 2005; Pérez-Jiménez et al. 2012). Interestingly and in accordance with the current results, Leveelahti et al. (2014) in a study on three fish species (the epaulet shark, threespine stickleback, and rainbow trout) exposed to hypoxia reported that in general, fish do not show an increase in redox-active antioxidant defense in response to oxidative stress associated with hypoxia. Rather, the changes in antioxidant defenses during hypoxia are very much specie- and tissue-specific and are not linked to the level of hypoxia tolerance of the fish species. It is well known that the response of MDA is very tissue-specific (Lushchak and Bagnyukova 2006) and depends on the type of stress.

Conclusions

In conclusion, results show that the inclusion of red beet and betaine on rainbow trout diets followed the normal pattern of any stress; an increase of cortisol, glucose, and lactate levels after the acute stress challenge followed by a decrease on these values after a recovery period. However, the inclusion of red beet and betaine did not enhance the tolerance to the acute stress challenge because no differences were observed compared to the control group. Soon after the stress challenge, MDA values showed significantly lower values on fish fed with 28% RB and 1.69% betaine than fish fed with 14% RB and 0.9% betaine. Also, high RB concentrations (28%) avoid the effect of stress on MDA after the stress challenge (30 min), while fish fed with the control diet and lower RB concentrations (14%) suffered the effect of the stress. Although the level of ingredients is important, it could be also be added that this effect suggests a possible antioxidant effect of red beet and betaine but further studies should be done to confirm this effect of the ingredient.

Acknowledgements This work has been co-funded with FEDER and INIA funds. Julia Pinedo has been granted with the FPI-INIA grant number 21 (call 2012, BOE-2012-13337).

Compliance with ethical standards

Ethical statement The rainbow trout *Oncorhynchus mykiss* (Walbaum) study complied with both European Union Council

Directive 2010/63/UE, which lays down minimum standards for the protection of animals, and Spanish national legislation (Spanish Royal Decree 53/2013) protecting animals used in experimentation and for other scientific purposes and approved by Animal Ethics Committee of Agro-Technological Institute of Castilla y León (Spain).

References

Aluru N, Vijayan MM (2006) Aryl hydrocarbon receptor activation impairs cortisol response to stress in rainbow trout by disrupting the rate limiting steps in steroidogenesis. Endocrinology 147:1895–1903

Ashley PJ (2007) Fish welfare: current issues in aquaculture. Appl Anim Behav Sci 104:199–235

Barton BA, Iwama GK (1991) Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annu Rev Fish Dis 1:3–26

Bertotto D, Poltronieri C, Negrato E, Majolini D, Radaelli G, Simontacchi C (2010) Alternative matrices for cortisol measurement in fish. Aquac Res 41:1261–1267

Bertotto D, Poltronieri C, Negrato E, Richard J, Pascoli F, Simontacchi C, Radaelli G (2011) Whole body cortisol and expression of HSP70, IGF-I and MSTN in early development of sea bass subjected to heat shok. Gen Comp Endocrinol 174:44–50

Chagas EC, Val AL (2006) Ascorbic acid reduces the effects of hypoxia on the Amazon fish tambaqui. J Fish Biol 69:608–612

Cui XJ, Zhou QC, Liang HO, Yang J, Zhao LM (2010) Effects of dietary carbohydrate sources on the growth performance and hepatic carbohydrate metabolic enzyme activities of juvenile cobia (*Rachycentron canadum* Linnaeus.) Aquac Res 42:99–107

Dabrowski K, Lee KJ, Guz L, Verlhac V, Gabaudan J (2004) Effects of dietary ascorbic acid on oxygen stress (hypoxia or hyperoxia), growth and tissue vitamin concentration in juvenile rainbow trout (*Oncorhynchus mykiss*). Aquaculture 233:383–392

Enes P, Panserat S, Kaushik S, Oliva-Teles A (2006) Rapid metabolic adaptation of European sea beass (*Dicentrarchus labrax*) juveniles fed different carbohydrate sources after heat shock stress. Comp Biochem Physiol A 145:73–81

Fast MD, Hosoya S, Johnson SC, Alfonso LOB (2008) Cortisol response and immune-related effects of Atlantic salmon (*Salmo salar* Linnaeus) subjected to short- and long-term stress. Fish Shellfish Immunol 24:194–204

Francis G, Makkar HPS, Becker K (2001) Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture 199:197–227

Ganessan B, Anandan R, Lakshmanan PT (2011) Studies on the protective effects of betaine against oxidative damage during experimentally induced restraint stress in Wistar albino rats. Cell Stress Chaperones 16:641–652

Gesto M, López-Patiño MA, Hernández J, Soengas JL, Míguez JM (2013) The response of brain serotonergic and dopaminergic systems to an acute stressor in rainbow trout: a time course study. J Exp Biol 216:4435–4442

Gesto M, López-Patiño MA, Hernández J, Soengas JL, Míguez JM (2015) Gradation of the stress response in rainbow trout exposed to stressors of different severity: the role of brain serotonergic and dopaminergic systems. J Neuroendocrinol 27:131–141

Hemre GI, Mommsen TP, Krogdahl Å (2002) Carbohydrates in fish nutrition: effects on growth, glucose metabolism and heptic enzymes. Aquac Nutr 8:175–194

Ings JS, Vijayan MM, Servos MR (2012) Tissue-specific metabolic changes in response to an acute handling disturbance in juvenile rainbow trout exposed to municipal wastewater effluent. Aquat Toxicol 108:53–59

Janssens PA, Waterman J (1988) Hormonal regulation of gluconeogenesis and glycogenolysis in carp (*Cyprinus carpio*) liver pieces cultured in vitro. Comp Biochem Physiol 91A:451–457

Jeney G, Galeotti M, Volpatti D, Anderson DP (1997) Prevention of stress in rainbow trout (*Oncorhynchus mykiss*) fed diets containing different doses of glucan. Aquaculture 154:1–15

Kaplan LA, Pesce AJ (1984) Clinical chemistry: theory, analysis and correlation. Mosby, St. Louis, pp 1032–1036

Krogdahl Å, Sundby A, Olli JJ (2004) Atlantic salmon (*Salmon salar*) and rainbow trout (*Oncorhynchus mykiss*) digest and metabolize nutrients differently. Effects of water salinity and dietary starch levels. Aquaculture 229:335–360

Kujala TS, Vienola MS, Klika KD, Loponen JM, Pihlaja K (2002) Betalain and phenolic composition of four beetroot (*Beta vulgaris*) cultivars. Eur Food Res Technol 214:505–510

Kumar N, Jadhao SB, Chandan NK, Kumar K, Jha AK, Bhushan S, Kumar S, Rana RS (2012) Dietary choline, betaine and lecithin mitigate endosulfan-induced stress in *Labeo rohita* fingerlings. Fish Physiol Biochem 38:989–1000

Leveelahti L, Rytkönen KT, Renshaw GMC, Nikinmaa M (2014) Revisiting redox-active antioxidant defences in response to hypoxic challenge in both hypoxia-tolerant and hypoxia-sensitive fish species. Fish Physiol Biochem 40:183–191

Lushchak VI, Bagnyukova TV, Lushchak OV, Storey JM, Storey KB (2005) Hypoxia and recovery perturb free radical processes and antioxidant potential in common carp (*Cyprinus carpio*) tissues. Int J Bichem Cell Biol 37:1319–1330

Lushchak VI, Bagnyukova TV (2006) Temperature increase results in oxidative stress in goldfish tissues. 1. Indices of oxidative stress. Comp Biochem Physiol C 143:30–35

Madaro A, Olsen RE, Kristiansen TS, Ebbeson LOE, Nilsen TO, Flik G, Gorissen M (2015) Stress in Atlantic salmon: response to unpredictable chronic stress. J Exp Biol 218:2538–2550

Ming J, Xie J, Xu P, Ge X, Liu W, Ye J (2012) Effects of emodin and vitamin C on growth performance, biochemical parameters and two HSP70s mRNA expression of Wuchang bream (*Megalobrama amblycephala* Yih) under high temperature stress. Fish Shellfish Immunol 32:651–661

Mommsen TP, Vijayan MM, Moon TW (1999) Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev Fish Biol Fish 9:211–268

Montero D, Tort L, Robaina L, Vergara JM, Izquierdo MS (2001) Low vitamin E in diet reduces stress resistance of gilthead seabream (*Sparus aurta*) juveniles. Fish Shellfish Immunol 11:473–490

Ortuño J, Esteban MA, Meseguer J (2003) Effect of dietary intake of vitamins C and E on the stress response of gilthead seabream (*Sparus aurata* L.) Fish Shellfish Immunol 14: 145–156

Øverli Ø, Sørensen C, Kiessling A, Pottinger TG, Gjøen HM (2006) Selection for improved stress tolerance in rainbow trout (Oncorhynchus Mykiss) leads to reduced feed waste. Aquaculture 261:776–781

Pérez-Jiménez A, Peres H, Rubio VC, Oliva-Teles A (2012) The effect of hypoxia on intermediary metabolism and oxidative status in gilthead sea bream (*Sparus aurata*) fed on diets supplemented with methionine and white tea. Comp Biochem Physiol C 155:506–516

Pichavant K, Maxime V, Thébault MT, Ollivier H, Garnier JP, Bousquet B, Diouris M, Boeuf G, Nonnotte G (2002) Effects of hypoxia and subsequent recovery on turbot (*Scophthalmus maximus*): hormonal changes and anaerobic metabolism. Mar Ecol Prog Ser 225:275–285

Pinedo-Gil J, Tomás-Vidal A, Larrán-García AM, Tomás-Almenar C, Jover-Cerdá M, Sanz-Calvo MA, Martín-Diana AB (2016) Enhancement of quality of rainbow trout (*Oncorhynchus mykiss*) flesh incorporating barley on diet without negative effect on rearing paramters. Aquacult Int 25:1005–1023. https://doi.org/10.1007/s10499-016-0091-0

Pinedo-Gil J, Tomás-Vidal A, Jover-Cerdá M, Tomás-Almenar C, Sanz-Calvo MA, Martín-Diana AB (2017) Red beet and betaine as ingredients in diets of rainbow trout (*Oncorhynchus mykiss*): effects on growth performance, nutrient retention and flesh quality. Arch Anim Nutr 71:486–505. https://doi.org/10.1080/1745039X.2017.1391503

Rabeh NM (2015) Effect of red beetroot (*Beta vulgaris* L.) and its fresh juice against carbon tetrachloride induced hepatotoxicity in rats. World Appl Sci J 33(6):931–938

Rollo A, Sulpizio R, Nardi M, Silvi S, Orpianesi C, Caggiano M, Cresci A, Carnevalli O (2006) Live microbial feed supplement in aquaculture for improvement of stress tolerance. Fish Physiol Biochem 32:167–177

Sadoul B, Leguen I, Colson V, Friggens NC, Prunet P (2015) A multivariate analysis using physiology and behaviour to characterize robustness in two isogenic lines of rainbow trout exposed to a confinement stress. Physiol Behav 140:139–147

Tan Q, Xie S, Zhu X, Lei W, Yang Y (2006) Effect of dietary carbohydrates sources on growth performance and utilization for gibel carp (*Carassius auratus*) and Chinese longsnout catfish (*Leiocassis Longirostris* Günther). Aquac Nutr 12: 61–70

Tintos A, Míguez JM, Mancera JM, Soengas JL (2006) Development of a microtitre plate indirect ELISA for measuring cortisol in teleosts, and evaluation of stress responses in rainbow trout and gilthead sea bream. J Fish Biol 68:251–263

Van Anholt RD, Spanings FAT, Koven WM, Nixon O, Wendelaar Bonga SE (2004) Arachidonic acid reduces the stress response of gilthead seabream, *Sparus aurata* L. J Exp Biol 207:3419–3430

Virtanen E (1995) Piecing together the betaine puzzle. Feed Min 3: 12–17

Wu XY, Liu YJ, TIan LX, Mai KS, Yang HJ (2007) Utilization of several different carbohydrate sources by juvenile yellowfin seabream (*Sparus latus*). J Fish China 31(4):463–471

Yoshida Y, Itoh N, Hayakawa M, Piga R, Cynshi O, Jishage K, Niki E (2005) Lipid peroxidation induced by carbon tetrachloride and its inhibition by antioxidant as evaluated by an oxidative stress marker, HODE. Toxicol Appl Pharmacol 208:87–97

Zeng L, Wang YH, Ai CX, Zheng JL, Wu CW, Cai R (2016) Effects of β-glucan on ROS production and energy metabolism in yellow croaker (*Pseudosciaena crocea*) under acute hypoxic stress. Fish Physiol Biochem 42:1395–1405

Zolderdo AJ, Algera DA, Lawrence MJ, Gilmour KM, Fast MD, Thuswaldner J, Willmore WG, Cooke SJ (2016) Stress, nutrition and parental care in a teleost fish: exploring mechanisms with supplemental feeding and cortisol manipulation. J Exp Biol 219:1237–1248

AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES.

Q1. Figures 1-3 contains poor quality of text in image. Otherwise, please provide replacement figure file.
Q2. Statements related to ethics/ethical standards must be presented in the back matter. Thus, the relevant text was copied and captured under "Compliance with...."