doi: 10.17221/8583-VETMED

Assessment of oxidative stress in Flathead mullet (*Mugil cephalus*) and Gilthead sea bream (*Sparus aurata*)

F. Fazio[1], G. Piccione[1], C. Saoca[1], A.R. Caputo[2], S. Cecchini[3]

[1]Department of Veterinary Science, University of Messina, Messina, Italy
[2]Council for Research and Experimentation in Agriculture, Research Unit for Livestock Extensive, Potenza. Italy
[3]Department of Sciences, University of Basilicata, Potenza, Italy

ABSTRACT: In this work we compared two species of fish with different feeding habits: Flathead mullet (*Mugil cephalus*) and Gilthead sea bream (*Sparus aurata*). The aim of this study was to evaluate total oxidant status (TOS), total antioxidant capacity (TAC) and TOS/TAC ratio (OSI), in order to highlight the presence of any differences and correlations in these two different species of fish. Thirty adult fish of *Mugil cephalus* and thirty of *Sparus aurata* were used. From each fish 0.6 ml of blood was collected. TOS and TAC indicators were measured in serum obtained from samples previously clotted and centrifuged. Our results showed statistically significant differences between the two species in TAC. TOS and OSI did not show significant differences between Gilthead sea bream and Flathead mullet. A positive relationship between TOS and TAC was found in Flathead mullet (*Mugil cephalus*), and a negative relation between TOS and TAC in Gilthead sea bream (*Sparus aurata*). Our study indicates that the oxidative status and the relationship between total oxidant status (TOS) and total antioxidant capacity (TAC) in serum are probably dependent on the fish species and are affected by different feeding habits.

Keywords: total oxidant status; total antioxidant capacity; teleost species; redox balance

List of abbreviations

FRAP = ferric reducing antioxidant potential, **ROM** = reactive oxygen metabolites, **ROS** = reactive oxygen species, **TAC** = total antioxidant capacity, **TOS** = total oxidant status, **OSI** = oxidative stress index (TOS/TAC ratio)

Oxidative stress, an unavoidable aspect of aerobic life, is the result of an imbalance between pro-oxidants and antioxidants (Nishida 2011). Pro-oxidants are chemical complexes that induce oxidative stress through the production of free radicals, including reactive oxygen species (ROS), or through inhibition of antioxidant systems. Mitochondrial respiration is the main endogenous source of ROS. The mechanisms by which free radicals interfere with cellular function are not understood, but elevated production of ROS can cause oxidation and damage to biological macromolecules such as membrane lipids, proteins and DNA and result in changes in cell redox status (Livingstone 2003). This cellular damage causes a shift in the net charge of the cell and changes the osmotic pressure, which leads to swelling and eventually cell death (Nijveldt et al. 2001). ROS also can play a beneficial role in cells by contributing to pathways of intracellular signalling and redox regulation (Grim et al. 2013). Their damage to the biological components is balanced by the activities of many cellular defence mechanisms (Stohs et al. 2000). The cellular antioxidant defence system is one of the important biochemical strategies protect cells against the deleterious effects of endogenous ROS by keeping their levels relatively low (Paital and Chainy 2010). Mechanisms of antioxidant defences in fish include the enzyme system

Supported partially by the University of Messina, Italy (Grant No. PON02 00451 3362185 INNOVAQUA – Technological innovation to support increased productivity and competitiveness Aquaculture Sicili).

doi: 10.17221/8583-VETMED

and low molecular weight antioxidants, similar to those in mammals, although the specific isoforms of enzymes in various fish species have not been well identified (Di Giulio and Meyer 2008). In fish, changes in antioxidant defence enzyme activities can be influenced both by intrinsic factors (age, feeding behaviour, food consumption) and by extrinsic factors, such as toxins present in the water, seasonal and daily changes in dissolved oxygen and water temperature (Bayir et al. 2011). In their natural habitats, fish often have periods of poor food supply as a result of lower environmental temperature, spawning, migration and reproduction (Furne et al. 2009), and changes of these variables are accompanied with seasonal fluctuations. Therefore, assays of antioxidant defence and oxidative damage parameters are used as biomarkers of oxidative stress.

In mammals, oxidant and antioxidant enzymes in blood showed a positive correlation (Po et al. 2013; Ranade et al. 2014). In fish, some authors showed that oxidation products and antioxidant defences were correlated in the muscle of several estuarine fish species (Madeira et al. 2013). Particularly, a correlation between ROS and FRAP (ferric reducing antioxidant potential) in different tissues (liver, muscle, digestive and gill) has been shown in *Sparus aurata* (Sanchez-Muros et al. 2013). Other researchers (Fazio et al. 2014) have shown a positive relationship between total oxidant status (TOS) and total antioxidant capacity (TAC) in serum from Flathead mullet analysed after different periods of storage (2, 24, 48 and 72 h) at 4 °C. *Sparus aurata* and *Mugil cephalus* are two species that differ from each other with respect to their habitat and feeding habits. *S. aurata* is a carnivorous fish and its diet consists of a wide variety of organisms, preferentially gastropods and bivalves (Pita et al. 2002). The habitat of *M. cephalus*, which is an omnivorous fish, is pelagic, usually inshore, in estuaries and lagoons. While juveniles feed on invertebrates, adults feed mostly on detritus, bottom algae and small organisms, occasionally on plankton. Metabolic activity is directly related with ROS production and dietary habits. These different habitats and feeding habits could influence the oxidative stress status and the relationship between oxidant and antioxidant serum enzymes.

The aim of this study was to assess oxidative stress and the possible correlation (Pearson, Spearman *r*) between TOS and TAC in the serum of two commercially important fish species (*S. aurata* and *M. cephalus*).

MATERIAL AND METHODS

All experimental procedures were approved by the Animal Ethics Committee of Messina University (Decree n. 39 of 19/03/2005) and were carried out in accordance with European legislation regarding the protection of animals used for experimental and other scientific purposes (Council Directive 2010/63/EU, as amended).

Thirty Flathead mullet (*M. cephalus*) (average weight 300.00 ± 27.14 g and length 30.00 ± 2.39 cm) were caught from Ganzirri Lake (Sicily, Italy). Thirty Gilthead sea bream (*S. aurata*) (average weight 295.00 ± 28.36 g and length 29.00 ± 2.72 cm) were obtained from farmed stock. All fish were considered healthy on the basis of an external examination for any signs of abnormalities or infestation. Fish were acclimated before sampling for three weeks in 800-l tanks with flowing seawater (temperature: 18 °C, salinity: 39 ppm and pH 7.5) to restore the effects of capture, handling and transport. The work was performed during May and June 2013. Blood samples were collected between 08:00 h and 12:00 h and feeding was stopped 24 h before blood collection. Flathead mullet and gilthead sea bream were quickly dip-netted from the tanks and immediately anaesthetised with 2-phenoxyethanol (1 : 300 v/v) in a 60-l bucket, before submitting them to blood collection from the caudal vein using a 2.5 ml syringe. To obtain the serum from each fish, 0.6 ml of blood were collected and the samples, stored in Eppendorf tubes with no additive, were left to clot. Each blood sample was centrifuged for 10 min at 3000 × *g* using a refrigerated centrifuge at 4 °C (Beckman Coulter, TJ25) to obtain the serum. Serum was collected and stored at –80 °C until analysis of TOS and TAC parameters. TOS, measured as ROMs (reactive oxygen metabolites), was evaluated using the radical cation *N,N'*-diethyl-para-phenylendiamine (DEPPD), as described by Alberti et al. (2000) with some modifications. Ten µl of samples in duplicate were added to wells of a microtitre plate. Subsequently, 200 µl of a solution containing 0.37mM DEPPD and 2.8mM iron (II) sulfate heptahydrate in 100mM acetate buffer, pH 4.8, were added to each well. After incubation (30 min at 25 °C) absorbance was recorded at 530 nm using a microplate reader (Model 550, BioRad). A standard curve was constructed using *tert*-butyl hydroperoxide (*t*-BHP) at concentrations ranging from 125 to 1000µM (Pearson's correlation

doi: 10.17221/8583-VETMED

coefficient: r = 0.99) without units of expression. TAC of plasma was evaluated using the FRAP assay as indicated by Benzie and Strain (1996). Firstly, 300mM sodium acetate buffer, pH 3.6, 10mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) in 40mM HCl and 20mM iron (III) chloride hexahydrate were mixed in a volume ratio of 10 : 1 : 1 to generate fresh FRAP solution. Subsequently, 10 µl of samples in duplicate were added to 300 µl of FRAP solution in wells of a microtitre plate and the absorbance of the reaction mixture was recorded at 593 nm after 5 min of reaction using a microplate reader. A standard curve was constructed using iron (II) sulphate 7·H_2O at concentrations ranging from 62.5 to 1000µM (Pearson's correlation coefficient: r = 1) without units of expression. Moreover, the TOS/TAC ratio was calculated as OSI (oxidative stress index), an indicator of redox balance. OSI indicates the degree of oxidative stress, and it is calculated as follows: OSI (arbitrary units) = TOS/TAC. Results were expressed as means ± standard error. A one-sample Kolmogorov-Smirnov test was used to determine if the data were normally distributed. Differences in TOS and TAC between *M. cephalus* and *S. aurata* were statistically analysed using Student's *t*-test. Relationships between variables (TOS and TAC) were determined using the Spearman correlation analysis. *P*-values less than 0.05 were considered statistically significant. All data were analysed using the statistical package PRISM 5.

RESULTS

Table 1 shows the values of TOS and TAC, measured as ROMs and FRAP, respectively, and OSI, together with statistical differences in Flathead mullet (*M. cephalus*) and Gilthead sea bream (*S. aurata*). Student's *t*-test unpaired data showed a statistically significant difference between the two species in the TAC (P < 0.04). TOS and OSI did not show any significant differences between the two species.

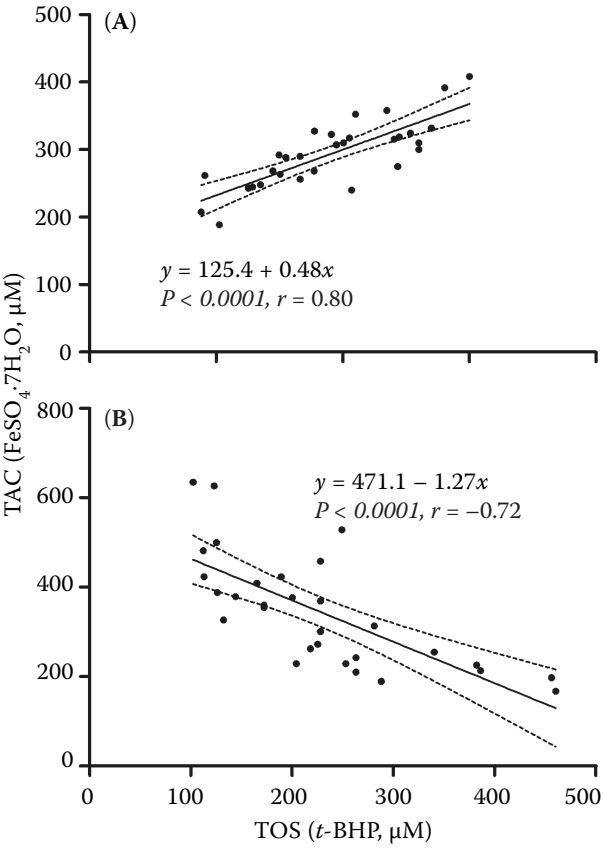

Figure 1. A positive correlation between TOS and TAC (r = 0.80, P < 0.0001) in Flathead mullet (*M. cephalus*) (**A**) and a negative correlation (r = –0.72, P < 0.0001) in Gilthead sea bream (*S. aurata*) (**B**)

Simple regression analysis showed a positive relationship between TOS and TAC in Flathead mullet (*M. cephalus*) whereas in Gilthead sea bream (*S. aurata*) it showed a negative relation between TOS and TAC (Figure 1A, B). The regression lines using TAC as outcome variable (y) and TOS as predictor variable (x) are shown in Figure 1A, B.

Table 1. Mean values ± standard error of oxidative stress biomarkers (TOS, TAC and OSI) in Flathead mullet (*Mugil cephalus*) and Gilthead sea bream (*Sparus aurata)*

Species	Oxidative stress biomarkers		
	TOS	TAC	OSI
Flathead mullet	191.83 ± 10.64[a]	294.50 ± 9.00[a]	0.65 ± 0.02[a]
Gilthead sea bream	227.60 ± 17.97[b]	345.10 ± 23.04[a]	0.84 ± 0.12[a]

TOS = total oxidant status (t-BHP, mM); TAC = total antioxidant capacity ($FeSO_4$·7 H_2O, µM); OSI = TOS/TAC ratio
Means with different letters in the same column are statistically different (*t*-test; P < 0.05)

doi: 10.17221/8583-VETMED

DISCUSSION

In Flathead mullet, the values of TOS, TAC and OSI do not differ from our previous results (Fazio et al. 2014). No studies were found with which to compare our data in Gilthead sea bream. Measurement of the TOS and TAC biomarkers revealed that the values are species-dependent. The major difference was found for TAC in Gilthead sea bream where a significantly higher level (345.10) with respect to Flathead mullet (294.50) was observed, an increase of about 15%. Differences in feeding habits could influence oxidative stress status in fish. In fact, lipid peroxidation tends to be lower in herbivorous fish than in omnivorous species, correlating with lower glutathione peroxidase and catalase activities, although the herbivorous species have a high superoxide dismutase activity. Carnivorous species, compared with herbivorous and omnivorous species, have very low glutathione peroxidase activity in the liver mitochondria, the highest catalase activity in the liver and kidney, and highest superoxide dismutase activity in the liver. These differences between carnivorous and herbivorous fish could influence the enzymatic antioxidant system in their serum. Our results showed a significant increase ($P < 0.0001$) of TAC in Gilthead sea bream compared to Flathead mullet. Differences in feeding habits influence antioxidant defence and oxidative status in fish (Martinez-Alvarez 2005). Some authors have reported that antioxidant enzyme activity was higher in the livers of fish fed diets with a high lipid level. Higher oxidation rates were observed in fish fed a diet containing raw carbohydrate (Rueda-Jasso et al. 2004). When comparing three species of fish with different feeding habits (herbivorous, omnivorous and carnivorous), similar levels of lipid peroxidation were found (Martinez-Alvarez 2005). In accordance with this research, our results showed no statistically significant differences in total oxidant status (TOS) in Gilthead sea bream and Flathead mullet. There was a significant positive correlation (Figure 1B) between TOS and TAC levels in Flathead mullet, and a negative correlation in Gilthead sea bream (Figure 1A). In Flathead mullet, our previous research (Fazio et al. 2014) showed a positive relationship between TOS and TAC following different storage times of serum. Other authors (Sanchez-Muros et al. 2013) showed a correlation between ROS and FRAP in different tissues (liver, muscle, digestive and gill) in unstressed *S. aurata*; this positive trend disappears in the stressed fish and may even become a negative trend in the organs of the fish. Correlative relationships between pro-oxidative parameters and antioxidant enzyme activities also conform to what is anticipated if the peroxidation process is subsequent to the exhaustion of the antioxidant defence system. From available data, it seems that the oxidative status and the relationship between TOS and TAC in serum are dependent on the fish species, and are affected by different feeding habits. Nutrition, including its characteristics, type and quality, and ratio of various nutrients, is one of the most significant aetiological factors for oxidative stress. Some factors intrinsic to the fish itself, such as phylogeny and feeding habits, together with environmental factors, play an important role in oxidative status. Studies on oxidative stress in fish should lead to a better understanding of fish physiology. Further studies are necessary to better understand the relationship between oxidative status and feeding habits and behaviour in different fish species.

REFERENCES

Alberti A, Bolognini L, Macciantelli D, Caratelli A (2000): The radical cation of N,N diethyl-para-phenylendiamine: a possible indicator of oxidative stress in biological samples. Research on Chemical Intermediates 26, 253–267.

Bayir A, Necdet Sirkecioglu A, Bayir M, Ibrahim Haliloglu H, Mahmut Kocaman E, Mevlut Aras N (2011): Metabolic responses to prolonged starvation, food restriction and refeeding in the brown trout, Salmo trutta: Oxidative stress and antioxidant defenses. Comparative Biochemistry and Physiology Part B 159, 191–196.

Benzie IFF, Strain JJ (1996): The ferric reducing ability of plasma (FRAP) as measure of "antioxidant power": the FRAP assay. Analytical Biochemistry 239, 70–76.

Di Giulio RT, Meyer JN (2008): Reactive oxygen species and oxidative stress. In: Di Giulio RT, Hinton DE (eds.): The Toxicology of Fishes. CRC Press, Taylor and Francis Group, New York. 273–324.

Fazio F, Cecchini S, Faggio C, Caputo AR, Piccione G (2014): Stability of oxidative stress biomarkers in flathead mullet, Mugil cephalus, serum during short-term storage. Ecological Indicators 46, 188–142.

Furne M, Garcia-Gallego M, Hidalgo MC, Morales AE, Domezain A, Domezain J, Sanz A (2009): Oxidative stress parameters during starvation and refeeding periods in Adri-

doi: 10.17221/8583-VETMED

atic sturgeon (Acipenser naccarii) and rainbow trout (Oncorhynchus mykiss). Aquaculture Nutrition 15, 587–595.

Grim JM, Simonik EA, Semones MC, Kuhn DE, Crockett EL (2013): The glutathione dependent system of antioxidant defence is not modulated by temperature acclimation in muscle tissues from striped bass, Morone saxatilis. Comparative Biochemistry and Physiolology, Part A 164, 383–390.

Livingstone DR (2003): Oxidative stress in aquatic organism in relation to pollution and agriculture. Revue de Medecine Veterinaire 154, 427-430.

Madeira D, Narciso L, Cabral HN, Vinagre C, Diniz MS (2013): Influence of temperature in thermal and oxidative stress responses in estuarine fish. Comparative Biochemistry and Physiology, Part A 166, 237–243.

Martinez-Alvarez RM, Morales AE, Sanz A (2005): Antioxidant defences in fish: biotic and abiotic factors. Reviews in Fish Biology and Fisheries 15, 75–88.

Nijveldt RJ, van Nood E, van Hoorn DEC, Boelens PG, van Norren K, van Leeuwen PAM (2001): Flavonoids: a review of probable mechanisms of action and potential applications. American Journal of Clinical Nutrition 74, 418–425.

Nishida Y (2011): The chemical process of oxidative stress by copper (II) and iron (III) ions in several neurodegenerative disorders. Monatshefte fur Chemie 142, 375–384.

Paital B, Chainy GB (2010): Antioxidant defenses and oxidative stress parameters in tissues of mud crab (Scylla serrata) with reference to changing salinity. Comparative Biochemistry and Physiology, Part C 151, 142–151.

Pita C, Gamito S, Erzini K (2002): Feeding habits of the gilthead seabream (Sparus aurata) from the Ria Formosa (southern Portugal) as compared to the black seabream (Spondyliosoma cantharus) and the annular seabream (Diplodus annularis). Journal of Applied Ichthyology 18, 81–86.

Po E, Williams C, Muscatello G, Celi P (2013): Assessment of oxidative stress biomarkers in exhaled breath condensate and blood of Thoroughbred foals. Veterinary Journal 196, 269–271.

Ranade R, Talukder S, Muscatello G, Celi P (2014): Assessment of oxidative stress biomarkers in exhaled breath condensate and blood of dairy heifer calves from birth to weaning. Veterinary Journal 202, 583–587.

Rueda-Jasso R, Conceicao LEC, Dias J, De Coen W, Gomes E, Rees JF, Soares F, Dinis MT, Sorgeloos, P (2004): Effect of dietary non-protein energy levels on condition and oxidative status of Senegalese sole (Solea senegalensis) juveniles. Aquaculture 231, 417–433.

Sanchez-Muros MJ, Villacreces S, Miranda-de la Lama G, de Haro C, Garcia-Barroso F (2013): Effects of chemical and handling exposure on fatty acids, oxidative stress and morphological welfare indicators in gilthead sea bream (Sparus aurata). Fish Physiology and Biochemistry 39, 581–591.

Stohs SJ, Bagchi D, Hassoun E, Bagchi M (2000): Oxidative mechanisms in the toxicity of chromium and cadmium ions. Journal of Environmental Pathology, Toxicology and Oncology 19, 201–213.

Received: 2015–03–25
Accepted after corrections: 2015–10–28

Corresponding Author:

Francesco Fazio, University of Messina, Department of Veterinary Science, Polo Universitario dell'Annunziata, 98168 Messina, Italy

E-mail: ffazio@unime.it