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

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ABSTRACT: Flathead mullet (Mugil cephalus) and gilthead sea bream (Sparus aurata) are two fish species that have distinct eating patterns, and we compared them in this paper. In order to identify any differences or connections between these two fish species, this research evaluated total oxidant status (TOS), total antioxidant capacity (TAC), and TOS/TAC ratio (OSI). We utilized 30 adult Sparus aurata fish and 30 adult Mugil cephalus fish. A little amount of blood (0.6 ml) was drawn from every fish. Serum TOS and TAC markers were evaluated in centrifuged and clotted samples. We found that the two species differed significantly with respect to TAC. There were no discernible differences between Flathead mullet and Gilthead sea bream on TOS and OSI. Flathead mullet (Mugil cephalus) showed a positive correlation between TOS and TAC, but gilthead sea bream (Sparus aurata) showed a negative correlation. According to our research, various fish species and their eating patterns likely influence serum oxidative state and the link between TOS and TAC concentrations.

Keywords: species of teleosts, redox balance, total antioxidant status, and total antioxidant capacity

INTRODUCTION:

List of abbreviations

FRAP = ferric reducing antioxidant potential, **ROM** = reactive oxygen metabolites, **ROS** = reactive oxygen spe- cies, **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-oxi- dants 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 inhibi- tion of antioxidant systems. Mitochondrial respira- tion 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 cellulardamage causes a shift in the and low molecular weight antioxidants, similar to those in mammals, although the specific isoforms of enzymes in various fish species

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 signal- ling 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 strate- gies protect cells against the deleterious effects of endogenous ROS by keeping their levels relatively low (Paital and Chainy 2010). Mechanisms of anti- oxidant defences in fish include the enzyme system

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have not been well identified (Di Giulio and Meyer 2008). In fish, changes in antioxidant defence enzyme activities can be influenced

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both by intrinsic factors (age, feeding behaviour, food consumption) and by ex- trinsic 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 sup- ply as a result of lower environmental temperature, spawning, migration and reproduction (Furne et al. 2009), and changes of these variables are accompa- nied with seasonal fluctuations. Therefore, assays of antioxidant defence and oxidative damage param- eters 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 cor- relation between ROS and FRAP (ferric reducing antioxidant potential) in different tissues (liver, mus- cle, digestive and gill) has been shown in *Sparus au- rata* (Sanchez-Muros et al. 2013). Other researchers (Fazio et al. 2014) have shown a positive relationship between total oxidant status (TOS) and total antioxi- dant 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 cepha- lus* 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. cepha- lus*, 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 differ- ent 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 com- mercially 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*) (aver- age 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*) (av- erage 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 in- festation. 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 trans- port. 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 se- rum. Serum was collected and stored at –80 °C until analysis of TOS and TAC parameters. TOS, measured as ROMs (reactive oxygen

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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 solu- tion containing 0.37mM DEPPD and 2.8mM iron (II) sulfate heptahydrate in 100mM acetate buf- fer, pH 4.8, were added to each well. After incuba- tion (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 correlationcoefficient: 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 hexahy- drate 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 con- structed using iron (II) sulphate $7{\cdot}H_2O$ at concen- trations 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 in- dicator of redox balance. OSI indicates the degree of oxidative stress, and it is calculated as follows: OSI (arbitrary units) = TOS/TAC. Results were ex- pressed 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. au-*

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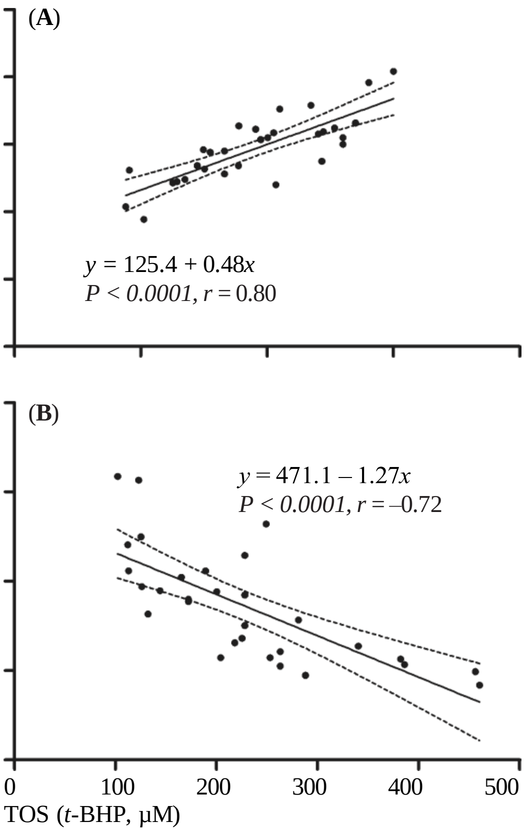

rata were statistically analysed using Student's *t*-test. Relationships between variables (TOS and TAC) were determined using the Spearman cor- relation analysis. *P*-values less than 0.05 were considered statistically significant. All data were analysed using the statistical package PRISM 5.

RESULTS

Table 1 shows the values of TOS and TAC, meas- ured as ROMs and FRAP, respectively, and OSI, to- gether 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.

Simple regression analysis showed a positive rela- tionship between TOS and TAC in Flathead mullet (*M. cephalus*) whereas in Gilthead sea bream (*S. au- rata*) 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.

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**)

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 ($FeSO_4 \cdot 7\ H_2O$, μM); OSI = TOS/TAC ratio Means with different letters in the same column are

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statistically different (t-test; $P < 0.05$)

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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 com- pare 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, li- pid peroxidation tends to be lower in herbivorous fish than in omnivorous species, correlating with lower glutathione peroxidase and catalase activi- ties, although the herbivorous species have a high superoxide dismutase activity. Carnivorous species, compared with herbivorous and omnivorous spe- cies, have very low glutathione peroxidase activ- ity in the liver mitochondria, the highest catalase activity in the liver and kidney, and highest super- oxide dismutase activity in the liver. These differ- ences 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 feed- ing habits influence antioxidant defence and oxida- tive 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 carbohy- drate (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 dif- ferences in total oxidant status (TOS) in Gilthead sea bream and Flathead mullet. There was a signifi- cant 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 disap- pears 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 oxida- tive 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 bet- ter understand the relationship between oxidative status and feeding habits and behaviour in different fish species.

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