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# Redox Balance Affects Fish Welfare

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## Abstract

Aquaculture is a growing industry that is increasingly providing a sizable proportion of fishery products for human consumption. Thus, in the last years, several efforts are made in improving fish welfare. As well as in the rest of vertebrates, fish welfare is sensible to a balanced redox status. Numerous inputs like diet and environmental factors could alter this balance. In this sense, the last feeding strategies are focused on developing a more sustainable aquaculture, trying to maintain a redox balance. On the other hand, under culture conditions, animals cannot migrate to more favourable conditions, and environmental stress is one of the most relevant inputs that could compromise redox balance. This chapter is focused on the review of last works in redox balance analysis in Mediterranean aquaculture species and is organized as follows: (1) redox reactions on poikilotherms versus homeotherms; (2) effect of feeding strategies and environmental stress in fish redox balance; and (3) wide vision in fish redox balance.

**Keywords:** antioxidant enzymes, glutathione redox cycle, lipoperoxidation, protein oxidation, Mediterranean aquaculture

## 1. Introduction

In the recent decades, the increase in demand of fish products has boosted the development of aquaculture, being today responsible of the major supply of fish for human consumption. Currently, aquaculture is growing faster than other major food production sectors. In 2016, 88%, more than 151 million tons (t) of the fish produced was destined for human consumption (FAO). Thus, the potential of oceans and inland waters could be fundamental for human nutrition in the coming years, due to the expected demographic increase.

Classically, the vast majority of aquaculture research have been focused on growth studies, feed efficiency, larval maturation or fillet quality. However, as in all intensive animal exploitation, fish culture presents different alterations and pathologies affecting fish welfare, causing great economic losses to the aquaculture sector. Rearing conditions in sea cages expose animals to several stressful inputs like crowding, feed competence, predators and environmental parameters (tides, salinity, temperature, etc.). These stressful conditions affect fish welfare, being more susceptible to infections or even triggering mortality [1, 2], causing important economic loss. To evaluate the individualized effect of each of the inputs and also to test new feeds, aquaculture research applies indoor models to evaluate the cellular and physiological responses. Therefore, a growing interest about fish welfare exists, being the oxidative stress and the fish redox status a new focus of research.

Like to the studies in mammals' species, the most of fish approaches on redox aquaculture subjects were focused in liver as the main physiological organ. Liver shows powerful enzymatic antioxidant machinery, it is involved on glutathione synthesis and it is a main target of reactive oxygen species (ROS). However, digestive tract, white and red muscle even plasma are of a novel targets for redox studies in fish.

Despite the bibliography reporting redox markers in the last years are increasing, there are only few works tackling on the redox balance and the consequences of its alteration in fish welfare. This chapter aims to evaluate the most relevant and recent studies, and the advances of oxidative status, antioxidant defences and global welfare in fish. Along the chapter we will be focused on the studies which report dietary effects and environmental challenges on fish culture.

## 2. Redox reactions on ectotherms versus endotherms

### 2.1 Definition of oxidative stress

Oxygen Free Radicals are highly reactive species which are known to be the major factor in oxidative cell injury via the oxidation and subsequent functional impairment of lipids, carbohydrates, proteins and DNA. In the 1950s, free radicals were first identified in biological systems and were proposed to be involved in pathological processes [3]. The major source of intracellular free radicals is mitochondria due to the presence of an electron transport chain [4, 5], which consumes 85–90% of the oxygen utilized by cell [4, 6]. While passing through the mitochondrial electron transport chain, up to 2% of the total oxygen consumed undergoes one-electron reduction to generate superoxide anion radicals ( $O_2^{\bullet-}$ ) and hydrogen peroxide ( $H_2O_2$ ). This hydrogen peroxide may lead to hydroxyl radical ( $OH^{\bullet}$ ), the most reactive free radical produced in biological systems, with the participation of transition metals in the Haber-Weiss reaction [7]. In addition, different stressors, particularly those induced by environmental physical and chemical factors were reported to increase levels of free radicals. As research on free radicals focused on oxygen radicals, with some other forms of non-radical active oxygen, they are collectively referred to as reactive oxygen species (ROS).

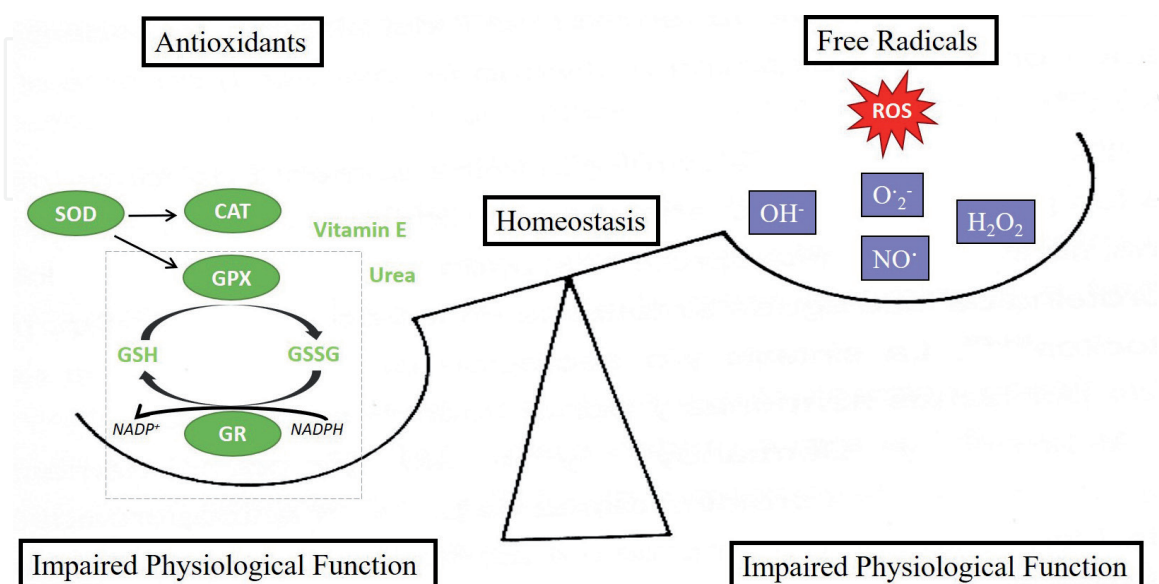
As the formation of reactive oxygen species (ROS) is a part of natural cellular oxidative metabolism, the question if living organisms possess regulated enzymatic systems to defend against ROS, suddenly arises. This was first confirmed by McCord and Fridovich [8] who discovered the enzyme superoxide dismutase (SOD) and demonstrated that living organisms have developed protective mechanisms against ROS. Over time, this was supported by continuing discoveries of several mechanisms by which ROS can be neutralized: antioxidant enzymes and low molecular mass antioxidants. During normal oxidative metabolism ROS are produced continually, but they are scavenged by superoxide dismutase (SOD), glutathione peroxidase and catalase [9]. Other small molecular antioxidants: glutathione, ascorbic acid and  $\alpha$ -tocopherol are also involved in the detoxification of free radicals. Those reported evidences lead to Helmut Sies [10] to first propose the following definition of oxidative stress as 'imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage'. Oxidative stress was considered to be harmful, while antioxidants provided defence and prevention of tissue damage. However, ROS were recently found to play signalling roles not only in ROS-related processes, but in many basic functions such as fertilization, growth, and differentiation [11–14].

## 2.2 Redox balance

We have described how reactive oxygen species (ROS) can be both harmful and beneficial. Consequently, under physiological conditions, the cellular redox equilibrium is tightly regulated on the one hand by pro-oxidants and on the other by enzymatic and non-enzymatic antioxidants (**Figure 1**). Due to the central role of ROS in many pathologies, restoring the redox balance forms an innovative target in the development of new strategies for treating several conditions. For example, Coenzyme-Q and its redox status -that was mostly found in the reduced form- have been proposed as an adaptation to different thermal environments in Antarctic fishes [15], and to reflect species-specific ecological habits and physiological constraint associated with oxygen demand represent an adaptation to environmental oxygen availability in coral reef fishes [16]. Recently, the determination of the redox balance of liver Coenzyme-Q from fish has been observed to have a potential, based on physiological principles, to be used as a practical biomarker for polycyclic aromatic hydrocarbon (PAH) contamination in aquatic biotopes [17].

The main marker of oxidative damage in fish tissues have been considered as lipid peroxidation (LPO, usually measured as ThioBarbituric Reactive Substances, TBARS) [18–20]. However, the application of mammals' techniques to measure the direct oxidation of the amino acid side chains, so-called the Advanced Oxidation Protein Products, (AOPPs) or/and the tissue accumulation of 4-hydroxynonenal (4-HNE) cross-linked proteins are novel approach in the study of oxidative insult in fish species [21].

To cope with the oxidative damage resulting from metabolism, animals use non-enzymatic defences, such as thiol groups and glutathione, and enzymes with antioxidant activity [1, 20, 22]. Thus, in fish as in mammals, antioxidant liver capacity has been classically measured via total glutathione, (tGSH) and its oxidized and reduced forms (GSH and GSSG, respectively), and via the main antioxidant enzyme activities: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GR).



**Figure 1.**  
*Homeostasis in redox balance.*

### 2.3 Comparative studies: ectotherms versus endotherms

The generic term 'fish' comprises an extremely diverse group of vertebrates, which represents 40% of the world's vertebrates. Fish are adapted to aquatic environment, showing a wide type of adaptations to different environmental conditions of temperature, salinity, oxygen level and water chemistry. To survive in such diverse environments, fish need high adaptive potential. If it is overwhelmed, organisms may enter stress conditions. The most studied environmental stress conditions in aquatic environments include changes in salinity, ion composition, and temperature and oxygen availability. Recently, they also included pollutants exposure due to human activity.

If the previous studies of comparative physiology were focused on the fish availability of O<sub>2</sub>, the generation of ROS and its harmful consequences are currently taken into account. In fishes, the main physiological source of ROS is also the mitochondria, and the mechanism of ROS production similar to that of mammal. However, the fraction of total mitochondrial electron flux that generate H<sub>2</sub>O<sub>2</sub> (the fractional electron leak), was far lower in rat than in all the ectothermic fishes assayed [23]. Results previously published concluded that mitochondria of true endotherms (such as birds and mammals) produce lower rates of ROS generation compared to fish and showed higher levels of antioxidant enzymes compared to fish [24].

Despite similarities in mitochondrial coupling mechanisms and proton leakage, differences in mitochondrial function between mammals and fish have been reported. A greater phospholipid unsaturation, the presence of cardiolipin and the absence of cholesterol in the mitochondrial membranes of fish can establish different aspects of mitochondrial functionality in fish. In fact, the presence of the phospholipid cardiolipine and the absence of cholesterol may confer a high structural flexibility in fish mitochondria. Moreover, differences in membrane phospholipid composition involving polyunsaturated fatty acids (PUFA) may play a role in the proton leak across the mitochondrial membranes (which can explain greater proton leak in endotherms when compared to ectotherms) and in the lipid peroxidation process. It has been previously demonstrated the correlation between the consumption of polyunsaturated fatty acid diet and the enhancement of endogenous lipid peroxidation, in rats [1, 25, 26]. As the increased unsaturation of fatty acids leads to increased parameters of oxidative stress damage, this increases the oxidative challenge to fish tissues. However, some important differences in the lipid composition of membranes have been reported for phylogenetic distinct marine fish species [27]. In the referred work, the elasmobranch *Raja erinacea* showed lower percentage of polyunsaturated fatty acids when compared to non-elasmobranch species. Fishes of the Antarctic seas showed relatively high proportion of polyunsaturated fatty acids in mitochondria [28], which make them prone to lipid peroxidation. Indeed, reported TBARS levels are much higher in fish liver than in mammals [24].

As reported above, all aerobic forms of life developed antioxidant defences. Both, low-molecular weight antioxidants and antioxidant enzymes like catalase, superoxide dismutase and glutathione-dependent enzymes, have been detected in different fish species [1]. Many studies confirm that enzymatic antioxidant activities in fish were lower than in endotherms. As fish refers to different evolved species, some studies have been performed to determine whether antioxidants correlate with phylogenetic position [29]. Marine fishes showed high levels of the antioxidant vitamin E [30] and elasmobranchs compensate limited antioxidant enzymes with high levels of glutathione and urea [31]. It seems that low molecular weight antioxidants appeared early in evolution to later develop enzymatic antioxidant systems.

### 3. Advances in redox balance of temperate fish species

#### 3.1 Redox balance in feeding strategies

Usually, the main aim of the diet trials in fish pursue to improve growth performance as well as food conversion and efficiency, guaranteeing a high-quality product, reducing fish mortality and with economic positive gains. Recently, feeding studies include redox balance markers as additional indicators of the fish physiological status. **Table 1** represents the most relevant feeding strategies in Mediterranean fish related to redox balance.

	Tissue	Diet strategy	Oxidants	Antioxidants
<b>Diet effect</b>				
<i>Dicentrarchus labrax</i>	Liver, intestine	Soy based diets and Taurine [40]	LPO	SOD, CAT
	Liver, intestine	Substitution diets and carbohydrates reduction [32]	LPO	SOD, CAT, GR, GPX, G6PDH, GSH and GSSG
<i>Sparus aurata</i>	Liver	Supplemented diet Met and Tea [41]	LPO	SOD, CAT, GR, GPX, G6PDH, GSH and GSSG
	Mucus, gut, skin	Palm fruit extract and probiotics <sup>+</sup> [43]	LPO, H <sub>2</sub> O <sub>2</sub>	SOD, CAT, GR
	Intestine	Sprayed diet porcine protein [42]	TBARS	CAT, GR, GST, GPX
	Liver, intestine	Soybean and wheat with arginine [39]	LPO	SOD, CAT, GR, GPX, G6PDH
	Liver, intestine	Soybean and wheat with glutamine [38]	LPO	SOD, CAT, GR, GPX, G6PDH
	Liver, intestine	Substitution diets and carbohydrates reduction [34]	LPO	SOD, CAT, GR, GPX, G6PDH, GSH and GSSG
	Liver	Soybean replacement [36]		SOD, CAT, GR, GPX, GST
Liver	Soybean replacement supplemented with Met and phosphate [37]		SOD, CAT, GR, GPX, GST	
<i>Dentex dentex</i>	Liver, white muscle	Different carbohydrates and carbohydrates amount [35]	LPO, carbonylated proteins	SOD, CAT, GR, GPX, G6PDH
<b>Diet to enhance welfare</b>				
<i>Dicentrarchus labrax</i>	Liver, intestine	Substitution diets and carbohydrates reduction <sup>*</sup> [33]	LPO	SOD, CAT, GR, GPX, G6PDH
<i>Solea senegalensis</i>	Liver	Reduced dietary proteins <sup>**</sup> [19]	LPO	SOD, CAT, GR, GPX, G6PDH
<i>Sparus aurata</i>	Liver, plasma	Lipid reduction <sup>**</sup> [21]	LPO, AOPP	SOD, CAT, GR, GPX, GSH and GSSG
<i>Scophthalmus maximus</i>	Liver	Prebiotics <sup>**</sup> [53]	LPO	SOD, CAT, GR, GPX, G6PDH

<sup>+</sup>Gene expression of antioxidant enzymes.

<sup>\*</sup>To enhance the response to handling stress.

<sup>\*\*</sup>To enhance the response to thermal stress.

**Table 1.**  
 Last decade works related to feeding strategies in Mediterranean teleost fish.

### 3.1.1 Fish oil reduction

During the last two decades several feeding strategies and economic needs have been promoted the reduction of total protein and the substitution of both fish meal and fish oil for other sources such as vegetable, algae and even insects or yeasts. However, a common strategy for the sector cannot be achieved due to the great variability within the biology of fish species concerning, for instance marine versus freshwaters; carnivorous versus herbivorous and omnivorous; temperate versus eurytherms, etc.

The replacement of fish oil (FO) by vegetal oil blend (VO) (20% rapeseed, 50% linseed, and 30% palm oils) would promote lower levels of lipoperoxidation (LPO) products in liver and intestine, together with higher enzymatic activities of GPX and GR in intestine of sea bass, *Dicentrarchus labrax* [32, 33]. When a stressful condition introduced, carbohydrate rich diets also diminished LPO products and increased GR and glucose 6-phosphodihydrogenase (G6PDH) and VO diets enhanced GPX and G6PDH activities [33]. The same diet strategy was conducted in other species such as the gilthead sea bream, *Sparus aurata*, evidencing that the enriched diets with starch carbohydrates promoted the antioxidant defences by reducing oxidized glutathione and lower LPO products [34]. By its way, in common dentex, *Dentex dentex*, higher carbohydrates levels inclusion in diets, increased GPX in liver and white muscle and GR in liver and 18 and 24% and decreased oxidative products as protein carbonylation in liver and LPO in liver and white muscle [35].

In addition to the studies of reducing FO consumption by reducing from VO substitution, some authors had considered that the lipid content of commercial diets must be lowered. Sánchez-Nuño et al. [21] evidenced that the reduction from 18–14% in the lipid content of the diet did not affect *Sparus aurata* growth, glutathione levels or enzyme activities, but did reduce the amount of LPO.

### 3.1.2 Fish sources reduction in diet formulation

The substitution (total and partial) of fish meal proteins by vegetable sources (soybean and wheat proteins as the main used products in the last decades) seems to be another relevant topic in diet substitution. Although a classic discrepancy exists on the benefits to substitute protein by carbohydrate and its effects on fish growth, for several fish species the carbohydrate inclusion to replace protein would benefit redox status, and if the growth performance is not affected, this strategy could be considered as beneficial for fish welfare.

In gilthead sea bream [36] fed with formulated diets replacing fish meal by soybean protein at 20, 40 and 60% showed a gradual increase of liver antioxidant enzymes activities (SOD, CAT, GPX, GR) according to higher levels of fish meal replacement. Despite any oxidized products were not evaluated, growth performance and immunity markers were negatively affected, suggesting that fish proteins are essential in diet formulation. However, when soybean diets were supplemented with methionine and phosphate fish redox status enhanced significantly [37]. In the same line, glutamine and arginine supplementation would improve deleterious effects of higher fish meal substitutions [38, 39] although with lower benefits observed with methionine inclusion. Taurine supplementation was also proposed to improve fish welfare when fish meal is replaced by vegetable oils but with not clear benefits [40].

Irrespective to sources substitution several studies approached specific supplements to improve the redox balance to benefit fish welfare. A combination of methionine and white tea dry leaves supplementation to a commercial diet were proposed

in gilthead sea bream by [41]. After 4 weeks feeding this diet liver SOD and CAT activities were increased, although no reduced LPO levels or higher glutathione amount were evident.

Other novel techniques and feed strategies were also assayed in fish nourishment studies. For instance, the inclusion of spray-dried plasma from porcine blood (SDPP) has been evaluated because of in mammals evidenced great results in immune and redox status. In fish fed with 3% of SDPP showed lower CAT, GR, and Glutathione S-Transferase (GST) enzymatic activity [42]. Other emerging strategy is to enrich diets with bacterial probiotics and to study its effects also in redox status. A recent study including *Shewanella putrefaciens*, *Pdp11* and *Bacillus* sp. evaluated the gene expression of redox balance markers in gill, intestine and epidermal mucosae [43]. Experimental diets alter the expression of the studied antioxidant genes, primarily in the gill and skin. Furthermore, the tested probiotics and mainly, the palm fruits extracts had significant antioxidant properties especially after feeding for 30 days.

### 3.2 Redox balance in response to environmental stress conditions

Another of the most relevant aspects in animal culture is the exposure to continuous environmental stressors and, in the most cases, fish are challenged to various types of abiotic or biotic stressors simultaneously. Whereas in the wildlife, fish have the freedom to migrate to locations where environmental conditions are within their tolerance range or to escape from disfavour ones, in culture conditions their confinement makes the escape impossible and entails the need to face up stressors with physiological responses. The classical abiotic and biotic stressors for fish culture are infections, parasites, changes in water salinity, exposure to dissolved heavy metals, the decrease in oxygen availability, the food access limitation, human handling, higher densities and mainly, at temperate latitudes, the natural and seasonal variations in water temperature. From the last two decades, the implications of physiological redox balance to face up culture conditions stressors are of the main interest for scientists and farmers. Some of the most relevant works are summarized in **Table 2** and discussed below.

	Tissue	Environmental stress	Oxidants	Antioxidants
<b>Starvation</b>				
<i>Dicentrarchus labrax</i>	Liver, intestine, white and red muscle	Starvation 1–2 months [44]	LPO	SOD, CAT, GPX
<b>Handling</b>				
<i>Dicentrarchus labrax</i>	Liver, intestine	Handling stress [33]	LPO	SOD, CAT, GR, GPX, G6PDH
<b>Salinity</b>				
<i>Dicentrarchus labrax</i>	Liver	Salinity and ammonia toxicity [45]	LPO, H <sub>2</sub> O <sub>2</sub>	SOD, CAT, GR, GPX, GSH and GSSG
<b>Hypoxia</b>				
<i>Sparus aurata</i>	Liver, heart	Hypoxia [47]	LPO	CAT, GR, GPX, GST
<b>Temperature</b>				
<i>Dicentrarchus labrax</i>	White muscle	18 versus 24 versus 28°C [52]	LPO	CAT
<i>Solea senegalensis</i>	Liver	12 versus 18°C [19]	LPO	SOD, CAT, GR, GPX, G6PDH



	Tissue	Environmental stress	Oxidants	Antioxidants
<i>Sparus aurata</i>	Liver	8 versus 20°C [50]	LPO NO	Liver proteome
	Liver, heart, white and red muscle	18 versus 24 versus 28°C and CO <sub>2</sub> [48]	LPO	SOD, CAT, GR
	Liver, heart, white and red muscle	Seasonal fluctuation [51]	LPO	SOD, CAT, GPX, XO
	Liver, plasma	14 versus 22°C [21]	LPO, AOPP	SOD, CAT, GR, GPX, GSH and GSSG
<i>Scophthalmus maximus</i>	Liver	15 versus 20°C [53]	LPO	SOD, CAT, GR, GPX, G6PDH

**Table 2.**

Last decade works related to environmental stress in Mediterranean teleost fish.

### 3.2.1 Food deprivation

Food deprivation in wild fish, is a common fact and the physiological impact tends to be less aggressive than in higher vertebrates. Starvation reduces energy metabolism and cellular activity, resulting to lower oxygen consumption which can lead to oxidative stress caused by hypoxia [44]. Due to the economical repercussion for the industry, fasting is widely described for the vast majority of fish, classically focusing in metabolism and growth. However, few studies approach to redox balance and on the consequences of an unbalanced oxidative context. For instance, long-term starvation (1–2 months) in sea bass, *Dicentrarchus labrax* altered the redox balance in red muscle, white muscle, intestine and liver. Moreover, refeeding period has been demonstrated as a crucial and additional stressful period [44]. Alterations in redox balance seem to be tissue dependent, but triggering LPO in liver. However, food deprivation and refeeding enhanced antioxidant enzyme activities in intestine, and decreased SOD activity in red and white muscle, and CAT and GPX in white muscle.

### 3.2.2 Salinity

In seawater, salinity variations influence physiological processes and condition fish species abundance, being in consequence, one of the most determinant environmental stressors. Some species are tolerant with variable salinities, euryhaline species, and could be related with its migratory behaviour. Thus, the physiological adaptative strategies to cope with the changing environmental salinities were very recurrent on fish biology studies. Recent studies suggested that changes in salinity may also induce oxidative stress compromising antioxidant defences. However, it becomes difficult to extrapolate a solution under culture conditions after evaluating the response to different stressors independently. For that reason, the current trend in salinity studies is to combine different stressors to evaluate the joint response. For instance, [45] studied the combined effects on the sea bass redox balance of the salinity and the High Environmental Ammonia (HEA) The results evidenced the antioxidant defences strength in this specie in low-saline seawaters (up to 10 ppt) remaining unaltered even with increased HEA levels. However, it seems to be a limit at hypo-saline environment (2.5 ppt) in combination with HEA exposure where antioxidant defence were compromised.

### 3.2.3 Hypoxia

As it was referred above hypoxia is one of the main factors resulting in an oxidative attack in fish as in mammals. However, the mechanisms of hypoxia-induced

oxidative stress remain still unclear. One of the hypotheses supports that a reduction in the mitochondrial electron-transport chain efficiency may contribute to ROS generation [46]. Sustained severe hypoxic conditions could trigger fish mortality. As an example, [47] demonstrated in *Sparus aurata* an increase of LPO, increased CAT activity and reduced GR and GPX hepatic enzymes in response to environmental O<sub>2</sub> fluctuations after an hypoxia exposure. All these effects seem to be mitigated by the dietary supplementation of seaweeds, suggesting its protective role against oxidative stress. The principal causes of hypoxia in the sea are crowding and the increase of water temperature, being the climate change one of the main effectors. Because climate change is currently a trending topic, some researchers have focused their efforts on finding new strategies to mitigate the effects of high temperatures [48].

### 3.2.4 Temperature

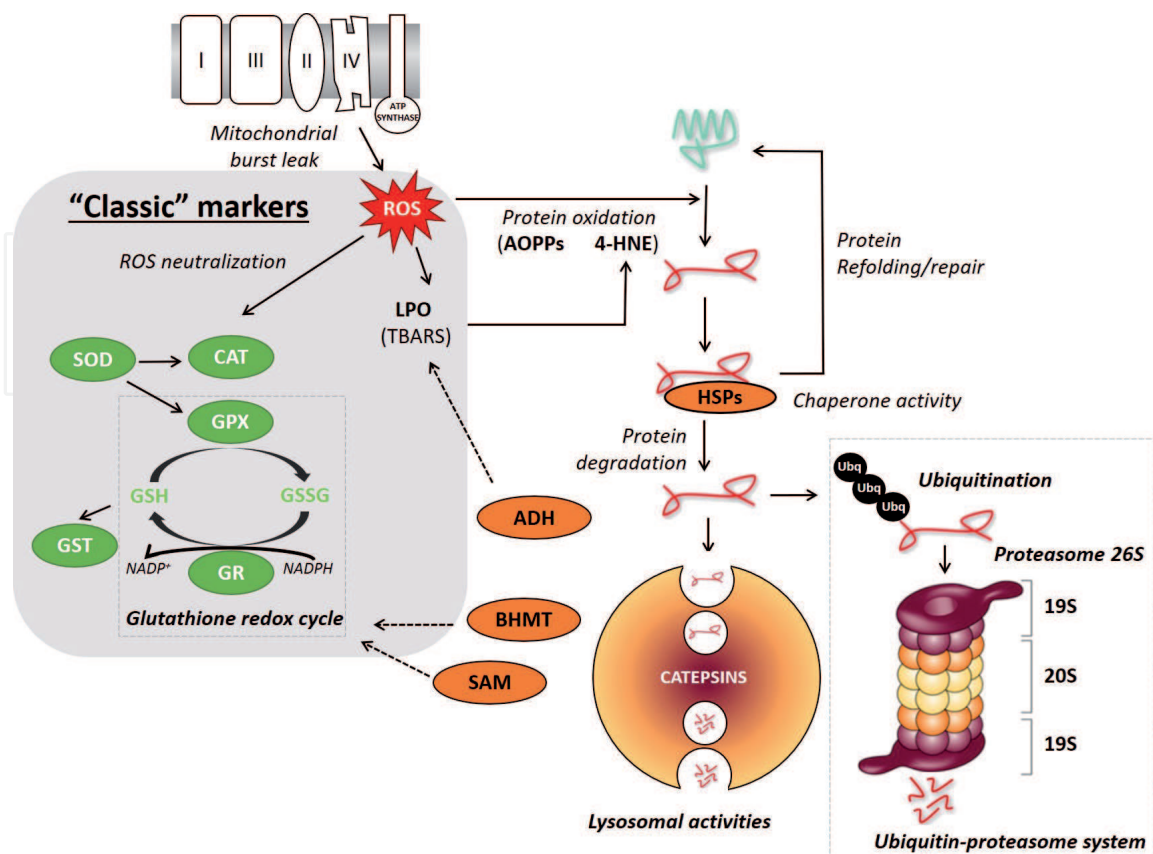
One of the most relevant environmental inputs is thermal variations, being a challenge for poikilothermic animals. When the seawater temperature escapes from the limits of intraspecific tolerance, wild animals can respond in different ways, being the physiological escape (migration) one of the most common responses [2]. Under culture conditions, animals are not able to migrate and are obligated to face these temperatures, forcing an adaptation and implying physiological changes. Focusing on the physiological 'symptoms', loss of appetite would be the first response to stress due to low temperatures [49]. Due to the productive interest and the evidences of the increasingly extreme seasonal temperatures, great efforts have been devoted to improving animal welfare during the thermal changes, being the influence on metabolism and redox balance analysis key topics. In last decade, several studies have introduced novel techniques in fish to evaluate the redox balance. Ibarz et al. [50] approached proteomics to evaluate the cold exposure in sea bream liver. Their results demonstrated that after 10 days at 8°C LPO increased by 50% and antioxidant proteins such as betaine-homocysteine-methyl transferase (BHMT, related with glutathione synthesis), GST and CAT were downregulated, suggesting that this species are very sensitive by low temperatures. The warming response was also evaluated after 10 days at 24 and 28°C, evidencing that growing temperatures also increase LPO and stimulates CAT and GR activities in several tissues like heart, muscle (white and red) and liver [48]. In addition to acute exposure to thermal stress, some studies have focused on the medium-long-term effect of temperature challenge, trying to understand the physiological response. In this way, Sánchez-Nuño et al. [21] described the redox balance behaviour (in liver and in plasma) after 50 days of cold exposure at 14°C. Cold exposure compromised antioxidant enzyme activities mainly CAT and GR, which subsequently affected the glutathione redox cycle and caused an acute reduction in total hepatic glutathione levels. During temperature recovery, antioxidant enzymatic machinery was gradually restored but the glutathione redox cycle was not recovered. Despite field studies are not very common, it was evaluated the effect of seasonal temperature fluctuation in heart, liver and muscle to understand the adapted physiological state, including redox balance [51]. Results evidenced clear seasonal metabolic patterns involving oxidative stress during summer as well as winter, but more prominent during warming because of the increased aerobic metabolism. During cold acclimatization and under increased temperatures LPO was higher.

By its way, [52] evaluated in sea bass white muscle the LPO and CAT activity when increasing temperature from 16–18, 24 and 28°C and maintained during 15 and 30 days, describing that temperature rise increase LPO and CAT activity. The exposure time conditioned the response, evidencing an acclimation after 30 days at

28°C. Moreover, it was evaluated the effect of rearing water temperature in turbot juveniles, *Scophthalmus maximus* (15 days at normal 15°C and higher 20°C), evidencing lower CAT and GPX enzyme activities [53]. In Senegalese sole, *Solea senegalensis*, cold exposure at 12°C increased GR activity and LPO while decreased SOD activity in liver [19]. Moreover, in this specie decreasing protein content in diet from 55–45% worsened redox balance. All these recent studies reinforce the idea that temperature challenges strongly compromise redox balance in fish temperate species.

#### 4. A wide vision of redox balance in fish: looking for new markers

Fish, like all other organisms, must have a balance between the production of oxidative substances (ROS and RNS) and antioxidant defences, and are of particular interest as they experience a multitude of above-mentioned stressors. To protect themselves against the potentially highly damaging oxidants, organisms have evolved a system to either prevent or repair the effects of oxidative stress. Prevention comes in the form of antioxidants, which can either be enzymatic (SOD, CAT, GPX, GR) or non-enzymatic molecules (mainly glutathione and vitamins C and E), carotenoids and other small molecules. These are the most commonly measured oxidative markers and antioxidants in fish biology (recently revised [54]). However, recent advances on redox studies in mammals suggest other markers to be considered widening to associated pathways of redox balance. Some of them should be also considered when studying redox balance in fish. **Figure 2** attempts to provide this broad range of markers also in fish: including markers of protein oxidation levels, metabolic enzymes related to glutathione synthesis, repair/refolding of oxidized proteins or main protein degradation processes (via ubiquitin-proteasome system, UPS, or lysosomal proteolytic fate).



**Figure 2.**  
'Wide view' of redox balance markers.

Beyond the LPO measurements (via TBARS analyses), it was recently reported that advanced oxidation protein products (AOPPs) could act as markers of oxidative stress, resulted from the direct oxidation of the amino acid side chains of proteins as a consequence of metal toxicity in freshwater species [55, 56] or produced at low temperatures in marine species [21]. Another marker recently used in mammals is the accumulation of conjugated proteins with 4-HNE as the major biomarker relating lipoperoxidation and protein oxidation [57, 58]. 4-HNE is the most toxic product of lipid peroxidation reported in mammals damaging proteins by adding covalent adducts and accelerating protein aggregation [59, 60]. Although no data exists in fish on HNE products analyses, a preliminary study in gilthead sea bream would demonstrate the existence of a singular pattern of 4-HNE oxidized proteins in liver with the proteins ranging 40–50 and 75–100 kDa the main target of lipid peroxidation (own data unpublished).

As proteins are the major constituents of cellular organization and metabolism, effects of oxidative attack on protein structure, stability and folding deserve careful consideration in fish species. Several cellular pathways exist which repair and eliminate damaged proteins and thus prevent their accumulation and aggregation. One of the first mechanisms to cope with protein damage is binding with chaperones or 'heat shock proteins' (HSPs). Thus, a few studies recently addressed the changes on HSPs levels/expression in fish when the redox balance is challenged. For instance, it was reported on several fish tissues the expression of HSP70 and HSP90 protein expression levels in response to temperature seasonal challenges [61, 62]. When a protein is irretrievably damaged, its fate is to be recycled via UPS degradation pathways, or to be removed/autophaged via a lysosomal degradation process. Protein degradation via UPS involves two discrete and successive steps: tagging of the substrate protein by the covalent attachment of multiple ubiquitin molecules, and the subsequent degradation of the tagged protein by the 26S proteasome, composed of the catalytic 20S core and the 19S regulator [63]. The capacity to remove damaged proteins by the proteasome may prevent oxidative stress and it has been suggested that this is also part of the antioxidant defences in mammals [64]. In fish which inhabiting permanently temperature-fluctuant aquatic environments, this enzymatic complex could play a key role in antioxidant defence systems [65]. The analysis of UPS system markers is still scarce or null in fish and to obtain a profile of protein-ubiquitination labelling according the MW or the analyses of main proteasome subunits (catalytic 20S core or 19S regulatory) should be of further interest on fish studies related to redox balance.

The aggregation of proteins as a result of the accumulation of cross-linked 4-HNE proteins cannot be degraded through the UPS even could block its correct functioning. Then, lysosome acts to eliminate protein aggregates. The lysosomal system has an elevated non-selective protein degradation capacity as a result of the combined random and limited action of various proteases, with the cathepsin family being one of the most important in mammals [59, 66] and also in fish [67]. Thus, as the fate of 4-HNE protein conjugates is preferably the lysosomal degradation pathway, the cathepsin activities should be another target on the study of redox balance.

Finally, we encourage fish biology researcher to study other enzymes which their activities are also strongly related with antioxidant defence. Both TBARS and HNE are aldehydic products, relatively stable and capable of roaming freely and attacking molecules, e.g. DNA, proteins, lipids far from their origin [68]. Thus, aldehyde dehydrogenase (ADH) is an enzyme involved in the oxidation pathway, and is complementary to the GST pathway, which reduces the potential damage of these peroxide products by oxidizing them and removing them from inside the cells. As the glutathione plays a central role in lipid peroxide detoxification, reducing the peroxides to their corresponding alcohols, the study of enzymes involved in

glutathione synthesis seems adequate to evaluate cell redox potential and response. The BHMT and the adenosine-methionine synthetase (SAM) are enzymes involved in antioxidant mechanisms through the synthesis of S-adenosylmethionine and through maintain its steady-state levels which is a crucial component of methylation reactions and a biosynthetic precursor of glutathione. Under acute cold stress the expression of these enzymes of glutathione synthesis as well as ADH are affected in gilthead sea bream [50].

Overall, we hope that the proposed markers in **Figure 2** from that 'wide view' on redox balance and related processes in fish. Can contribute to expanding knowledge of the relevant oxidant products (LPO, AOPPs, HNE), the classic enzymes studied (SOD, CAT, GPX, GR, GST) and the associated processes affected such as protein repairing/protection machinery and protein turnover by the oxidant insult.

## 5. Conclusions


In the last years, several efforts have been made in the Aquaculture industry to improve fish welfare. Changes in environmental conditions and diets can alter the redox balance, thus affecting the welfare state. Most redox studies in fish focuses on classical markers such enzymatic and non-enzymatic antioxidants, and lipoperoxidation products as oxidative damage markers. In this paper we present evidence that the study should be extended to the associated pathways of redox balance including: markers of protein oxidation levels, metabolic enzymes related to glutathione synthesis, repair/refolding of oxidized proteins or main protein degradation processes.

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