Universidade de Aveiro Departamento de Biologia

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Mercury contamination and oxidative stress responses in the eye of wild fish (*Liza aurata*)

Contaminação por mercúrio e respostas de stresse oxidativo nos olhos de peixes selvagens (*Liza aurata*)

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Patrícia Alexandra Oliveira Pereira Kowalski, investigadora de pós-doutoramento da Universidade de Aveiro, e do Professor Doutor Mário Guilherme Garcês Pacheco, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro) da Universidade de Aveiro.

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NEUTOXMER

“Mysteries don't lose their poetry because they are solved:
The solution often turns out more beautiful than the puzzle.”

- Richard Dawkins

o júri

presidente

Professor Doutor Carlos Miguez Barroso,
Professor Auxiliar da Universidade de Aveiro

Doutora Joana Raimundo Pimenta,
Investigadora de Pós-Doutoramento do Instituto Português do Mar e da Atmosfera.

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Under thy shade, Tree of Wisdom

palavras-chave

mercúrio, contaminação, peixes, *Liza aurata*, resposta antioxidante, stress oxidativo, bioacumulação

resumo

Todos os animais usam os olhos para monitorizar o ambiente circundante e manter a homeostase. O Mercúrio (Hg) (incluindo o metilmercúrio - MeHg) é um contaminante pernicioso e omnipresente em águas naturais, mas a avaliação do seu potencial para interferir com olhos de peixe é um problema maioritariamente inexplorado. Esta tese irá contribuir para preencher a lacuna de conhecimento correspondente à avaliação da acumulação de mercúrio na parede do olho de tainha-garrento (*Liza aurata*), juntamente com a avaliação de parâmetros bioquímicos relacionados com o estado de stresse oxidativo. Esta abordagem foi complementada pela caracterização dos perfis de contaminação ambiental (tanto na água como no sedimento). O estudo foi realizado na Ria de Aveiro (Portugal), onde um gradiente bem estabelecido de contaminação histórica de mercúrio fornece um bom plano de fundo para o estudo de acumulação de mercúrio e os seus efeitos tóxicos sob condições realistas. A amostragem foi realizada em dois locais, distanciados em cerca de 10 km: Largo do Laranjo (LAR), a área mais contaminada, e São Jacinto (SJ), localizado mais perto da entrada da lagoa, tendo sido selecionado como o local de referência. Considerando que a temperatura da água pode afetar os níveis de Hg acumulado e as consequentes respostas bioquímicas, a amostragem foi realizada tanto no inverno (Fevereiro de 2013) e verão (Junho de 2013). Espécimes de *L. aurata* (n = 20) foram capturados em cada local/estação e o olho foi removido e preservado no campo. Estas amostras foram analisadas em relação a níveis totais de Hg e de MeHg e em relação à resposta antioxidante (catalase-CAT, SOD – superóxido dismutase, glutationa peroxidase-GPx, glutationa redutase-GR, glutationa-S-transferase - GST) e dano peroxidativo (LPO). Os níveis de mercúrio inorgânico foram estimados a partir dos níveis de mercúrio total e metilmercúrio. Mercúrio total, mercúrio inorgânico e metilmercúrio no olho foram superiores em LAR (em relação a SJ) tanto no inverno como no verão, refletindo diferenças espaciais ambientais na coluna de água e no sedimento superficial. Além disso, os peixes recolhidos em LAR no inverno demonstraram uma diminuição significativa na atividade da CAT e SOD, enquanto GST e GR exibiram a mesma tendência, mas sem suporte estatístico. A quantidade de LPO aumentou significativamente, e foram também registados danos peroxidativos indicados pelo aumento de LPO no olho de peixes recolhidos em LAR no inverno. No verão foi registado um padrão espacial muito mais homogéneo para esses parâmetros biológicos, uma vez que apenas o aumento da GR e da GPx foram registados em LAR. As relações de causa-efeito entre acumulação de mercúrio (inorgânico e metilmercúrio) foram pesquisadas através de uma análise de componentes principais (PCA). Uma associação entre as duas formas de mercúrio, GPx e LPO foi evidenciada pelo PCA. Dados atuais apontam para a aparente vulnerabilidade dos olhos de peixe a contaminantes da água, ou seja, mercúrio. Este neurotóxico pode acumular no olho, levando a alterações na proteção celular contra o stresse oxidativo. Tais repercussões eventualmente podem comprometer o desempenho do peixe e a sua sobrevivência.

keywords

mercury, contamination, fish, *Liza aurata*, antioxidant response, oxidative stress, bioaccumulation

abstract

Every animal uses the eyes to monitor the surrounding environment and maintain homeostasis. Mercury (Hg) (including methylmercury - MeHg) is a pernicious and ubiquitous contaminant in natural waters but the assessment of its potential to interfere with fish eyes is an almost unexplored issue. This thesis will contribute to fill this knowledge gap by the evaluation of mercury accumulation in grey mullet eye wall (*Liza aurata*) together with the assessment of biochemical endpoints related with the oxidative stress status. This approach was complemented by the characterization of environmental contamination profiles (both in water and sediment). The study was performed at Aveiro lagoon (Portugal), where a well-established mercury historical contamination gradient provides a good background for the assessment of mercury accumulation and its toxic effects under realistic conditions. Sampling was conducted in two sites distancing around 10 km, namely: Largo do Laranjo (LAR) located in the most contaminated area, and São Jacinto (SJ) closer to the lagoon inlet and selected as reference site. Keeping in view that water temperature could affect Hg accumulated levels and biochemical responses, sampling was carried out both in winter (February 2013) and summer (June 2013). *L.aurata* specimens (n=20) were caught at each site/season and eye wall was removed and preserved in the field. Eye wall was analysed for total Hg and MeHg levels, as well as for antioxidant responses (catalase- CAT, SOD – superoxide dismutase, glutathione peroxidase- GPx, glutathione reductase- GR, glutathione–S-transferase - GST) and peroxidative damage (LPO). Inorganic mercury levels were estimated from total mercury and methylmercury levels. Total mercury, inorganic mercury and methylmercury in eye wall were higher at LAR than SJ in winter and summer, reflecting environmental spatial differences of water column and surface sediments. Moreover, fish caught at LAR in winter showed a significant decrease of CAT and SOD, while GST and GR exhibited the same tendency but without statistical support. Peroxidative damage was also recorded as indicated by the LPO enhancement. A much more homogenous spatial pattern was recorded for those biological endpoints in summer, since only the increment of GR and GPx was noticed at LAR. Cause-effect relationships between accumulated mercury (inorganic and methylmercury) were searched by the principal component analysis (PCA). An association between both forms of mercury and GPx and LPO was discerned by PCA. Current data point out the vulnerability of fish eyes to water contaminants, namely mercury. This neurotoxicant can be accumulated in eye wall leading to alterations in the cellular protection against oxidative stress. Such repercussions could eventually compromise fish performance and survival.

I

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1. Introduction

1.1 Mercury as a global threat and its occurrence in aquatic systems

Mercury (Hg) is considered to be a widespread natural and anthropogenic contaminant with no biological function, being responsible for highly negative impacts at the organism and ecosystem levels (Mieiro et al., 2011). While most Hg in the air and surface water is present in inorganic form (iHg), sulfur-reducing bacteria in the sediment of aquatic environments can methylate iHg compounds into methylmercury (MeHg) (Sandheinrich and Weiner, 2011), which in turn can cause adverse genotoxic, reproductive, and behavioral effects in aquatic fauna (Marr et al., 2013). The study of Hg is mainly driven by the fact that it poses a very serious threat to organisms and to the proper function and balance of ecosystems, which makes Hg contamination a danger to the environment. Several known cases of Hg-related contamination and pollution have been reported, the Minamata bay incident (1960) being the most well-known by the general public (George, 2001).

Despite the fact that the main sources of Hg in the environment are natural processes from the Earth's biogeochemical cycles, global Hg concentrations have been progressively increasing at a relatively high rate since the Industrial Revolution period, due to anthropogenic influence (UNEP, 2013). Certain human activities cause a significant increase of the concentration and distribution of Hg in the biosphere to a point when pollution phenomena are generated in certain areas. The presence of Hg in ecosystems can be traced to four different origins (UNEP, 2013):

- **Natural releases** due to the mobilization of occurring Hg from the Earth's crust, such as volcanic activity and weathering of rocks. The presence of MeHg in the aquatic environment is mostly due to biological processes in which some species of bacteria biotransform iHg molecules into organic Hg compounds, generally described as the most toxic chemical forms of Hg (Wiener et al., 2003).
- **Anthropogenic releases due to the mobilisation of Hg impurities in raw materials** such as fossil fuels (particularly coal, and to a lesser extent gas and oil) and other extracted, treated and recycled minerals. Oil deposits are known to contain Hg, generally at low concentrations (U.S.EPA, 2013).
- **Anthropogenic releases resulting from Hg used intentionally in products and processes**, due to releases during manufacturing, leaks, disposal or incineration of spent products, and especially mining activity (particularly of gold, which uses large amounts of liquid Hg that seeps into ground and eventually contaminates aquatic ecosystems (Tchounwou et al., 2003). Other examples are the industries of chloride and sodium hydroxide production through the electrolysis of brine, the incineration

of waste and/or bituminous coal, and the production of electricity, measurement instruments (barometers), fluorescence tubes, alkaline batteries and usage in dental medicine (UNEP, 2013).

- **Remobilisation of historic Hg deposits** of anthropogenic origin in soils, sediments, landfills and waste/tailings piles.

1.2 Mercury properties and toxicity

Scientific data shows that Hg is cytotoxic, mutagenic, carcinogenic, and affects a wide variety of tissues and organs, being primarily known for its neurotoxic properties (Wiener et al., 2003; UNEP, 2013). Its presence in the global environment is considerably increased by anthropogenic activities (Hutchenson et al., 2014), and despite being considered to have higher toxicity in organic forms, Hg is overall more toxic than any other nonradioactive element (Korbas et al., 2013). Hg found in the environment can be divided in four different main forms: iHg, MeHg (Figure 1) and other organic forms such as ethylmercury and diethylmercury, which are less prevalent. All these chemical forms of Hg have different rates of solubility, reactivity and toxicity (Clarkson et al., 2003; Aschner et al., 2007). Particularly in aquatic ecosystems, two main forms are present, namely elemental mercury (Hg^0) dissolved as particulate ions (Hg^{2+} and Hg^{+}) or as methyl or ethyl-mercury ($MeHg^{+}$) in dissolved or particulate forms (Mason e Fitzgerald, 1993). Organic forms of Hg are considered as being the most toxic (Leong et al. 2001; Holmes et al., 2009). The diagram of the Hg cycle, as displayed in Figure 1, illustrates the fact that Hg can be brought to the sediments by particle settling and then later released by diffusion (or resuspension). It can enter the food chain, or it can be released back to the atmosphere by volatilization (Hudson et al., 1995). The exact mechanism(s) by which Hg enters the food chain remain largely unknown, and probably varies among ecosystems. However, it is known that certain bacteria play an important early role. Studies have shown that bacteria that process sulfate (SO_4^{2-}) in the environment take up iHg, and through metabolic processes convert it to MeHg (River, 1975).

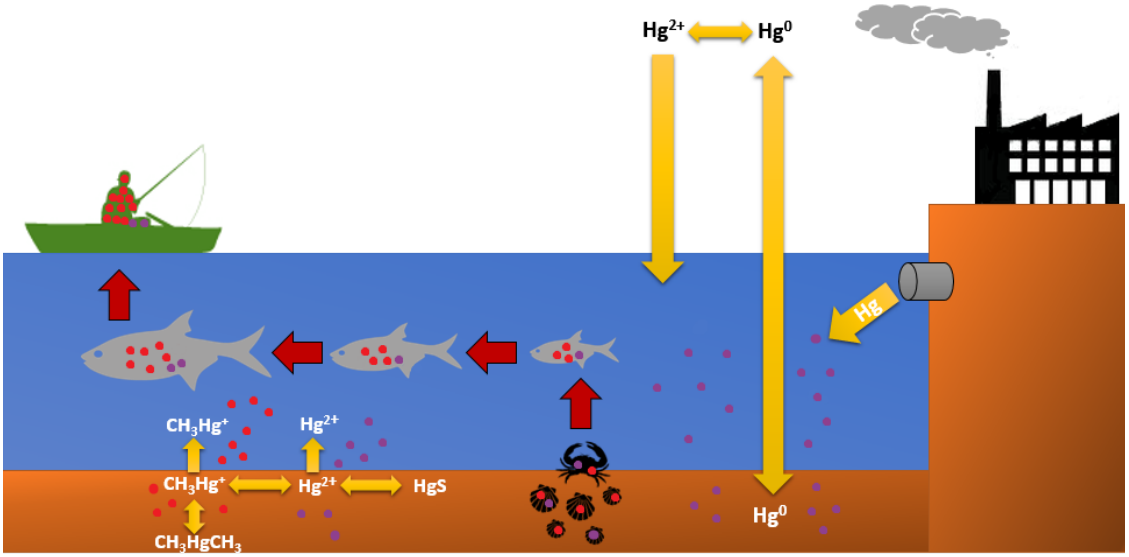


Figure 1 - Representative diagram of mercury cycle, showing the differences of bioaccumulation throughout the food chain and the different chemical transformations of organic and inorganic Hg (red and purple, respectively). Adapted from Mieiro (2011).

1.3 Fish as biossentinel organisms of aquatic contamination and models in neurotoxicology

Fish may provide extremely useful biomarkers in several steps of the risk assessment process: effect, exposure and hazard assessment, risk characterization or classification, and monitoring the environmental quality of aquatic ecosystems (Van der Oost et al., 2003). This is related with a favorable amount of key biological and ecological characteristics of fish for ecotoxicological studies. Fish are considered relevant components of the trophic chains, also playing an important role signaling water pollution, due to the fact that they react with relatively great sensitivity to changes in aquatic systems (Van der Oost et al., 2003; Guilherme et al., 2008; Mieiro et al., 2010). They can be found virtually everywhere in the aquatic environment, and function as a carrier of energy from lower to higher trophic levels (Beyer, 1996). This makes the understanding of toxicant uptake, behavior and responses in fish crucial to understanding environmental health in aquatic systems. However, there is considerable variation in both the basic physiological features and the responsiveness of certain biomarkers between different fish species towards environmental pollution. Despite this and other limitations, such as a relatively high mobility, fish are generally considered to be good sentinel organisms for ecotoxicological studies in aquatic environments.

There are numerous studies that quantified organic and inorganic Hg in fish organs (liver, gills, brain) in order to assess environmental quality and establish causal relationships

with fish health (Zorita et al., 2008; Mieiro et al., 2011; Pereira et al., 2010 and 2014). The eyes have been generally disregarded in that research framework. However, the few existing studies found that both iHg and MeHg could reach fish eyes (Pereira et al., 2014) and particularly that MeHg target the photoreceptors cells of retina (Mela et al., 2012; Korbas et al., 2013).

1.3.1 Exposure pathways of fish to Hg

Mercury may be accumulated in fish through different pathways, namely directly from water, via uptake from suspended particles and sediment, or by the consumption of lower trophic level organisms. The former is an essential point to consider in evaluating adverse effects on ecosystems (Van der Oost et al., 2003). In view of that, there are several works that use the accumulation of Hg in fish as mean to assess the environmental health status (Van der Oost et al., 2003; Fernandes et al., 2007; Pereira et al., 2009 and 2014; Mieiro et al., 2010 and 2011). Hg absorption by fish involves their transfer to the blood through the epithelial barrier of gills, digestive organs or skin. Dissolved Hg is mainly taken up by exposed body surfaces such as the gills. Because of direct exposure in the water medium and wide surface contact area, it has been accepted that the gills are the main organ of trace elements uptake (Karan et al., 1998; Dalzell and Macfarlane., 1999) According to Klinck et al. (2004), accumulation of non-essential waterborne metals through the gills of fish is generally thought to occur when cationic trace elements (such as Hg) are taken up inadvertently by transport processes designed for essential cations (e.g., Hg^{2+} uptake instead of Ca^{2+}uptake), causing adverse effects in the organism, such as oxidative stress (Arabi, 2004). Gills' epithelium is regularly subject to exfoliation and erosion, which is counteracted by an intense cell division rate (Potter et al., 1997). This high renewal rate of the branchial tissue could be unfavorable trial to environmental health assessment.

In that perspective, fish eyes could provide complementary information of environmental quality. In fact, the eye wall of fish was already described as a gateway organ for Hg uptake (Korbas et al., 2013; Pereira et al., 2014). The eyes of fish are in constant and direct contact with the aquatic medium, which makes them prone to bioaccumulated Hg. However, the accumulation of Hg in the eyes through direct contact with contaminated water is generally small. This may result from its mucus membrane protection named conjunctiva that has the purpose of protecting the eye wall (Kulczycka, 1965). Mercury can also enter the organism through feeding processes that involve the ingestion of contaminated food (Wiener and Spry, 1995; Watras et al., 1998). Thereafter, Hg could reach the eye wall by transport throughout the bloodstream and by passing the blood-retinal

barrier (BRB), which is a biological barrier composed by two distinct layers (Eriksson et al., 2007): (i) an inner barrier, formed by endothelial cells lining the retinal blood vessels;(ii) outer barrier formed by the retinal pigment epithelium (RPE), which is a layer of epithelial cells between the retina and non-neuronal choroid. Crossing the BRB is the only biological pathway for xenobiotics reach the eye wall through the bloodstream. MeHg can easily pass the BRB due to its high affinity with the sulfhydryl groups of organic molecules, passing through organic barriers by connecting to organic molecules present in the organism, such as cysteins (Quig, 1998).

1.3.2 Oxidative stress involvement on mercury toxicity

Fish populations can be adversely affected by the presence of Hg in their tissues, which emphasizes the importance of assessing its distribution and subsequent retention (Guilherme et al., 2008), as well as other biological adverse effects like inhibition of biotransformation enzymes, reproductive alterations (Van der Oost et al., 2003), oxidative stress (Berntessen et al., 2003) and genotoxicity (Guilherme et al., 2008). Although the toxicity mechanism of Hg is still unclear, especially in fish (Mieiro et al., 2011) it was previously associated with the occurrence of oxidative stress and the formation of reactive oxygen species (ROS) (Berntssen et al., 2003; Mieiro et al., 2010 and 2011; Pereira et al., 2014). Antioxidants play an extremely important role in maintaining cell homeostasis, and when their activity is adversely altered, the formation of ROS can lead to oxidative damage expressed by lipid peroxidation of the cellular membranes, DNA damage, enzymatic inactivation and cell aging (Stohs and Bagchi, 1995; Hirata et al., 2004; Guilherme et al., 2008). This would eventually lead to an enzymatic response in the cytosol designed to decrease the rate of oxidative damage. It is known that Hg forms covalent bonds with glutathione (GSH) and a single Hg ion can bind to two GSH molecules leading to this complex cellular excretion (Franco et al. 2009). The releasing of Hg-GSH conjugates results in diminishing the intensity of the antioxidant response due to the lack of GSH, resulting in greater activity of the free Hg ions disturbing GSH metabolism and damaging cells (Franco et al. 2009). Additionally, the binding of Hg to GSH will promote the accumulation of ROS that would normally be eliminated by GSH. This oxidative cellular environment would probably lead to the activation of other antioxidant defenses, such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and glutathione reductase (GR). Their interaction with ROS is illustrated in Figure 2.

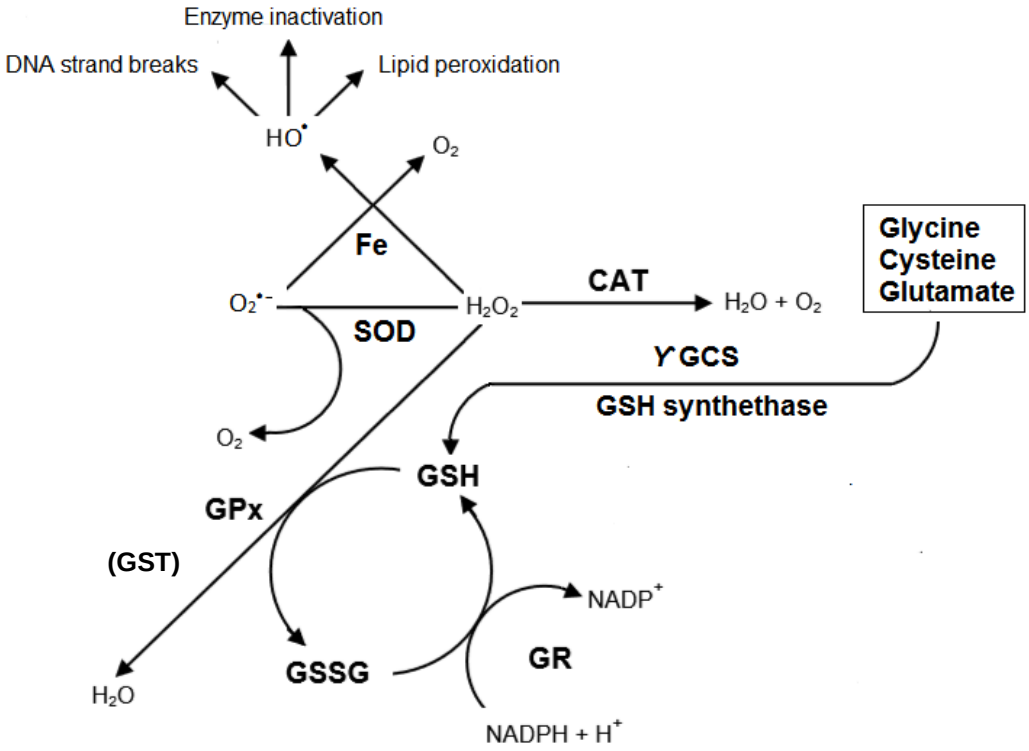


Figure 2 - Representative diagram of the interactions between the antioxidant enzymes measured in this study. Adapted from Macdonald et al. (2003)

The most well documented antioxidant enzymes in terms of mechanisms and effects are:

- **SOD:** catalyzes the dismutation of the superoxide ion ($O_2^{\bullet -}$) in hydrogen peroxide (H_2O_2) (Peskin et al., 2000);
- **CAT:** degrades hydrogen peroxide into water and oxygen molecules (Rojkind et al., 2002);
- **GR:** catalyses the transformation of GSSG to GSH with the concomitant oxidation of NADPH to $NADP^+$. Therefore, GR maintains the GSH/GSSG homeaostasis under oxidative stress conditions (Meister, 1988);
- **GPx:** Detoxifies organic and inorganic peroxides, by using GSH as a cofactor. Is an integral part of the mechanisms that contribute to diminish the rate of lipid peroxidation (Epp et al., 1983);
- **GST**: Metabolizes several xenobiotic compounds and is responsible for conjugating electrophyllic compounds with GSH for the purpose of detoxification, and may also play an important role in deactivating the products of lipid peroxidation and their derivates (Cnubben et al., 2001).

- **GSH:** Responsible for the chelation of xenobiotic molecules, which in turn makes them chemically inert. GSH is also a cofactor of other enzymes such as GST and GPx (Cnubben et al., 2001);

Hg contamination also induces lipid peroxidation (LPO) consisting in the oxidative degradation of lipids, in which free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage (Muller et al., 2007).

Oxidative stress was already identified as a chief process in humans' visual deterioration corroborating the relevance of this issue for neurotoxicology. For instance, a significant proportion of lenses and aqueous humor taken from cataract patients have elevated H_2O_2 levels. Since H_2O_2, at concentrations found in cataract, can cause lens opacification and produces a pattern of oxidation similar to that found in cataract, it was concluded that H_2O_2 is the major oxidant involved in cataract formation (Spector, 1995). Moreover, growing evidence supports the involvement of oxidative stress as a common component of glaucomatous neurodegeneration in different subcellular compartments of retinal ganglion cells (Tezel, 2006).

1.4 What it is known about mercury accumulation in fish eye and associated biological effects?

Fish eyes possess a complicated morphological and neural retinal organization, in comparison to other animals. The retina of most vertebrates (including fish) is duplex, containing both rods and cones. Fish may have a scotopic system, with input from the rods that is responsible for achromatic, high sensitivity, low acuity vision, while a photopic system using the cones is responsible for color vision, low sensitivity, and high acuity vision at higher light intensities (Kusmic and Gualtieri, 2000).

Uptake of essential metals such as Ca, Cu, Fe and Zn, often involves specific pathways - calcium channels and specific membrane carriers for Fe and Cu (Sunda and Huntsman, 1998). Mercury is a non-essential element that uses specific uptake mechanisms of other essential metals to enter the organism (Sunda and Huntsman, 1998). Zebrafish larvae (*Danio rerio*) was previously exposed to waterborne MeHg, revealing that this form is preferentially accumulated in the eyes, specifically in the outer layer of the lens and retina (Korbas et al., 2010 and 2013). Higher levels were detected in photoreceptor layer, in the outer and inner plexiform layers, distributed evenly throughout the retina. In fact, the retinal pigment epithelium is considered a metal-chelating tissue that is capable of binding essential and toxic metals due to their high affinity to melanin in retinal pigment epithelium

melanosomes (Erie et al., 2005). A more detailed study of the same research group showed that MeHg accumulates in the secondary lens fibers immediately underlying the lens epithelial cells (Korbas et al., 2013). iHg deposits were also found in the photoreceptor layer, as well as in the inner and outer nuclear layers of fish (Mela et al., 2010). This is in agreement with several studies in mammals (including humans) that reported the accumulation of metals in eyes (e.g. Erie et al., 2005) as well as a visual deterioration associated with Hg (e.g. Warfvinge and Bruun, 1996; Erie et al., 2005). However, more research is necessary in order to assess the permeability of the outer membrane to MeHg and iHg.

A toxicokinetic experiment with iHg revealed an increase of accumulated Hg in eye wall of the white seabream (*Diplodus sargus*) after 7 days of exposure. It was also found a temporal delay between gills and eye wall for iHg load increase, which could be attributed to the distinct tissues nature and physiology (Pereira et al., submitted). Despite the direct contact of fish eye with water, this organ seems to be, in some extent, impervious to the dissolved iHg, being thus physiologically protected. The epidermal mucus secretions covering fish eyes can be a first line of defense against metals. Several mucus constituents of fish skin such as the sialic acid and other glycoprotein components may bind and immobilize iHg, preventing its direct uptake by eyes. Additionally, data suggested that water is not the main vehicle of iHg to fish eyes, pointing to the occurrence of an alternative pathway for iHg to reach eye wall. iHg can be distributed through the blood to eye wall and this seems to be the preferential uptake route. Such distribution was previously observed for MeHg in zebrafish (Korbas et al., 2013) and invoked to explain the iHg accumulation in wild fish (Pereira et al., 2014). Indeed, blood is the main vehicle of mercury (re)distribution in fish body (similarly to other xenobiotics). To reach the eye wall by this pathway Hg need to cross the BRB, as previously described.

Fish use a variety of mechanisms to sequester Hg and thus reducing its toxicity. There are some non-enzymatic antioxidants that could be specifically found in eyes, namely the carotenoid pigments lutein and zeaxanthine. These compounds were also found in eyes of fish, particularly in the macular region (Goodrich et al., 1941).

Fish are considerably vulnerable to disturbances on sensory processing due to environmental Hg exposure (Baatrup et al., 1990) as well as behaviour changes, implying that both MeHg and iHg may be damaging for the central nervous system of fish (Berntssen et al., 2003). Despite Hg being recognized as a highly toxic, persistent and ubiquitous contaminant and/or pollutant in aquatic environments, the available knowledge regarding its potential to induce neuronal and sensory dysfunctions in aquatic animals is a

considerably unexplored issue (Pereira et al., 2014). Neurological effects associated with sensorial and behavioral disturbances in fish have been observed with Hg accumulation in the nervous system (Hawryshyn & Mackay, 1979; Baatrup, 1991). Some of those behavioral disturbances are: schooling behavior and delayed spawning (Hammerschmidt et al., 2002; Webber & Haines, 2003), impairments in prey-capture behavior (Smith & Weis, 1997). It was also observed alterations in serotonin levels and anxiogenic-like behavior (Maximino et al., 2010).

The existing studies about the toxic effects of Hg contamination in the neurosensory system are mainly centered in the study of the high impact of Hg contamination in brain tissues, which are primary target for Hg, especially in its organic form (Mieiro et al., 2010 and 2011; Pereira et al., 2014). Also, the significant accumulation of Hg in the astrocytes of fish, present in the brain and spinal cord can lead to complications in the proper functioning of the nervous system and eventual death (Davis et al. 1994; Charleston et al. 1996) due to elevated extracellular glutamate levels and MeHg-induced excitotoxicity (Aschner et al., 2007). Hg-related oxidative damage has also been shown to occur in other sensorial organs such as the inner ear of fish when treated with MeHg. Deposits of this potential neurotoxicant were identified in the apical part of both the receptor cells and the supporting cells of the inner ear, while the iHg deposits were located along the borders of neighboring cells in the sensory epithelium and along the basal membrane in fish exposed to iHg (Sakak and Baatrup, 1993). That study also reported an abnormal swimming behavior and disequilibrium of Hg exposed fish, possibly associated with an impaired inner ear function (Sakak and Baatrup, 1993). Finally, there are only a few studies reporting damages on the vision system of fish due to Hg exposure. Hg contamination has been proven to occur in the ocular areas of fish, especially in the eye wall and lens, with the possibility of causing visual impairment or blindness in the affected organisms (Korbas et al., 2013), possibly related to oxidative stress and ROS formation since mercury induced oxidative stress has already been demonstrated in other organs of fish (Guilherme et al., 2008; Mieiro et al., 2010 and 2011), and adverse effects of oxidative stress have already been assessed in the eyes of other organisms (Spector, 1995; Vinson, 2006). Additionally, Tanan and co-authors (2006) showed electrophysiological anomalous responses on horizontal cells of eyes due to MeHg, while Bonci et al. (2006) found losses of immunoreactivity of specific eye cells after MeHg exposure.

1.5 Thesis outline and scientific impact

The current work was designed to clarify the occurrence of oxidative stress in fish eyes after Hg exposure (iHg and MeHg) under realistic field conditions. For this purpose, an integrative approach was established that combines iHg and MeHg exposure levels, iHg and MeHg accumulation levels and oxidative stress endpoints (enzymatic antioxidants and peroxidative damage) in fish eyes. This approach was replicated in two contrasting seasons (winter vs. summer) in order to cover distinct environmental conditions and availability of both Hg counterparts. The golden grey mullet (*Liza aurata*) was selected as sentinel, since it is a representative fish species of Ria de Aveiro. Fish were captured in two areas of the lagoon with distinct Hg contamination levels, Laranjo basin and São Jacinto. The first area corresponds to the historical hotspot of Hg contamination. The enzymatic parameters measured in order to assess the intensity of the antioxidant response to iHg and MeHg contamination were CAT, SOD, GPx, GST and GR. This study contributes to better understand the underlying mechanisms of Hg toxicity at fish eyes, providing also valuable information for human health. It will be also clarified if the vulnerability of fish eyes to oxidative stress would change in winter and summer conditions in association with accumulated iHg and MeHg levels. Since oxidative stress is a chief event in neurotoxicology, relevant insights are also provided for this scientific field. One of the main impacts of this thesis is related with the Hg neurotoxic potential. After Hg accumulation in eye wall it can lead to alterations in the cellular protection against oxidative stress. Such repercussions could eventually compromise fish performance and survival.

2. Materials and methods

2.1. Study area

The Ria de Aveiro is a large natural water body located in the Northwest (NW) coast of Portugal (40º 38´N, 8º 45´W), and is composed by three main channels: São Jacinto-Ovar, Mira and Ílhavo. It is characterized by narrow channels and by large areas of mud flats and salt marshes. The Ria de Aveiro is a mesotidal lagoon (Davies, 1964) that houses many different aquatic species of flora and fauna, partially or throughout the entirety of their life cycle (depending on the species), playing an important ecological role. The lagoon is about 45 km long and 10 km wide, with an average depth of about 1m, but some of the artificial channels can be as deep as 30 meters (Dias et al., 2000). The chosen areas for sampling were São Jacinto as the control site and the Laranjo Basin (Figure 3), a location widely known for its historical Hg contamination and pollution due to high mercury concentrations that can still be found in the fine surface sediments of this basin despite the fact that industrial mercury discharges have not been made since 1994 (Coelho et al. 2005). This location has been subjected to effluent discharges from a chlor–alkali industry during several decades, which in turn resulted in the accumulation of about 25.4 tons of Hg in the basin and the upstream channel, most of it in the sediment. Although effluent releases stopped in 1994, high Hg concentrations are still found in the surface sediments of this area, and a contamination gradient is observable (Coelho et al., 2005), which makes this area very important for field studies regarding the ecotoxicological effects of Hg.

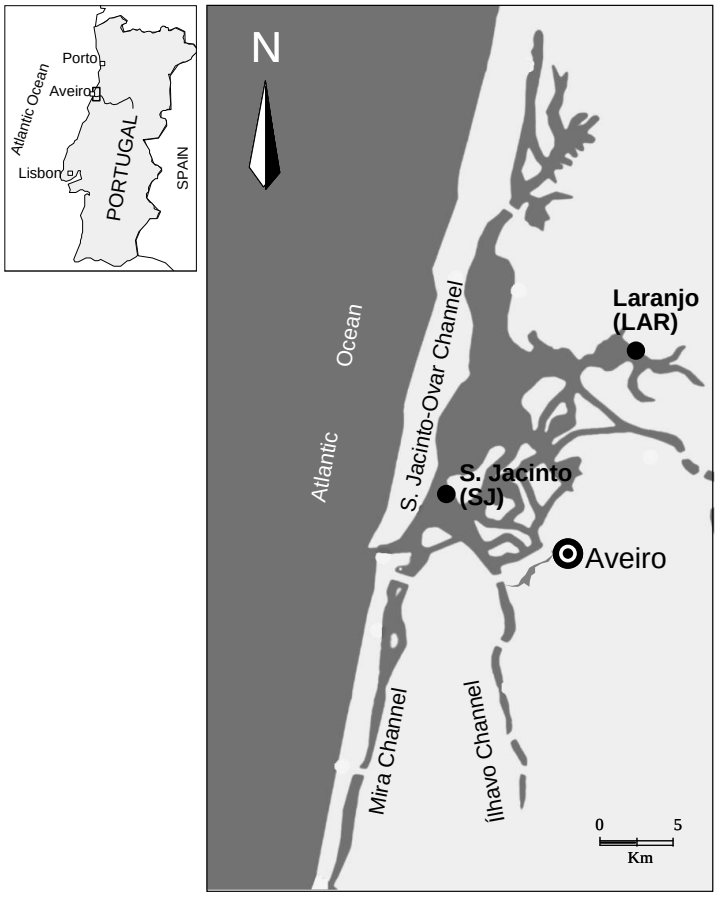


Figure 3 - Location of the sampling sites at Aveiro lagoon (Portugal): São Jacinto (SJ) (40°41′ 00″ N, 8°42′44″W); Laranjo (LAR) (40°43′28.98″ N, 8°37′35.80″ W).

2.2. Sampling

Two surveys were carried out at Aveiro lagoon (Figure 3), in winter (February 2013) and summer (June 2013), during low-tide, and juveniles of the golden grey mullet (*Liza aurata*) were collected (n=20) using a traditional beach-seine net. Two sampling sites were selected taking into account previous ecotoxicological studies (Guilherme et al., 2008; Mieiro et al., 2010): Laranjo (LAR) in the most contaminated area; São Jacinto (SJ) as the reference site. In winter at LAR and SJ, fish total length was 12.4±0.63 and 11.9±0.15 cm, respectively, while in summer it was 13.6±2.1 and 16.5±2.1 cm, respectively. Immediately after catching, fish were anesthetized, sacrificed and properly bled, and then eyes were removed. Eyes were carefully washed with distilled water and gentle rubbing (to remove adherent particles) and dissected for lens removal. The remaining components of the eye, encompassing eye wall (retina, sclera, cornea, ciliar body, etc.), chambers' content (vitreous and aqueous humours) and other small structures (hereafter collectively called "eye wall", to simplify) were stored. In the field, eye wall were instantly frozen in liquid nitrogen. In the

laboratory, samples were preserved at -80 ºC until further processing for Hg and oxidative stress determinations.

Sub-surface water (at 0.2 m depth) was sampled in triplicates to polypropylene bottles for the determination of total Hg (tHg) and MeHg in the dissolved fraction of water column. At the same depth, temperature, salinity and dissolved oxygen were measured *in situ* in triplicates with an YSI 650 meter (Yellow Springs, USA). Surface sediments (approximately 2 cm depth) were collected in the two sites for tHg and MeHg determinations.

2.3 Analytical procedures

2.3.1 Mercury determinations

The technique of atomic absorption spectrometry (AAS) with thermal decomposition following gold amalgamation was used for the quantification of tHg values in the sediment. Using a Hg analyzer (AMA) LECO 254 (Costley et al., 2000), MeHg amounts were determined in dry sediments by alkaline digestion (KOH/MeOH), organic extraction with dichloromethane (DCM) pre-concentration in aqueous sulphide solution, back-extraction into DCM and quantification by GC-AFS in a Agilent Chomatograph coupled with a pyroliser unit and a PSA florescence detector (Canário et al., 2004). Recoveries and the possible MeHg artifact formation were evaluated by spiking several samples with Hg (II) and MeHg standard solutions with different concentrations. Recoveries varied between 97 and 103% and no artifact MeHg formation was observed during this procedure. Precision of Hg analysis, expressed as relative standard deviation (RSD) of 4 replicate samples, was less than 4% ($p<0.05$). Certified reference materials (MESS-2, IAEA-405 and BCR-580) were used to ensure the accuracy of the procedure. Levels of tHg and MeHg obtained in the reference materials were consistent within the ranges of certified values.

After the water samples were briefly preserved by addition of 0.5% BrCl until analysis, which was performed less than one week after collection. The values of total dissolved mercury (tHg) were assessed according to the U.S.EPA method 1631 (U.S. EPA 2002). The samples were then analyzed by cold-vapour atomic fluorescence spectrometry (CV-AFS) with a PSA model Merlin 10.023 equipped with a detector PSA model 10.003 using $SnCl_2$ reduction. BCR-579 reference material was used to control the accuracy of the procedure. The MeHg in water samples was determined following U.S.EPA method 1630 (U.S.EPA, 2001), by distillation of 50 mL sub-samples, after addition of 1% $C_5H_9NS_2.NH_3$ as a complexing agent. Flowingly, Hg was ethylated with $NaB(C_2H_5)_4$, purged with argon, collected on Tenax^{TM} traps, separated with a GC, thermally desorbed to Hg(0) for detection of MeHg with a Brooks Rand Model III CV-AFS. All sets of samples analyzed for MeHg

included at least one method replicate, and at least three analytical replicates of certified reference material (SQC-1238) (Sigma-Aldrich RTC).

For the quantification of MeHg in the test subjects an adaptation of the Westöö (1967) method was used. Approximately 2 mL of Milli-Q water and 3 mL of KOH (6 M) solution were added to 200 mg of dried sample, and the resulting mixture was shaken for 2 hours followed by the adding of 3 mL of HCl (6 M) and 4 mL of a $KBr/CuSO_4$ (3:1) solution. After 10 minutes of shaking, 5 mL of DCM was then added, the mixture centrifuged in a refrigerated centrifuge (Eppendorf 5415R) and finally the organic phase separated. A slight sulphide solution (≈ 0.06 mM) was used to extract MeHg from the organic phase and then MeHg was back extracted to DCM. MeHgin DCM was quantified by GC-AFS using the chromatographic equipment described above. The possible MeHg artifact formation was evaluated by spiking several samples with Hg(II) and MeHg standard solutions of different concentrations. Recoveries varied between 92 and 103% and no artifact MeHg formation was observed. For all the analysis, precision expressed as the relative standard deviation of 3 replicate samples, was less than 2% ($p<0.05$). Certified reference materials (DORM-3, DOLT-4) were used to ensure the accuracy of the procedures.

A crude estimation of the total inorganic iHg concentrations in the eye wall was done by subtracting tHg levels by the corresponding MeHg concentrations, assuming that MeHg is the only organic Hg compound that is bioaccumulated in fish (Zhang and Adeloju, 2012).

2.3.2 Oxidative stress endpoints determination

Tissue samples were homogenized, using a Potter-Elvehjem homogenizer, in chilled phosphate buffer (0.1 M, pH 7.4) (1 g of tissue/10 mL buffer). This homogenate was then divided in two aliquots, one for LPO and protein determination and another for post-microsomal preparation (PMS). The PMS fraction was obtained by centrifugation in a refrigerated centrifuge (Eppendorf 5415R) at 13,400 g for 20 min at 4 ºC. Aliquots of PMS were divided in microtubes and stored at -80 ºC until analyses, which consisted on the following procedures:

- **Catalase (CAT) activity measurement -** Assayed in PMS by Claiborne (1985) method (at 25 ºC) as described by Giri et al. (1996). Briefly, the assay mixture consisted of 1.95 mL phosphate buffer (0.05 M, pH 7.0), 1 mL hydrogen peroxide (0.030 M) and 50 µl of sample in final volume of 3 mL. The absorbance was read every 30 s for a period of 3 min using a spectrophotometer (Jasco UV/VIS, V-530). Change in absorbance was recorded at 240 nm and CAT activity was calculated in

terms of µmol H_2O_2 consumed $min^{-1}mg^{-1}$ protein using a molar extinction coefficient of 43.5 $M^{-1}cm^{-1}$.

- **Superoxide dismutase (SOD) activity measurement -** Measured in PMS (at 25º) using a spectrophotometric enzymatic kit (RANSOD TM, Randox) according to the method of Wolliams et al. (1983). This methodology employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. Changes in absorbance were recorded in 30 second cycles for 3.5 minutes using a spectrophotometer (Jasco UV/VIS, V-530) at 505 nm, and SOD activity was calculated in terms of the percentage of inhibition of the reaction. One unit of SOD is the amount that causes a 50% inhibition of the rate of reduction of INT, under the conditions of the assay. Results were expressed as SOD units/mg^{-1} protein.
- **Glutathione peroxidase (GPx) activity measurement -** Determined in PMS according to the method described by Mohandas et al. (1984) as modified by Athar and Iqbal (1998) (at 25 ºC). The assay mixture consisted of 0.72 mL phosphate buffer (0.05 M, pH 7.0), 0.05 mL EDTA (1 mM), 0.05 mL sodium azide (1 mM), 0.025 mL GR (1 IU/mL), 0.05 mL reduced glutathione (GSH; 10 mM), 0.05 mL NADPH (0.8 mM), 0.005 mL H2O2 (1.0 mM) and 0.05 mL of PMS in a total volume of 1 mL. GPx activity was determined by monitoring the oxidation of NADPH to $NADP^+$, resulting in an absorbance decrease at 340 nm. The absorbance was read every 30 s for a period of 3 min using a spectrophotometer (Jasco UV/VIS, V-530). GPx activity was calculated in terms of nmol NADPH oxidized $min^{-1}mg^{-1}$ protein using a molar extinction coefficient of 6.22x103 $M^{-1}cm^{-1}$.
- **Glutathione reductase (GR) activity measurement -** Assayed by the method of Cribb et al. (1989) with some modifications (at 25 ºC). Briefly, the assay mixture contained 0.025 mL of PMS fraction and 0.975 mL of NADPH (0.2 mM), glutathione dissulfide (GSSG - 1 mM) and diethylene triamine pentaacetic acid (DTPA - 0.5 mM). The enzyme activity was quantified by measuring the disappearance of NADPH at 340 nm during 3 min using a spectophotometer (Jasco UV/VIS, V-530). The enzyme activity was calculated as nmol NADPH oxidized $min^{-1}mg^{-1}$ protein using a molar extinction coefficient of 6.22×103 $M^{-1}cm^{-1}$.
- **Glutathione-S-transferase (GST) activity measurement -** Determined using CDNB (1-chloro-2,4-dinitrobenzene) as substrate according to the method of Habig et al. (1974) (at 25 ºC). The assay was carried out in a quartz cuvette with a 2 mL mixture of phosphate buffer (0.189 M, pH 7.4), CDNB (0.2 mM) and 0.2 M GSH. The

reaction was initiated by addition of 10 µl of PMS and the increase in absorbance was recorded at 340 nm during 3 min using a spectophotometer (Jasco UV/VIS, V-530). Enzyme activity was calculated as nmol CDNB conjugate formed $min^{-1}mg^{-1}$ protein using a molar extinction coefficient of 9.6 $mM^{-1}cm^{-1}$.

Protein content was determined using the previously prepared homogenate, according to the Biuret method (Gornall et al., 1949), using bovine serum albumin (E. Merck-Darmstadt, Germany) as a standard. Absorbance was measured at 550 nm using a SpectraMax 190 microplate reader. An estimation of lipid peroxidation (LPO) was determined, also in the the previously prepared homogenate, as adapted by Filho et al. (2001) after Bird and Draper (1984). Briefly, to 0.05 mL of homogenate, 0.045 mL of phosphate buffer (0.1 M, pH 7.4) and 0.005 mL of 1:1 butylated hydroxytoluene (4 % in methanol) was added and well mixed, followed by the addition of 1 mL of 12 % TCA in aqueous solution, 0.90 mL Tris–HCl (60 mM, pH 7.4, and 0.1 mM DTPA) and 1 mL 0.73 % TBA were added and well mixed. This mixture was heated for 1 h in a water bath set at boiling temperature and then cooled to room temperature, decanted into 2 mL microtubes and centrifuged at 15,800 g for 5 min using an Eppendorf 5415R centrifuge. Absorbance was measured at 535 nm, and LPO was expressed as nanomoles of thiobarbituric acid reactive substances (TBARS) formed per milligram of protein using a molar extinction coefficient of $1.56\times105\ M^{-1}\ cm^{-1}$.

2.4 Data analysis

Statistical software (Statistica 8.0) was used for statistical analyses. All data subsets were first tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) to meet statistical demands. In order to assess the existence of statistical differences, a non-parametric test (Mann Whitney) was performed on the GR and MeHg parameters of the winter season, and a parametric test (T-Student) was performed on all the other parameters, both applied for post-hoc comparison. Differences between means were considered significant when $p<0.05$.

Associations between Hg accumulated levels (iHg and MeHg) and oxidative stress endpoints (CAT, SOD, GPx, GR, GST and LPO) were searched by a multivariate analysis namely by a Principal Component Analysis (PCA). This analysis was performed using the first two factors of the factor plane (PC1 and PC2), which represents a total of 62.09% of variance.

3. Results

3.1 Environmental data

Water temperature was higher at LAR and SJ in summer than winter, while salinity was lower at LAR than SJ, particularly in winter (Table 1). Dissolved oxygen was around 100% but under saturation was recorded at LAR in summer. LAR presented higher levels of total dissolved Hg and MeHg in water than SJ, being these differences accentuated in winter. In this season, an enhanced proportion of Hg was present in the MeHg form relatively to summer at LAR (Table 1).

Table 1 – Water temperature (**T**), salinity, dissolved oxygen (**DO**), total dissolved mercury (**tHg**), total dissolved methylmercury (**MeHg**) and the percentage of MeHg with respect to total mercury. Water samples were collected at low-tide in winter and summer, at São Jacinto (**SJ**) and Laranjo (**LAR**) in Aveiro lagoon. Mean and associated standard deviations are presented. Not determined = n.d.

Season	Site	T (ºC)	Salinity	DO (%)	tHg (ng L^{-1})	MeHg (ng L^{-1})	MeHg (%)
Winter	SJ	13±0.15	31±0.13	94±0.50	<0.1	<0.01	n.d.
	LAR	12±0.00	4.9±0.01	87±0.84	4.4±0.90	1.0±0.24	23±2.4
Summer	SJ	18±0.12	33±0.11	102±1.9	1.0±0.02	0.016±0.007	1.4±0.79
	LAR	18±0.05	21±0.07	65±0.50	1.5±0.77	0.040±0.008	3.0±0.83

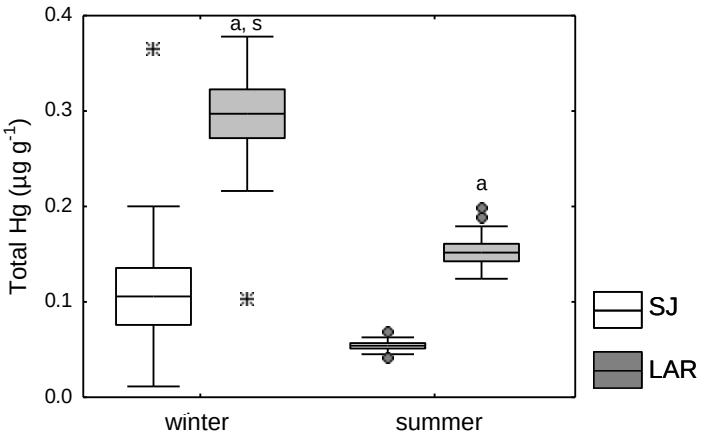
Surface sediments from LAR exhibited higher levels of tHg and MeHg than SJ both in winter and summer (Table 2). Seasonal differences were recorded for tHg and MeHg in sediments, showing levels one order of magnitude higher in winter than summer at LAR. The percentage of Hg in the MeHg form was maxima at LAR in winter.

Table 2 – Total mercury (**Total Hg**), methylmercury (**MeHg**) and the percentage of MeHg with respect to total Hg in surface sediment. Winter and summer data are presented for São Jacinto (**SJ**) and Laranjo (**LAR**) at Aveiro lagoon, and mean and associated standard deviations are presented.

Season	Site	tHg ($\mu g\ g^{-1}$)	MeHg ($\mu g\ g^{-1}$)	MeHg (%)
Winter	SJ	0.021±0.010	0.00005	0.38
	LAR	2.9±0.37	0.029	0.95
Summer	SJ	0.025±0.005	0.0001±0.00002	0.44±0.04
	LAR	0.44±0.25	0.008±0.003	1.9±0.42

3.2 Mercury levels in eye wall

Eye wall from LAR showed significantly higher accumulation of tHg, MeHg and iHg than SJ in both seasons (Figure 4).The percentage of MeHg exhibited the same spatial variation trend. Differences between winter and summer were found for tHg and MeHg with higher levels in winter than summer at LAR. The percentage of MeHg was significantly higher in winter than summer in both sampling sites, while no seasonal differences were found for iHg.



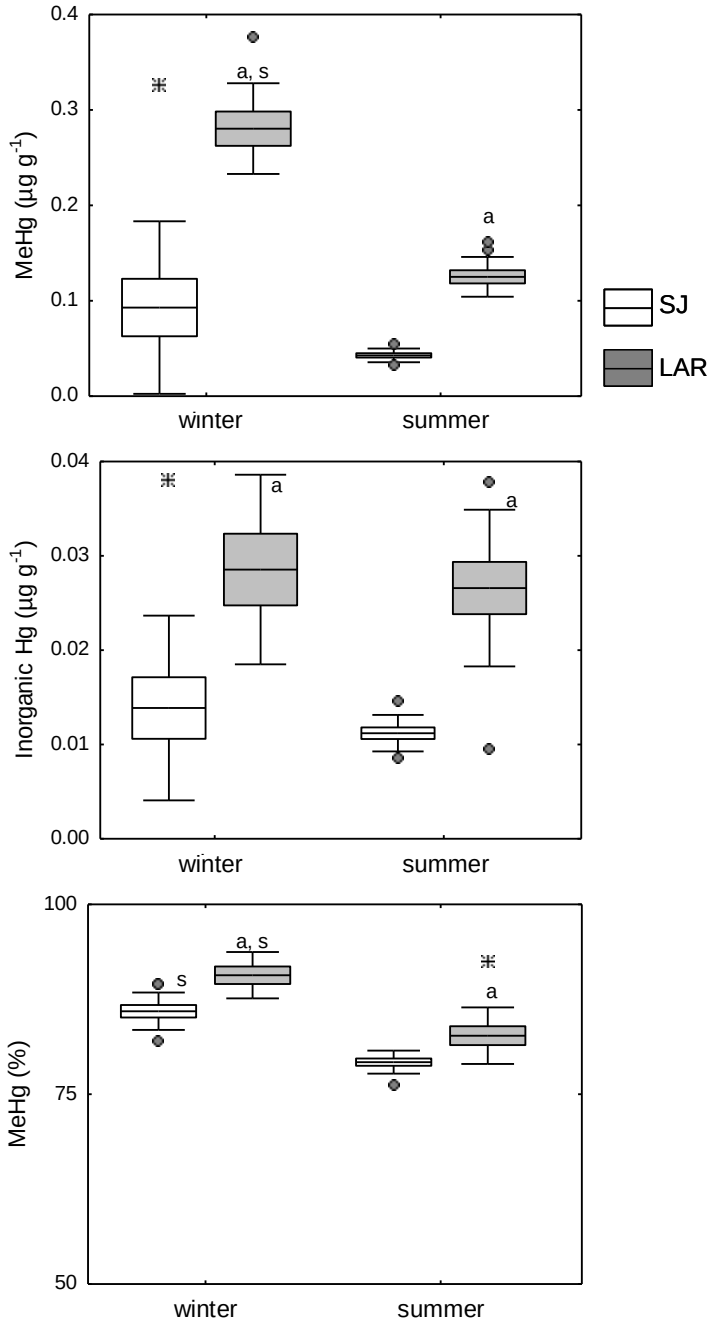
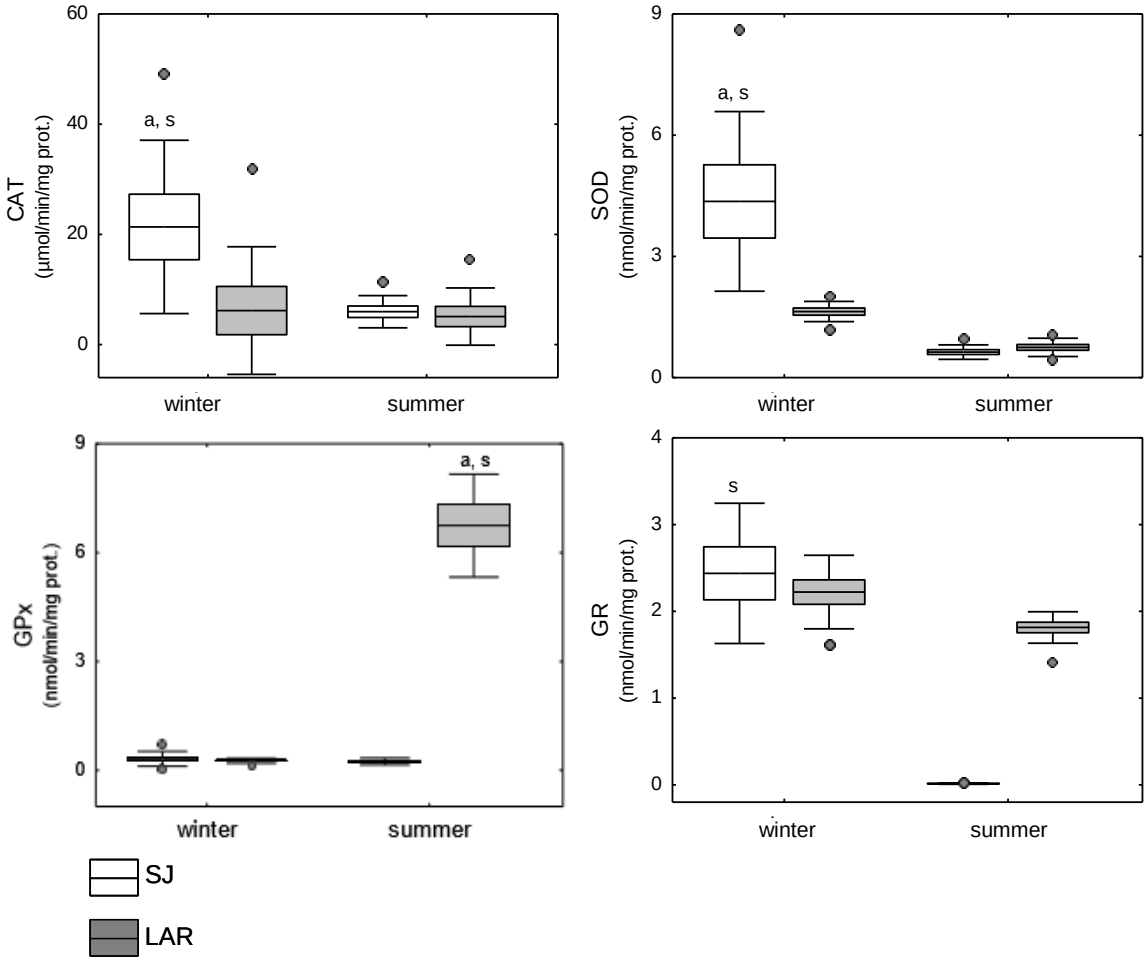


Figure 4 - Total Hg, MeHg, inorganic Hg (µg g-1, dry weight) and % of MeHg (in relation with total Hg) in the eye wall of *L. aurata* captured in winter and summer in Laranjo (**LAR**) and São Jacinto (**SJ**) at Aveiro lagoon. Mean, standard deviation, standard error, outliers (**grey dots**) and extreme values (※) are presented. **a** indicates significant differences between sites (within the same season) and **s** denotes seasonal significant differences (within the same site).

3.3 Oxidative stress profile in eye wall

As displayed on Figure 5, CAT and SOD activities decreased significantly in eye wall of fish from LAR in winter, whereas LPO was significantly higher at LAR in winter, when compared to SJ. No significant spatial differences were found for GR, GPx and GST in eye wall in winter. In summer, a distinct spatial pattern was found, being characterized by significantly higher activities of GPx and GR at LAR in comparison with SJ. Moreover, no significant changes were recorded for CAT, SOD, GST and LPO in summer. CAT, SOD and GR activities were higher in winter than summer in fish from SJ, while GPx was enhanced in summer at LAR.



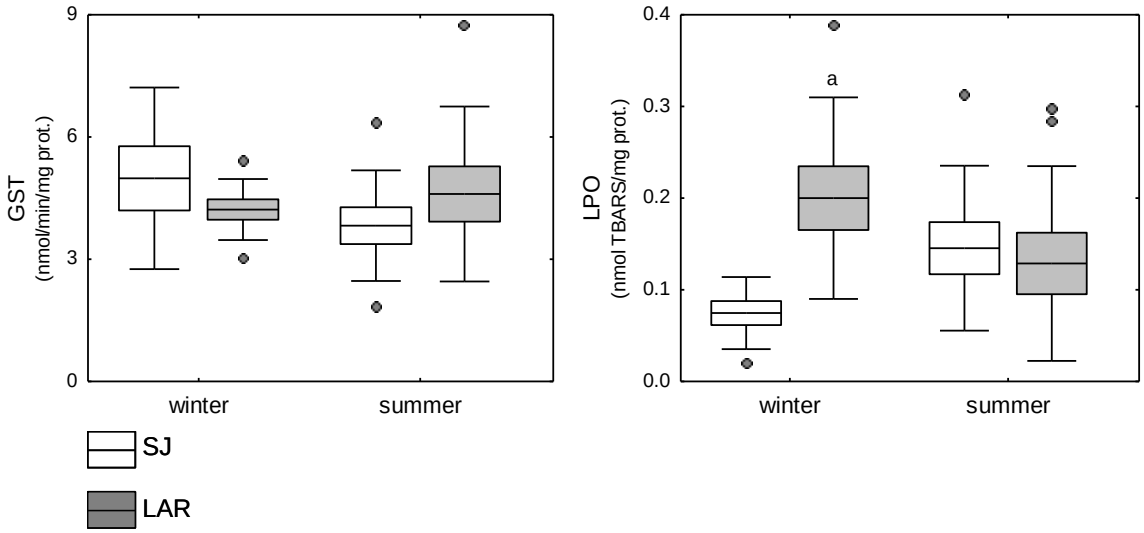


Figure 5 - Measurements of lipid peroxidation and the enzymatic activities of CAT, SOD, GPx, GST, GR and levels of LPO in the eyes of fish from SJ and LAR. Mean, standard deviation, standard error and outliers (**grey dots**) are presented. **a** indicates significant differences between sites (within the same season) and **s** denotes seasonal significant differences (within the same site).

3.4 Association between mercury accumulation and oxidative stress endpoints eye wall

The samples located in the upper right quadrant of the plot (mainly LAR in winter) were associated with higher accumulation levels of iHg and MeHg in eye wall, together with lower CAT, enhanced activity of GPx and higher levels of LPO. In the upper right quadrant it was mainly plotted summer data of SJ, which were mainly characterized by the lower activities of SOD, GR and GST. Samples of SJ in winter were mainly separated by the PC2 being positioning in both lower quadrants of the PCA plot.

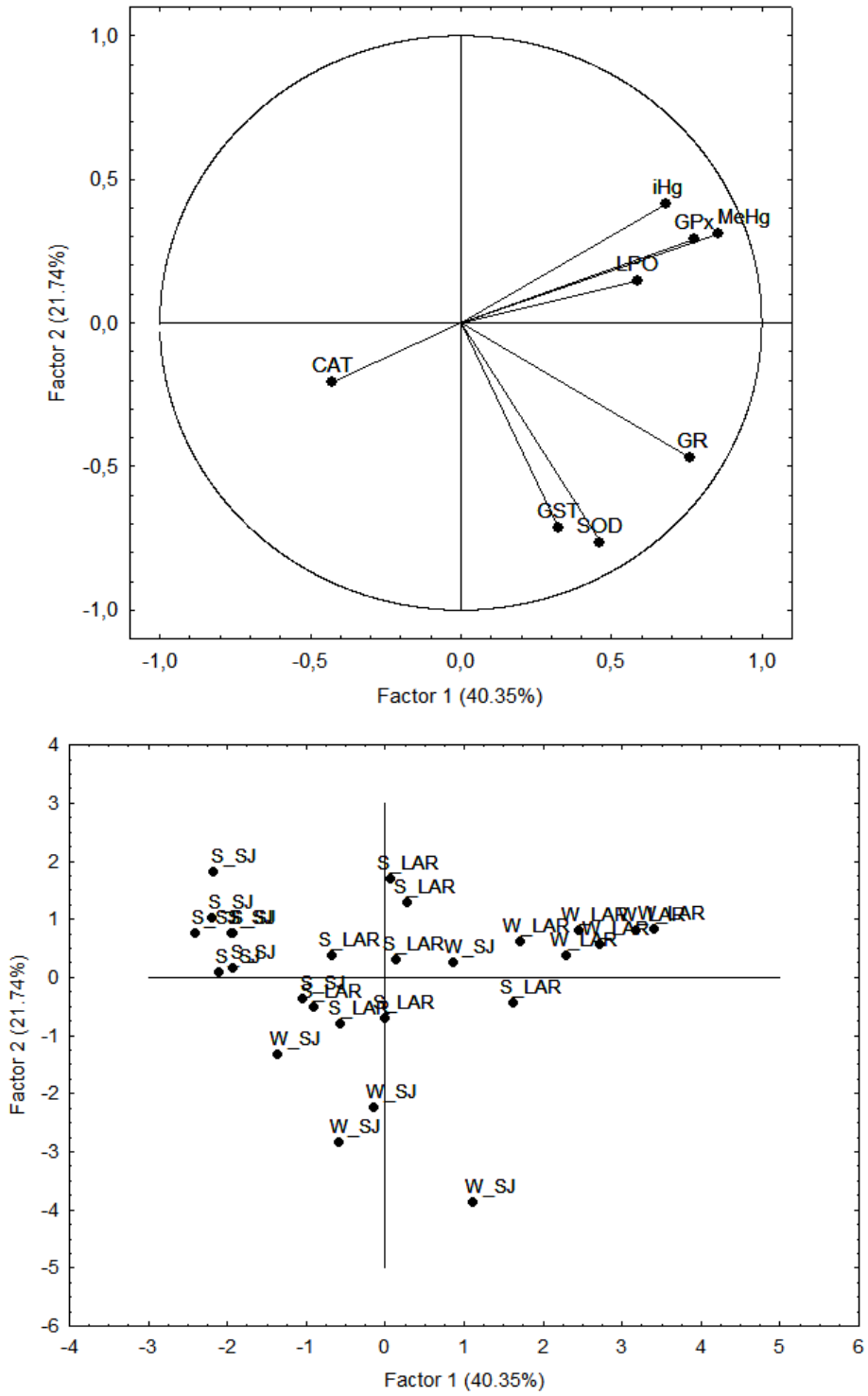


Figure 6 – Principal component analysis based on the oxidative stress responses and accumulated Hg (iHg and MeHg) in eye wall of fish from São Jacinto (SJ) and Laranjo (LAR) in winter and summer. The vectors in Figure 6a represent the variables whereas in Figure 6b are plotted the cases considered in the analysis, i.e. the eye samples collected at LAR in winter (W_LAR) and summer (S_LAR) and the eye samples collected at SJ in winter (W_SJ) and summer (S_SJ).

4. Discussion

4.1. Mercury levels in eye wall and association with environmental availability

LAR exhibited a higher availability of total dissolved Hg (tHg) and MeHg than SJ in both seasons, which is in line with spatial differences previously observed (Mieiro et al., 2011). Differences between the two sampling sites regarding Hg in water were accentuated in winter relatively to summer (44 and 100 times higher for tHg and MeHg in winter, respectively while values only doubled in summer). This is probably due to the higher re-suspension of Hg enriched sediments at Laranjo basin in winter. In fact, sediments from LAR exhibited also higher levels of Hg in winter than in summer (tHg was almost 7 times higher in winter than in summer, while MeHg enhanced 4 times). The spatial contamination trend recorded in water was also found in the sediments with higher levels of tHg and MeHg recorded at LAR than SJ. It was previously documented that LAR sediments are heavily contaminated by Hg and the depth variation reflects the industrial discharges evolution during the last decades (Ramalhosa et al., 2001).

Fish eyes are in permanent and direct contact with dissolved metals and those linked with re-suspended sediment particles. In fact, the significantly higher accumulation of MeHg and iHg (and likewise tHg) in fish eye wall (all ocular components except lens) measured at LAR in winter and summer indicated an enhanced uptake. Inorganic Hg and MeHg levels in eye wall were around 2 to 3 times higher at LAR than SJ in both seasons. These results are in agreement with a previous study that found higher levels of trace elements in fish eyes from a contaminated area of the Tagus estuary (Portugal) (Pereira et al., 2013). Korbas and co-authors (2013) investigated the uptake and accumulation of MeHg in zebrafish larvae and found the highest levels in the secondary lens fibers underlying the lens epithelium. It was also reported that MeHg targets photoreceptors which are directly involved in visual perception (Korbas et al., 2013). Abundant deposits of Hg were also found in the photoreceptor layer and in inner and outer nuclear layers of retina after MeHg exposure of *Danio rerio* (Mela et al., 2010).

Eye wall of *L. aurata* accumulated higher levels of MeHg (and tHg) in winter than summer at LAR. This is in agreement with the higher environmental availability of Hg (including in the MeHg form) in winter relatively to summer. Besides that, the influence of water salinity on Hg accumulation should be considered since values at LAR were 4-fold lower in winter than those recorded in summer. It was previously reported that tHg accumulation in crabs from Aveiro lagoon was favored by low salinity (Pereira et al., 2006), consistent with the current data on fish. Mercury is able to form strong inorganic complexes with chloride at saline and oxygen-rich waters (Conaway et al., 2003). In line, current data revealed a higher percentage of

dissolved MeHg at LAR in winter (when salinity was lower) than in summer. Since MeHg counterparts are highly accumulated in fish comparing to chloride Hg forms (Korbas et al., 2012), water salinity could indirectly influence the accumulated Hg levels. Elevated water temperature can also increase metals accumulation associated with the higher metabolism of ectothermic organisms (Sokolova and Lanning, 2008). Current data revealed a higher accumulation in winter relatively to summer but only at LAR. This suggests a minor role of temperature on Hg accumulation.

Despite the direct contact of fish eye with water, this organ seems to be, in some extent, impervious to the dissolved iHg, being thus physiologically protected due to the mucus membrane that protects the outer layer of the eye that is in direct contact with the water column (Kulczycka, 1965). Mercury data from an unpublished experiment (Pereira et al.) revealed that this element mainly targets eyes through the bloodstream, by which we can infer that blood is probably the main vehicle of iHg to fish eyes. Blood distribution was also previously identified as the main pathway of MeHg to zebrafish eyes (Korbas et al., 2013) and invoked to justified iHg and MeHg accumulation in wild fish (Pereira et al., 2013). Despite the similar pathway of iHg and MeHg transport to fish eyes, the toxicological role of both mercury counterparts remains a matter of debate. Some authors stated that the different forms of Hg share the same toxic entity, being the toxicity of iHg *versus* MeHg mainly dependent on bioavailability (DeFlora et al., 1994). In opposition, other authors stated that iHg and MeHg have different physical-chemical properties and toxicity profiles (Clarkson, 1997). Nevertheless, both Hg forms can induce a wide range of toxic effects in fish (Crespo-López et al., 2007). Oxidative stress has been described as one of the chief mechanism of mercury toxicity in fish but little is known about its occurrence in eyes related with iHg and MeHg accumulation.

4.2. Oxidative stress profile in eye wall

4.2.1. Spatial variations

The formation of ROS has been identified has a chief mechanism of Hg toxicity (Shanker and Aschner, 2003; Roos et al., 2009). Also, the depletion of GSH and antioxidant enzymes has been referred as a consequence of the pro-oxidative ability of Hg (Stringari et al., 2008; Roos et al., 2009). In accordance, CAT and SOD activities depletion were currently detected in *L. aurata* eye wall from the most contaminated area (LAR) (in winter period). Such depletion was in line with the higher accumulation of iHg and MeHg (and consequently tHg). This inhibitory effect of Hg on critical steps of the antioxidant process would potentially exacerbate the pro-oxidant properties of Hg. It is important to highlight that would be an indirect effect since Hg is not a redox active metal (Ercal et al., 2001). The inhibition of CAT will probably

lead to the enhancement of cellular levels of hydrogen peroxide, while SOD will result in the accumulation of superoxide anion radicals that could not be converted into oxygen. The increment of those oxygen free radicals would promote cellular oxidative stress conditions in eye wall of fish. Indeed, CAT of rabbit eye affords protection to the lens from hydrogen peroxide and it also protects superoxide dismutase of lens from inactivation by hydrogen peroxide. Superoxide dismutase, in turn, protects the lens from the superoxide radical (Bhuyan and Bhuyan, 1978). Additionally, the concomitant inhibition of CAT and SOD in rabbit eyes would lead to the production of the highly reactive oxidant, the hydroxyl radical, which would occur under pathological conditions such as eye cataracts (Bhuyan and Bhuyan, 1978). Accordingly, the inactivation of CAT and SOD may result in an elevation of H_2O_2 and $O_2.^-$ levels in human lens, which may be responsible for the oxidative modification of lens proteins observed in cataracts (Fecondo and Augusteyn, 1983). Based on the concurrent inhibition of CAT and SOD in eye wall, an identical pathology could be occurring in fish from LAR related with Hg accumulation suggesting Hg as a potent cataractogenic agent.

CAT activity was not previously reported in fish eye wall. However, current data of winter season are in agreement with recent studies conducted in the same contaminated system with fish brain (Mieiro et al., 2012). Indeed, it was also found a significant decrease of CAT activity in brain of fish from LAR, being associated with higher Hg accumulation. In fact, CAT has being described as a very sensitive parameter concerning ROS formation (Ahmad et al., 2009; Maria et al., 2009) and its decrease was normally associated with a depletion of antioxidant defenses (Bagnyukova et al., 2005a). This is the first report of SOD in fish eyes which prevents the comparison of current data with previous ones. Differently from CAT, no reports are available for SOD in fish brain from Aveiro lagoon.

It is well known that the action of an antioxidant enzyme can be replaced by the activity of other antioxidants (Bagnyukova et al., 2005a). CAT inhibition at LAR in winter could be compensated by an increment of GPx activity since both enzymes catalyse hydrogen peroxide. However, GPx activity did not increase significantly in fish eye wall from LAR (in winter). Such enzymatic compensation was already hypothesized to being occurring in fish brain at Aveiro lagoon due to mercury exposure but similarly it was not confirmed (Mieiro et al., 2012). Despite the key role of GR in glutathione recycling in order to maintain the proper GSH redox status, no significant changes were recorded at LAR in winter. The same spatial pattern was found for GST.

Free radical reactions in biological membranes could form lipid hydroperoxides that decompose double bonds of unsaturated fatty acids, destructing lipid membranes (Van der Oost et al., 2003). This complex process is known as lipid peroxidation (LPO) that can provide

information on the organ threshold limits and being thus an endpoint of toxicity expression. Current data revealed higher LPO in eye wall of fish from LAR (in winter), which is an indication of oxidative stress. The breakdown of the redox-defence system depicted in the inhibition of CAT and SOD in eye wall from LAR could be on the basis of LPO occurrence. This hypothesis is supported by the PCA analysis output that displayed in the upper right quadrant LPO together with iHg and MeHg in opposition to CAT (lower right quadrant). In fact, some authors acknowledge that the pro-oxidant properties of metals, including Hg, are intensified by their inhibitory effects on antioxidant processes (Stohs and Bagchi, 1995), thereby enhancing the risk of LPO. The occurrence of peroxidative damage in eyes of fish captured at LAR in winter reinforces the previous statement that Hg is a cataractogenic agent.

The analysis of response profiles in parallel with iHg and MeHg in eye wall may provide relevant information on the organ-specific threshold limits to express signs of toxicity. In this view, the PCA revealed an association between iHg and MeHg loads and the vulnerability towards the breakdown of the redox-defense system, since iHg and MeHg were negatively related with CAT in eye wall.

In summer, GPx and GR induction signaled a pro-oxidant challenge in eye wall of fish from the most contaminated area, which is in agreement with the higher accumulation both of iHg and MeHg. In fish, the principal peroxidase is a selenium-dependent tetrameric cytosolic enzyme (GPx) that employs GSH as a cofactor. GPx catalyses the metabolism of hydrogen peroxide to water with the concomitant conversion of reduced glutathione (GSH) to its oxidized form - glutathione disulfide (GSSG). The enhanced GR activity in eye wall from LAR pointed out higher glutathione recycling in order to maintain the proper GSH redox status and avoid its depletion. The restoration of GSH pools is responsible for continuously providing GSH as substrate for GPx and GST, and thus indicating high precocity and sensitivity to Hg contamination (Mieiro et al., 2012).

In summer, no LPO occurred despite the accumulation of iHg and MeHg in eye wall. This indicates that the activities enhancements of GPx and GR were sufficient to counterbalanced the excessive ROS production related with Hg accumulation. Besides the protective role of GSH, suggested by GR induction, other protective mechanisms could be acting as previously invoked by Mieiro and co-authors for fish brain. Firstly, the increment of other non-enzymatic antioxidant defenses may have a protective effect against LPO, namely cystein, alpha tocopherol and ascorbic acid. Moreover, the production of metallothioneins (MTs) in eye wall of fish, or allocation from other tissues, could be an important protective mechanism. In fact, the binding of Hg to Mts represents a sequestration process that unables its interaction with cellular key molecules (Navarro et al., 2009). It was previously described that MTs mitigate cell

destruction induced by oxidative stress in the eye by capturing and neutralizing free radicals through cysteine sulfur ligands (Vasák, 2005). Indeed, it was found an over-expression of MTIIa in human lens epithelial cells related with oxidative stress caused by cadmium (Hawse et al., 2006). The eye is also highly protected by lutein that exerts both antioxidant and anti-inflammatory effects (Kim et al., 2012). The carotenoid lutein is preferentially accumulated in the macular region of the human retina where it is known to protect the eyes against light damage, preventing the development of degenerative conditions in the eye including age-related macular degeneration (AMD) (Kim et al., 2012).

4.2.2. Winter-summer changes

The distinct patterns observed in winter and summer for enzymatic antioxidants and lipid peroxidation stimulated the discussion about toxicity of iHg vs. MeHg in fish eye wall. At LAR, fish eye wall presented significantly higher levels of MeHg (and tHg) in winter than summer, while no seasonal changes were recorded for iHg. Such higher levels of MeHg in eye wall inhibited both CAT and SOD, allowing LPO occurrence. This indicates that under enhanced environmental availability of MeHg, this Hg counterpart is highly accumulated in the eye wall and consequently oxidative stress occurs probably due to the depletion of enzymatic antioxidants. Contrastingly, in summer, no cellular damage (measured as LPO) was noticed in eye wall at LAR and only GPx and GR were enhanced. This is in agreement with lower levels of MeHg accumulation. Winter-summer changes were previously found in oxidative stress endpoints in *L. aurata* brain from the Aveiro lagoon (Mieiro et al., 2012). The winter-summer profiles of oxidative stress suggest that the MeHg levels measured in the eye wall of *L. aurata* in winter exceeded the threshold with respect to oxidative defence impairment.

Fish eye wall was able to detect the higher contamination at LAR than SJ. Moreover, environmental health assessment should be performed in distinct temporal periods due to changes on availability of contaminants and organisms' physiology. Eye wall signalized faithfully winter-summer differences of iHg and MeHg in water column and sediment. From the analytical perspective, eye wall displayed relatively high Hg levels (mainly MeHg) and a sufficient amount of mass for analysis. Eyes are interesting due to the possibility of Hg determinations in different compartments (like lens in the eyes) and the quantification of distinct organic counterparts (e.g. MeHg).

5. Conclusions

According to the results, it can be concluded that:

- The oxidative stress responses of *L. aurata* eye wall were able to detect inter-site differences, reinforcing that LAR is a critical area in the Aveiro lagoon. Higher levels of accumulated iHg and MeHg can be on the basis of the pro-oxidant challenge at LAR;
- Winter-summer variations were prevalent in eye wall of *L. aurata*, which exhibited a higher accumulation of MeHg in winter as well as higher vulnerability towards oxidative stress. In fact, peroxidative damage occurred at LAR in winter probably due to MeHg inactivation of antioxidant defenses (CAT and SOD);
- This thesis pointed out the importance of evaluating changes in eye wall at structural and functional levels in order to examine in what extent accumulated Hg could compromise neurosensory processes.

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