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Mercury Accumulation, Structural Damages, and Antioxidant and Immune Status Changes in the Gilthead Seabream (*Sparus aurata* L.) Exposed to Methylmercury

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Abstract In aquatic systems, mercury (Hg) is an environmental contaminant that causes acute and chronic damage to multiple organs. In fish, practically all of the organic Hg found is in the form of methylmercury (MeHg), which has been associated with animal and human health problems. This study evaluates the impact of waterborne-exposure to sublethal concentrations of MeHg (10 μg L^{-1}) in gilthead seabream (*Sparus aurata*). Hg was seen to accumulate in liver and muscle, and histopathological damage to skin and liver was detected. Fish exposed to MeHg showed a decreased biological antioxidant potential and increased levels of the reactive oxygen molecules compared with the values found in control fish (nonexposed). Increased liver antioxidant enzyme activities (superoxide dismutase and catalase) were detected in 2 day-exposed fish with respect to the values of control fish. However, fish exposed to MeHg for 10 days showed liver antioxidant enzyme levels similar to those of the control fish but had increased hepato-somatic index and histopathological alterations in liver and skin. Serum complement levels were higher in fish exposed to MeHg for 30 days than in control fish. Moreover, head–kidney leukocyte activities increased, although only phagocytosis and peroxidase activities showed a significant increase after 10 and 30 days, respectively. The data show that 30 days of exposure to waterborne MeHg provokes more significant changes in fish than a short-term exposure of 2 or 10 days.

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Methylmercury (MeHg), the most common and toxic form of organic mercury (Hg), is an environmental contaminant produced from metallic, inorganic, or organic Hg by sulphate-reducing microorganisms present in sediments (Benoit et al. 1998). Hg is released into the environment as a result of anthropogenic and natural activities; it is transformed into MeHg by anaerobic bacteria and rapidly taken up by living organisms and biomagnified through the marine food chain. MeHg represents a hazard to higher trophic level organisms, including humans, that largely accumulate Hg through the consumption of fish (Bourdineaud et al. 2011; Nøstbakken et al. 2012).The European Food Safety Authority recommends certain restrictions concerning the consumption of marine species, especially predatory species, which tend to be long live and accumulate Hg over their lifetime (Falcó et al. 2006).

In fish, practically all organic Hg is in the form of MeHg (Baatrup 1991). Four possible routes of metal absorption by fish exist: food intake, drinking water, through the gills, and by absorption through the skin (Sindayigaya et al. 1994). As a result, heavy metals can accumulate in multiple tissues and organs (e.g., liver, kidney, and muscle) (Sandheinrich and Wiener 2011; Depew et al. 2012). Several studies have shown that MeHg dietary intake or injection can affect fish biology. For example, MeHg has been seen to alter several functions in fish brain including cell

structural degeneration, calcium homeostasis, oxidative system, metabolic markers, and visual deficits (Berntssen et al. 2003; Weber et al. 2008; Berg et al. 2010). Furthermore, exposure to MeHg was seen to have toxic effects on reproductive organs (Drevnick et al. 2006; Klaper et al. 2006). Other effects of MeHg include alterations in the mitochondrial energy metabolism in skeletal muscle (Cambier et al. 2009), inhibition of the thioredoxin system in liver (Branco et al. 2011), delayed mortality syndrome (Samson et al. 2001), decreased larval swimming speed (Murphy et al. 2008), and impaired survival of larvae after maternal exposure to MeHg (Alvarez et al. 2006). It has been shown that MeHg causes delayed growth (Houck and Cech 2004), hyperplasia in gill epithelium, and alterations in the gene-expression profiles of several organs (including liver, gonad, and muscle) (Gonzalez et al. 2005; Klaper et al. 2006; Cambier et al. 2009).

To date, very few vertebrate studies have assessed the effects of Hg on the immune system (reviewed by Sweet and Zelikoff 2001). Low and Sin (1998) did, however, show that exposure to low or high Hg concentrations produce immune activation or depression, respectively. For example, in vitro exposure to 0.045 mg L^{-1} $HgCl_2$ (165 nM) induced lymphocyte mitosis in blue gourami (*Trichogaster trichopter*), whereas higher concentrations inhibited it. Regarding MeHg, its in vitro EC_{50} dose after 24 h of exposure for seabream head–kidney (HK) leukocytes and the SAF-1 cell line was 12 and 15 μM, respectively (Morcillo et al. 2016, 2015). In cells of the SAF-1 cell line incubated with MeHg, an increase in the production of reactive oxygen species (ROS) and apoptosis cell death was observed. Furthermore, the expression of different genes pointed to induction of the metallothionein protective system, cellular and oxidative stress, and apoptosis after 24 h of exposure (Morcillo et al. 2016). Increased production of free oxygen radicals and the percentage of phagocytic cells, but a decrease in the number of ingested particles and respiratory burst activity, were observed in HK leukocytes of gilthead seabream exposed to Hg (in the form of both MeHg and $HgCl_2$) (Morcillo et al. 2015). The increased production of macrophage-activation factor and higher respiratory burst and phagocytic activities were also shown in HK macrophages of European sea bass (*Dicentrarchus labrax*) exposed to 2–20 μM Hg chloride (Sarmento et al. 2004).

In the available in vivo studies performed in fish, acute toxicity effects were observed at very different concentrations of MeHg, although they were always more severe in the case of the $HgCl_2$ form. For example, waterborne exposure to 0.05 ppb $HgCl_2$ inhibited phagocytosis, respiratory burst activity, lymphocyte mitogenesis, and immunoglobulin M (IgM) production and increased serum lysozyme activity in the rainbow trout (*Oncorhynchus mykiss*) (Sanchez-Dardon et al. 1999). However, these activities remained unchanged in exposed Japanese medaka (*Oryzias latipes*) (Zelikoff 1998).

Very little is known about the effects of MeHg exposure in fish, and the comparison of any results is very difficult and sometimes contradictory because different investigators have used a variety of administration methods as well as concentrations of MeHg. For example, waterborne exposure to 9 ppb MeHg decreased specific antibody titres in the blue gourami (Roales and Perlmutter 1974). However, increased blood leukocyte and neutrophil counts were observed in tigerfish (*Hoplias malabaricus*) intraperitoneally injected with 0.075 μg g^{-1} (Oliveira Ribeiro et al. 2006). The dietary administration of 2 mg kg^{-1}MeHg altered the expression of some immune-related genes in the Atlantic cod (*Gadus morhua*) (Yadetie et al. 2013). Exposure to levels ranging from 4 to 34 μg MeHg L^{-1} resulted in rainbow trout death after 100 and 2 days, respectively (Niimi and Kissoon 1994). Finally, Samson et al. (2001) observed delayed mortality syndrome in zebrafish (*Danio rerio*) embryos exposed for short periods of time to concentrations of 5–15 μg L^{-1}MeHg.

Taking into consideration the results of previous available studies and the Hg concentrations reported for the Mediterranean Sea (0.05–2.87 μg L^{-1}) (Hamed et al. 2013), we exposed gilthead seabream (*S. aurata* L.) specimens to sublethal concentrations of waterborne MeHg [10 μg L^{-1} (equivalent to 40 nM or 10 ppb)] to ascertain the effect of MeHg on the main parameters of the immune system in this fish. Furthermore, its accumulation in liver and muscle, as well as its histopathology in liver, skin, and liver antioxidant enzymes, were also determined.

Materials and Methods

Animals

Thirty-six (weight 121 ± 30 g and length 20 ± 1.5 cm) specimens of the hermaphroditic protandrous seawater teleost gilthead seabream obtained from the Instituto Español de Oceanografía (Mazarrón, Spain), were kept in recirculating seawater aquaria (250 L) in the Marine Fish Facility at the University of Murcia. The water temperature was maintained at 20 ± 2 °C with a flow rate of 900 L h^{-1} and 28 ‰ salinity. The photoperiod was of 12 h of light to 12 h of darkness, and fish were fed with a commercial pellet diet (Skretting, Spain) at a rate of 2 % body weight (bw) day^{-1}. Fish were allowed to acclimatise for 15 days before the start of the experimental trial. All experimental protocols were approved by the Ethical Committee of the University of Murcia.

Experimental Design

Fish were randomly assigned and divided into two identical tanks: Fish in one tank were maintained unexposed (control group), and fish in the other tank were exposed to 10 μg L^{-1} (40 nM) of MeHg(II)chloride (CH_3HgCl; Sigma, Spain). Six fish per tank and group were sampled after 2, 10, or 30 days of exposure.

Sample Collection

Specimens were weighted; blood samples were collected from the caudal vein with an insulin syringe and the skin; and HK, liver, and spleen were dissected. The liver and spleen were weighted. Fragments of liver and muscle were obtained and stored at −80 °C for later determination of Hg accumulation. Blood samples were left to clot at 4 °C for 4 h, and later the serum was collected after centrifugation (at 10,000 g for 5 min at 4 °C) and stored at −80 °C until use. Fragments of liver and skin were processed for light microscopy analysis. Liver fragments were also processed for evaluating antioxidant enzymes. HK samples were cut into small fragments and transferred to 8 ml of sRPMI (RPMI-1640 culture medium; Gibco, USA) supplemented with 0.35 % sodium chloride (to adjust the medium's osmolarity to seabream plasma osmolarity of 353.33 mOs), 2 % foetal calf serum (Gibco, USA), 100 IU ml^{-1} penicillin (Sigma, Spain) and 100 μg ml^{-1} streptomycin (Sigma, Spain) for leukocyte isolation (Esteban et al. 1998). Cell suspensions were obtained by forcing fragments of the organ through a nylon mesh (mesh size 100 μm), washed twice (at 400 g for 10 min), counted (Z2 Coulter Particle Counter), and adjusted to 10^7 cells ml^{-1} in sRPMI. Cell viability was >98 % as determined by trypan blue exclusion test. All of the cellular immune functions were performed only in viable cells.

Determination of Organo-Somatic Indexes

Whole body, liver, and spleen were weighted, and the organo-somatic index (OSI) for liver and spleen was calculated using the following formula: OSI = (g tissue g $body^{-1}$) × 100 (Grinwis et al. 2000).

Liver and Muscle Analysis of Total Hg and Selenium

Samples were analyzed for the content of Hg and selenium (Se) by inductively coupled plasma–mass spectrometry (Agilent 7500CE) with collision/reaction cell using pure helium cell gas. Liver and muscle samples (0.2 g) were treated with trace mineral–grade nitric acid, 69 % HNO_3PA-ISO (Hiperpure, Panreac, Spain), and 33 % H_2O_2 (Suprapure, Merck, Germany) in special Teflon reaction tubes, treated in a microwave digestion system (UltraWave, Milestone) for 40 min at an increasing temperature range (100–220 °C), and finally diluted to 25 ml with double-deionized water MilliQ. Isotope and internal standard, respectively, were 202 and thallium-205 (Hg) and 78 and gallium-71 (Se). Three readings for each sample were made, and the concentration values were obtained from the mean of the readings. Calibration standards (LGC Standard, VHG Labs, USA) were PSEN-100 (Se) and PHGN-100 (Hg), both in 5 %HNO_3, and blank samples (1 sample for every 10 samples) were also analysed. The detection limit was 0.05 and 0.48 ng g^{-1} for Hg-202 and Se-78, respectively. All concentrations are expressed as microgram per gram (ppm) of tissue wet weight.

Oxidative Stress

Oxidative stress was determined in both serum and liver homogenates. Serum antioxidant and pro-oxidant status was measured by biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) kits according to the manufacturer (Diacron International S.R.L., Italy). Briefly, 10 or 5 μl of serum for BAP or d-ROMs measurement, respectively, were used in the kit, and the absorbance was read at 505 nm (BOECO, S-22 UV/VIS). BAP values were expressed as μmol antioxidant substance L^{-1} of vitamin C, which was used as an iron-reducing agent reference. In case of d-ROMs, free radicals were expressed in Carratelli units (1 unit = hydrogen peroxide concentration of 0.08 % mg).

Liver homogenates were used to determine superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR) enzymatic activities Liver samples were homogenized in 50 mM potassium phosphate buffer (pH 7.0) and centrifuged (at 10,000 g for 10 min) to obtain the supernatants, which were used to determine the enzymatic activities. SOD activity was determined by the inhibition of the reduction of cytochrome C (McCord and Fridovich 1969). The superoxide radical anion was generated in situ by the xanthine oxidase reaction and detected by monitoring the formation of the reduced form of cytochrome C at 550 nm (Thermo Scientific model Evolution 300 dual-beam spectrophotometer). CAT activity was measured by monitoring the consumption of H_2O_2 at 240 nm (Aebi 1984). This method is based on the principle that the absorbance will decrease due to the decomposition of hydrogen peroxide by CAT at 240 nm in a spectrophotometer. The amount of H_2O_2 converted into H_2O and O_2 in 1 min under standard conditions was accepted as the enzyme-reaction rate. GR was measured by the method modified by Carlberg and Mannervik (1975). The reaction was initiated by the addition of 0.1 mM nicotinamide adenine dinucleotide phosphate to the mixture of enzyme in 50 mM potassium phosphate buffer (pH 7.0) containing

2 mM ethylene diamine tetra acetic acid and 0.5 mM glutathione disulfide. The change in absorbance was monitored at 340 nm for 3 min in a spectrophotometer. One unit of GR activity is defined as the amount of enzyme that catalyzes the reduction of 1 μmol of NADPH/min (ε340 nm for NADPH = 6.22 mM^{-1} cm^{-1}).

Histological Analysis

For histological analysis, liver and skin samples were fixed with 10 % neutral buffered formalin (Panreac, Spain) at room temperature for 24 h, embedded in paraffin (Thermo Scientific), and sectioned at 5 μm. Sections were dewaxed, rehydrated, and stained with haematoxylin-eosin (HE). Slides were analysed by a light microscope (Leica 6000B), and images were acquired with a Leica DFC280 digital camera.

Immune Parameters

Natural Hemolytic Complement Activity

The activity of the alternative complement pathway was assayed using sheep red blood cells (SRBC; Biomedics, Spain) as targets (Ortuño et al. 1998). Equal volumes of SRBC suspension (6 %) in phenol red-free Hank's buffer (HBSS) containing Mg^{+2} and ethylene glycol tetra acetic acid were mixed with serially diluted serum to give final serum concentrations ranging from 10 to 0.078 %. After incubation for 90 min at 22 °C, the samples were centrifuged (400 g for 5 min at 4 °C) to avoid unlysed erythrocytes. The relative haemoglobin content of the supernatants was assessed by measuring their optical density (OD) at 550 nm in a plate reader (BMG Labtech). The values of maximum (100 %) and minimum (spontaneous) haemolysis were obtained by adding 100 μl of distilled water or HBSS to 100 μl samples of SRBC, respectively. The degree of haemolysis (Y) was estimated, and the lysis curve for each specimen was obtained by plotting $Y(1-Y)^{-1}$ against the volume of serum added (ml) on a log–log scaled graph. The volume of serum producing 50 % haemolysis (ACH_{50}) was determined as was the number of ACH_{50} units ml^{-1} obtained for each experimental fish.

Serum and Leukocyte Peroxidase Activity

The peroxidase activity in serum or leukocytes was measured according to Quade and Roth (1997). Briefly, 15 μl of serum were diluted with 135 μl of HBSS without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. Fifty microliters of 20 mM 3,3′,5,5′- tetramethylbenzidine hydrochloride (TMB; Sigma, Spain) and 5 m MH_2O_2 were added. To determine the leukocyte peroxidase content, 10^6 HK leukocytes in sRPMI were lysed with 0.002 % cetyltrimethylammonium bromide (Sigma, Spain) and, after centrifugation (at 400 g for 10 min), 150 μl of the supernatants were transferred to a fresh 96-well plate containing 25 μl of 10 mM TMB and 5 mM H_2O_2. In both cases, the colour-change reaction was stopped after 2 min by adding 50 μl of 2 M sulphuric acid, and the OD was read at 450 nm in a plate reader. Standard samples without serum or leukocytes, respectively, were used as blanks.

Serum IgM Level

Total serum IgM levels were analyzed using enzyme-linked immunosorbent assay (Cuesta et al. 2004). Thus, 20 μl/well of 1/100 diluted serum were placed in flat-bottomed 96-well plates in triplicate, and the proteins were coated by overnight incubation at 4 °C with 200 μl of carbonate-bicarbonate buffer [35 mM $NaHCO_3$ and 15 mM Na_2CO_3 (pH 9.6)]. After three rinses with PBT [20 mMTris-HCl, 150 mMNaCl and 0.05 % Tween 20 (pH 7.3)], the plates were blocked for 2 h at room temperature with blocking buffer containing 3 % bovine serum albumin (Sigma, Spain) in PBT followed by three rinses with PBT only. The plates were then incubated for 1 h with 100 μl/well of mouse anti-gilthead seabream IgM monoclonal antibody (Aquatic Diagnostics Ltd., UK) (1/100 in blocking buffer), washed, and incubated with the secondary antibody antimouse IgG-HRP (1/1000 in blocking buffer, Sigma, Spain). After exhaustive rinsing with PBT, the plates were developed using 100 μl of a 0.42 mM TMB solution, prepared daily in a 100 mM citric acid/sodium acetate buffer (pH 5.4) containing 0.01 % H_2O_2. The reaction was allowed to proceed for 10 min and was stopped by the addition of 50 μl of 2 M H_2SO_4; the plates were read at 450 nm. Negative controls consisted of samples without serum or without primary antibody whose OD values were subtracted for each sample value.

Respiratory Burst Activity

The respiratory burst activity of gilthead seabream HK leukocytes was studied by a chemiluminescence method (Bayne and Levy 1991). Briefly, samples of 10^6 leukocytes in sRPMI were placed in the wells of a flat-bottomed 96-well microtiter plate, to which 100 μl of HBSS containing 1 μg ml^{-1}phorbolmyristate acetate (PMA; Sigma, Spain) and 10^{-4} M luminol (Sigma, Spain) were added. The plate was shaken and the luminescence immediately read in a plate reader (BMG Labtech) for 1 h at 2-min intervals. The kinetics of the reactions were analysed, and the maximum slope of each curve was calculated. Luminescence backgrounds were calculated using reagent solutions containing luminol but not PMA.

Phagocytic Activity

The phagocytosis of *Saccharomyces cerevisiae* (strain S288C) by gilthead seabream HK leukocytes was studied by flow cytometry (Rodríguez et al. 2003). Heat-killed and lyophilized yeast cells were labelled with fluorescein isothiocyanate (FITC; Sigma, Spain), washed, and adjusted to 5×10^7 cells ml^{-1} of sRPMI. Phagocytosis samples consisted of 125 µl of labelled yeast cells and 100 µl of HK leukocytes in sRPMI (ratio of 6.25 yeast cells to 1 leukocyte). Samples were mixed, centrifuged (at 400 g for 5 min at 22 °C), resuspended, and incubated at 22 °C for 30 min. At the end of the incubation time, samples were placed on ice to stop phagocytosis and 400 µl ice-cold PBS was added to each sample. The fluorescence of the extracellular yeasts was quenched by adding 40 µl ice-cold trypan blue (0.4 % in PBS). Standard samples of FITC-labelled *S. cerevisiae* or HK leukocytes were included in each phagocytosis assay.

All samples were analysed in a flow cytometer (Becton–Dickinson) with an argon-ion laser adjusted to 488 nm. Analyses were performed on 3000 cells, which were acquired at a rate of 300 cells s^{-1}. Data were collected in the form of two-parameter side scatter (granularity) (SSC) and forward scatter (size) (FSC), and green fluorescence and red fluorescence dot-plots or histograms were made on a computerised system. The fluorescence histograms represented the relative fluorescence on a logarithmic scale. The cytometer was set to analyse the phagocytic cells showing highest SSC and FSC values. Phagocytic ability was defined as the percentage of cells with one or more ingested bacteria (green FITC-fluorescent cells) within the phagocytic cell population, whilst the phagocytic capacity was the mean fluorescence intensity. The quantitative study of the flow cytometric results was made using the statistical option of the Lysis Software Package (Becton–Dickinson).

Statistical Analysis

All measurements were performed on three replicates. The results in figures are expressed as mean ± SE (SEM) ($n = 6$). Data were statistically analysed by Student t test using SPSS 19 (SPSS, Chicago, Illinois, USA) to determine differences between unexposed and MeHg-exposed groups. Normality of the data were previously assessed using Shapiro–Wilk test, and the homogeneity of variance was also verified using Levene test. Nonnormally distributed data were log-transformed before analysis, and nonparametric Kruskal–Wallis test, followed by multiple-comparison test, was used when data did not meet parametric assumptions. Differences were considered statistically significant at $p \leq 0.05$.

Results

Hg Accumulation

The Hg concentration in liver samples of unexposed seabream specimens was low (<0.12 µg g^{-1} or ppm), whilst the concentration in MeHg-exposed fish was always significantly higher reaching a maximum (approximately 1 µg g^{-1} or ppm) at 2 days, after which the concentration decreased (Fig. 1). In the case of muscle samples, the Hg concentration was significantly increased in all seabream specimens exposed to MeHg for 10 or 30 days (approximately 0.3–0.4 µg g^{-1}, respectively) (Fig. 1). Of note, levels of Hg present in the muscle of exposed fish were always lower than those in liver. Hg concentrations in exposed fish were higher in liver than in muscle, although no significant variations were observed between the experimental groups (unexposed and MeHg-exposed fish) during the experimental trial (data not shown).

Effects of MeHg on Oxidative Status

The oxidative status of fish was analysed in serum and liver of seabream specimens exposed to waterborne MeHg (Fig. 2). In serum, the BAP (BAP test) was significantly lower and the presence of d-ROMs (d-ROMs test) significantly higher in fish exposed to MeHg for 10 and 30 days compared with the values obtained for fish of the control group (unexposed) (Fig. 2).

Liver SOD and CAT antioxidant enzymes showed a significant increase in fish exposed for 2 days to MeHg compared with the values obtained for control fish (Fig. 3). No significant changes were obtained in these activities in the liver of fish exposed for 10 or 30 days. GR activity was

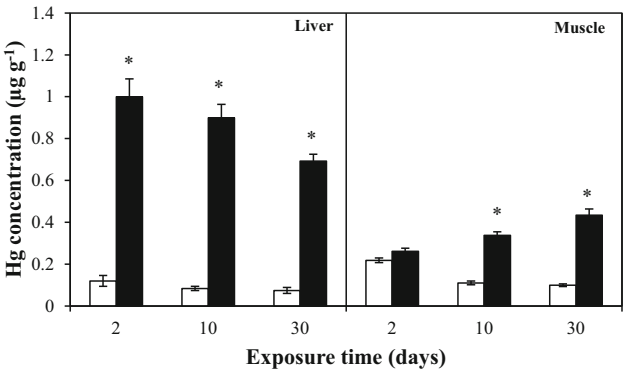


Fig. 1 Hg levels (µg g^{-1}) in liver and muscle samples of gilthead seabream specimens unexposed [control (*white bars*)] or exposed to waterborne MeHg [10 µg L^{-1} (*black bars*)]. *Bars* represent the mean ± SEM ($n = 6$). *Asterisk* denotes significant differences according to Student t test between unexposed and MeHg-exposed groups ($p \leq 0.05$)

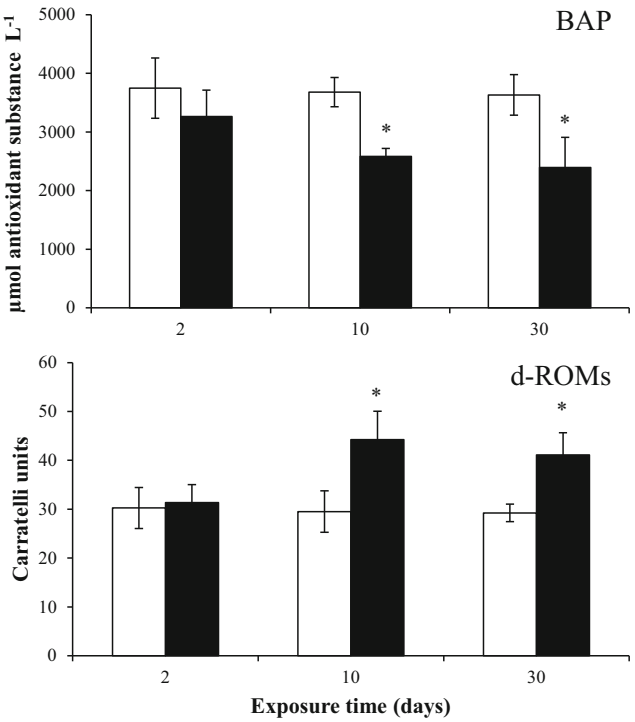


Fig. 2 BAP (µmol antioxidant substance L^{-1}) and d-ROMs (expressed as Carratelli units) in serum of gilthead seabream specimens unexposed [control (*white bars*)] or exposed to waterborne MeHg [10 µg L^{-1} (*black bars*)]. Bars represent the mean ± SEM ($n = 6$). *Asterisks* denote significant differences according to Student *t* test between unexposed and MeHg-exposed groups ($p \leq 0.05$)

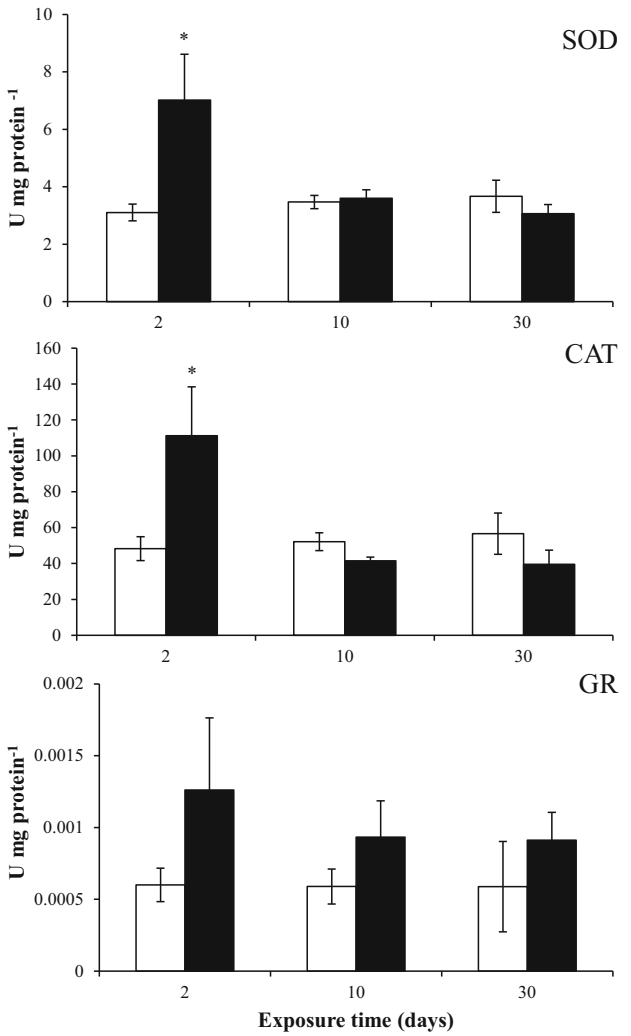


Fig. 3 SOD, CAT, and GR activities (U mg $protein_{-1}$) in liver of gilthead seabream specimens unexposed [control (*white bars*)] or exposed to waterborne MeHg [10 µg L^{-1} (*black bars*)]. *Bars* represent the mean ± SEM ($n = 6$). *Asterisks* denote significant differences according to Student *t* test between unexposed and MeHg-exposed groups ($p \leq 0.05$)

always higher in the liver of exposed fish than in control fish, although not to a statistically significant extent (Fig. 3).

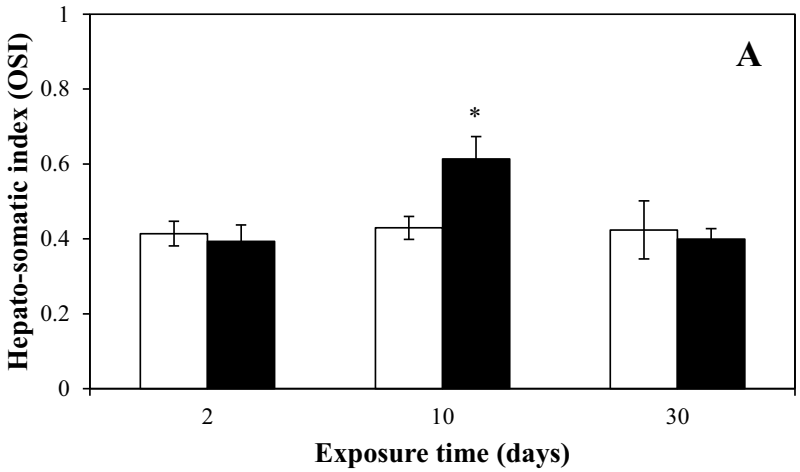
Somatic Indexes and Histopathology

Liver and spleen alterations were evaluated by studying the somatic indexes. The hepato-somatic index of seabream specimens exposed for 10 days to waterborne MeHg was higher than in the control fish (Fig. 4a), whereas the spleen-somatic index of exposed specimens remained unaltered with respect to the values found for control fish at any time tested (data not shown).

Waterborne exposure to MeHg altered the liver and the skin morphology of gilthead seabream specimens after 2 days of exposure with the alterations being much more evident after 10 and 30 days (Figs. 4, 5).

Regarding the liver, normal gilthead seabream hepatocytes are located between the sinusoids (which usually have circulating cells, mostly erythrocytes, in the lumen) forming cord-like structures known as "hepatic cell cords." Hepatocytes have a roundish polygonal cell body containing a clear spherical nucleus with usually one nucleolus (Fig. 4b). Hepatocyte distribution was gradually altered on exposure of the seabream to MeHg (Fig. 4c–e). The main alterations observed in liver were loss of the parenchyma organization, increased hepatocyte vacuolation, displacement of the nuclei of the hepatocytes to the cell periphery, and congestion of blood sinusoids (Fig. 4c–e). Liver from specimens exposed for 2 days showed vacuolated hepatocytes, whilst hypertrophied hepatocytes were also observed after 10 and 30 days of MeHg exposure (Fig. 4d, e).

The possible effects of waterborne MeHg on the skin were also studied. Normal gilthead seabream skin has the typical organization of teleost skin and is composed of three well-defined layers—the epidermis, dermis, and hypodermis—overlying a fat layer that varies in thickness. The nonkeratinized epidermis is a squamous stratified



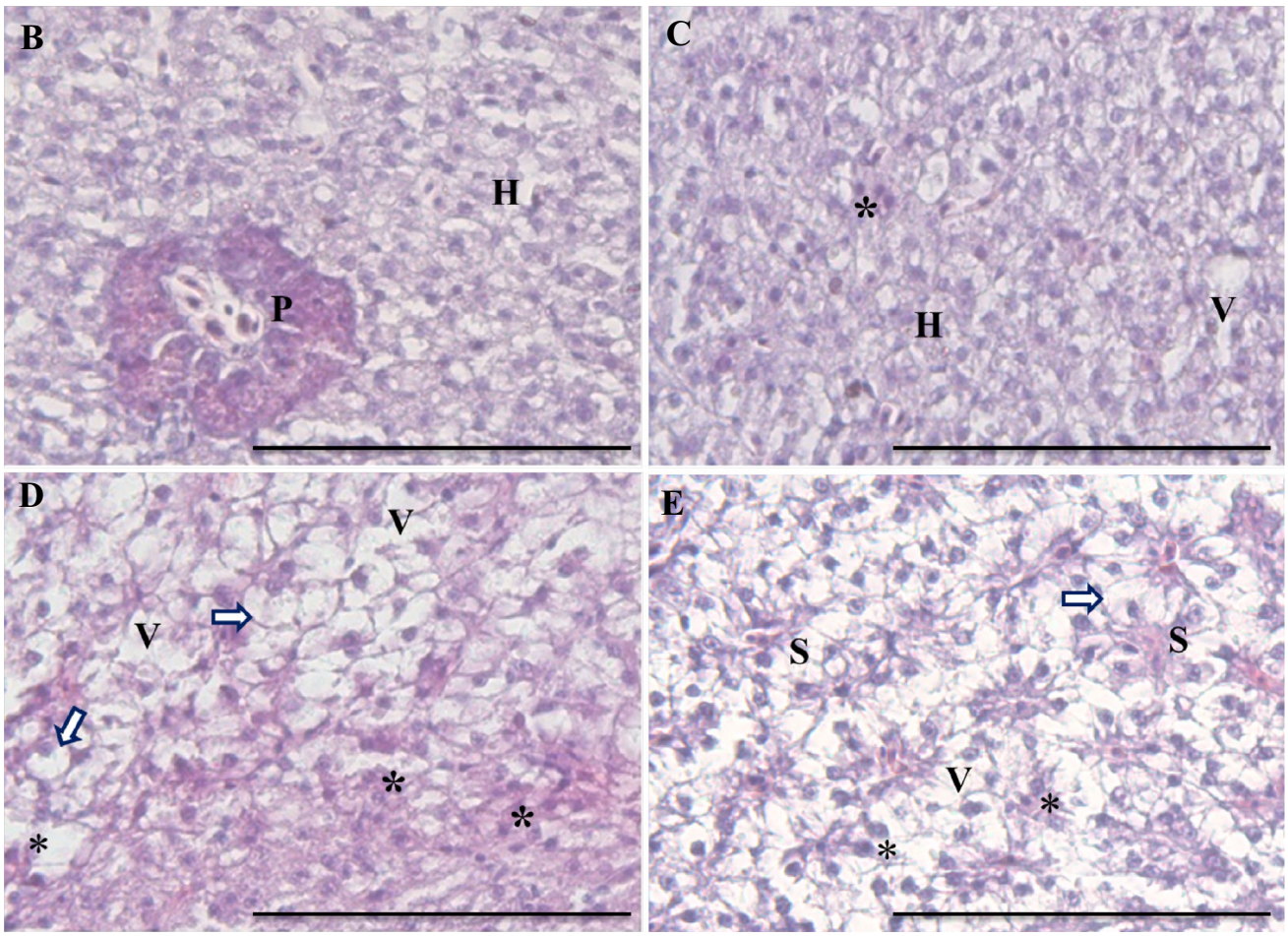


Fig. 4 Alterations in liver of gilthead seabream exposed to waterborne MeHg [10 μg L^{-1} (*black bars*)]. **a** Hepatosomatic index. Bars represent the mean ± SEM ($n = 6$). *Asterisks* denote significant differences according to Student *t* test between unexposed and MeHg-exposed groups ($p \leq 0.05$). (**b**–**e**) Histopathological study. Representative micrographs of liver sections from gilthead seabream unexposed (**b**) and exposed to waterborne MeHg for 2 (**c**), 10 (**d**), and 30 (**e**) days stained with hematoxylin-eosin. *H* hepatocyte, *S* sinusoid, *arrow* hypertrophic hepatocytes, *Fd* fatty degeneration, *V* vacuolization. *Bar* = 100 μm. *Focal necrosis

epithelium with goblet cells, whereas the dermis and the hypodermis are connective tissue (Fig. 5). The epidermis (5–10 cells thick) consists entirely of live cells, most of which are squamous cells and the rest mucous cells. The hypodermis consists of loosely organized collagen fibres with a rich supply of vessels (Fig. 5a). The epidermis from MeHg-exposed seabream specimens was thicker and showed a more disorganized cell arrangement (more

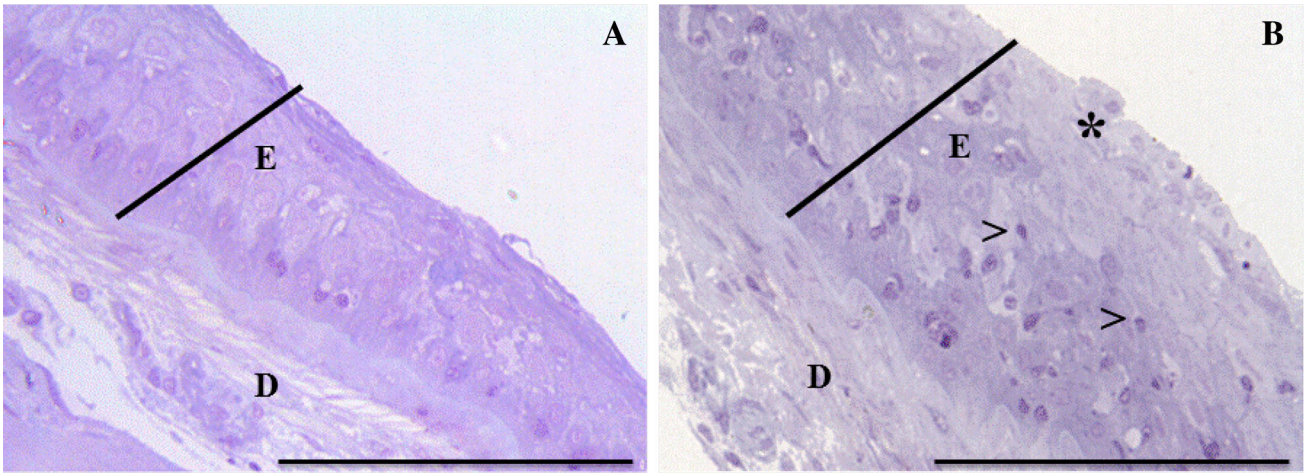


Fig. 5 Micrographs of skin sections from gilthead seabream unexposed (control) (**a**) and exposed to waterborne MeHg (10 μg L^{-1}) (**b**) for 30 days stained with hematoxylin-eosin. *E* epidermis, *D* dermis, > picnotic nuclei. *Bar* = 100 μm

evident in the basal part of the epithelium) than the epidermis of control (unexposed) fish (Fig. 5b). In the epithelium, cell degeneration, characterized by swollen epidermal cells (intracellular oedema) with pyknotic (condensed) nuclei, was observed (Fig. 5b).

Immune Status

Gilthead seabream humoral immune parameters were affected by MeHg exposure (Fig. 6). Overall, seric complement and peroxidase activities, as well as total IgM levels, were higher in specimens exposed to MeHg than in control fish, although only the increase in complement activity in serum from fish exposed for 30 days was statistically significant.

Similarly, innate cellular immune parameters of gilthead seabream specimens exposed to MeHg were higher than those observed in control fish. Although the phagocytic ability (Fig. 7a) and capacity (Fig. 7b) of HK leukocytes were significantly increased in fish exposed for 10 days to MeHg, leukocyte peroxidase content was increased in fish exposed for 30 days (Fig. 7c) in both cases with respect to the values recorded for control fish. Furthermore, the respiratory burst activity of HK leukocytes of fish exposed to waterborne MeHg was higher than the activity observed in control fish, although this increase was not statistically significant (Fig. 7d).

Discussion

The level of heavy-metal bioaccumulation in fish tissues is influenced by many biotic and abiotic factors (Zhang and Wong 2007; Has-Schön et al. 2008). The digestive tract is the main entrance route for Hg, but other tissues—such as gill and skin—are also important absorption sites. In the case of muscle, Hg is stored in the protein fraction of this tissue by binding to thiol groups (Harris et al. 2003). From a toxicological point of view, perhaps the most important organs are the liver followed by gills and kidney (Has-Schön et al. 2008). The liver can accumulate large quantities of pollutants from the external environment, and it also plays an important role in the storage, redistribution, detoxification, and transformation of pollutants (Evans et al. 1993). Our results agree with previous data in several fish species, and the Hg concentration in gilthead seabream liver was approximately two-fold the amount detected in muscle of *Carassius auratus*, *D. rerio*, and *D. labrax* (Abreu et al. 2000; Kennedy 2003; Gonzalez et al. 2005). In fact, high accumulation of Hg in liver is considered the primary signal of metal exposure (Olsson et al. 1998). Nevertheless, the levels found in liver of seabream exposed for 10 or 30 days were lower than the levels detected in fish exposed for 2 days. These results suggest that Hg is accumulated in the liver by short-term exposures but is then transported to muscle where it accumulates (0.434 μg g^{-1} after 30 days). These values in muscle are far from the concentration limit for total Hg in predatory fish intended for human consumption (1 μg g^{-1}ww) but close to the limit permitted for nonpredatory species (0.5 μg g^{-1}ww) such as gilthead seabream (FAO/WHO 1991; EC 2001). However, the WHO (1990) recommended a safety guideline value of 0.5 μg MeHg g^{-1} bw for all fish and a limit of 0.2 μg g^{-1} bw for vulnerable groups (such as pregnant women, individuals <15 years old, and frequent fish consumers). Therefore, our results point to levels close to the concentration limit for nonpredatory species and higher than the safety limit for vulnerable groups.

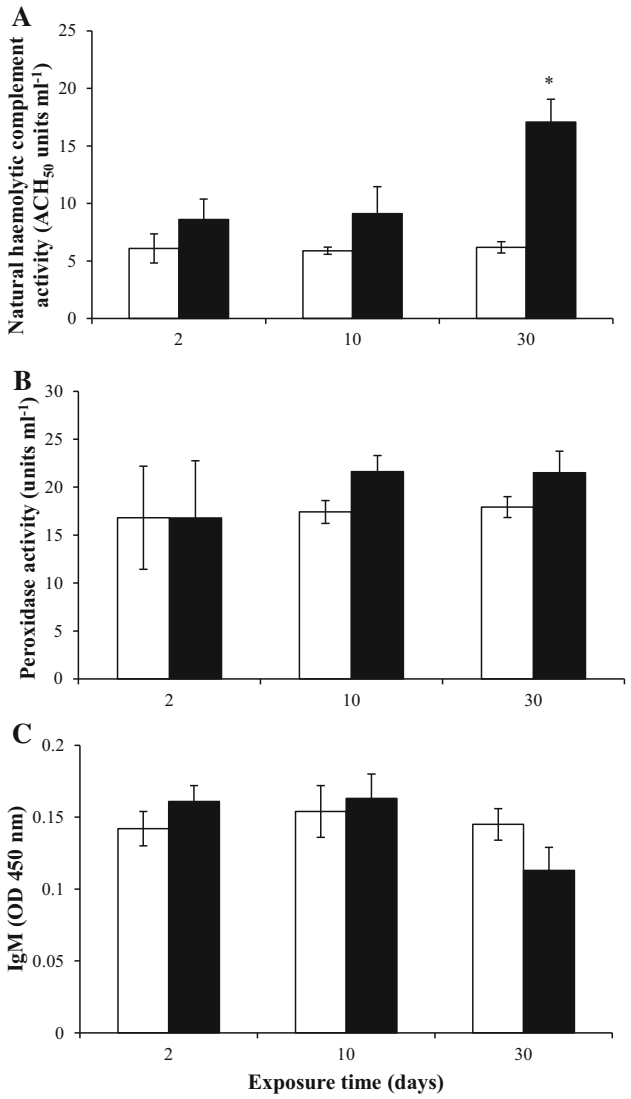


Fig. 6 Humoral innate immune parameters of serum from gilthead seabream specimens unexposed [control (*white bars*)] or exposed to waterborne MeHg [10 μg L^{-1} (*black bars*)]. **a** Natural haemolytic complement activity. **b** Peroxidase activity. **c** IgM. *Bars* represent the mean ± SEM ($n = 6$). *Asterisk* denotes significant differences according to Student *t* test between unexposed and MeHg-exposed groups ($p \leq 0.05$)

Similarly, several studies have detected high Hg value in fillets of species destined for human consumption, including gilthead seabream and European sea bass (approximately 70–100 μg kg^{-1}, respectively) (Yabanli et al. 2012), but they were always lower than the permissible level mentioned previously. However, Minganti et al. (2010) observed a mean of Hg in muscle of gilthead seabream of 120 μg kg^{-1} in farmed specimens and 540 μg kg^{-1}in wild fish with this last concentration being higher than the limit proposed by FAO/WHO. In addition, antagonistic relationships between Hg and Se have been described in European catfish (*Silurus glanis*), in which Se levels in liver were much higher than those of Hg (Squadrone et al. 2015). The data obtained in the present work do not agree with the previous observations, and new studies are needed to clarify the possible correlation between the accumulation of Hg and Se in fish liver.

New data suggest important changes in other tissues apart from the brain, one of the main targets for Hg. In this respect, organ-somatic indexes are valuable as simple and useful biomarkers of toxicity in fish (Schlenk et al. 2008). Thus, seabream specimens exposed to waterborne MeHg showed an increased hepato-somatic index, which is in agreement with our previous data concerning arsenic (As) or cadmium (Cd) toxicity (Guardiola et al. 2013a, b). Similarly, histopathology is known as an important methodology for assessing the effects of pollutants in vital processes because it identifies early changes in cells, tissues, and organs. Thus, histopathological biomarkers have often been used in fish to identify and evaluate toxic effects of exposure to pollutants (Oliveira Ribeiro et al. 2006). In the present work, histopathological changes were observed in liver of seabream exposed to waterborne MeHg, which drastically increased with the exposure time. Similarly, it has been observed that dietary doses of MeHg can also induce lesions and injuries in different fish organs such as liver, kidney, and gills (Oliveira Ribeiro et al. 2002, 2006). Furthermore, the dietary intake of MeHg also was seen to provoke some histological changes in the gut (Mela et al. 2014). The waterborne exposure of inorganic Hg severely affected the liver of *Trichomycterus brasiliensis* and, only after 4 h of exposure, the hepatocytes showed hyaline sites around the nuclei, the proliferation of smooth endoplasmic reticulum, modified mitochondria, and nuclei that were less electron dense and lacking a nucleolus. Furthermore, after 24 h of exposure, necrosis was almost complete, and blood came out of all of the capillaries (Oliveira Ribeiro et al. 2002). However, acute exposure to waterborne inorganic Hg had a limited effect in the liver of arctic charr (*Salvelinus alpinus*), whereas gills (an organ more directly exposed to the Hg) presented drastic changes (Oliveira Ribeiro et al. 2002). To the best of our knowledge, no study has focused on the effects of waterborne MeHg on the skin, which is one of the first tissues that comes into contact with the water compounds. The epidermis of the seabream specimens exposed to MeHg was thicker than the skin of unexposed (control) fish. Furthermore, a more disorganized cell arrangement (particularly in the basal part of the epithelium) was evident in the epidermis of exposed fish compared with the epidermis of the control fish. The morphological alterations observed in skin from MeHg-exposed specimens were correlated with epidermal degeneration, which was characterized by swollen epidermal cells (intracellular oedema) with pyknotic (condensed) nuclei. Such alterations not only affect the skin as a barrier

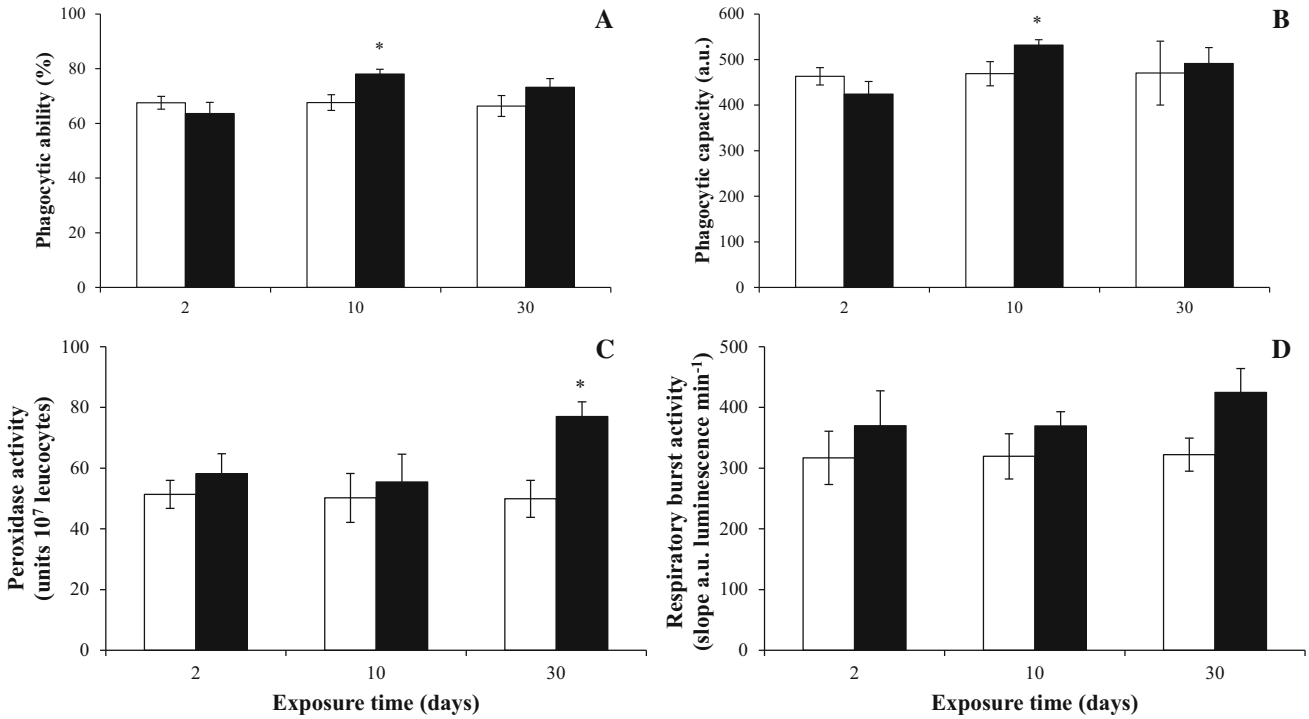


Fig. 7 Cellular innate immune parameters of HK leukocytes from gilthead seabream specimens unexposed [control (*white bars*)] or exposed to waterborne MeHg [10 μg L^{-1} (*black bars*)]. **a** Phagocytic ability. **b** Phagocytic capacity. **c** Peroxidase activity. **d** Respiratory burst activity. *Bars* represent the mean ± SEM ($n = 6$). *Asterisks* denote significant differences according to Student *t* test between unexposed and MeHg-exposed groups ($p \leq 0.05$)

and important organ for homeostasis maintenance but also affect fish immune status because the skin is the first line of defence and a very important portal of entry for many microorganisms. In fact, altered protein profiles and immune parameters, including increased enzymatic and bactericidal activities as well as the binding of lectins, were previously shown in the skin mucus of MeHg-exposed gilthead seabream (Guardiola et al. 2015). However, no differences in mucus production were detected among the fish from the different experimental groups, and studies should be developed to establish the real implications of this important organ in fish toxicology.

Regarding the oxidative stress response, SOD, CAT, GR, glutathione peroxidase, glutathione S-transferase, and thioredoxins are major antioxidant enzymes involved in the protection against the deleterious effects of free radicals (Ni et al. 2010). There is some evidence that MeHg toxicity produces oxidative stress both by increasing the production of ROS (Ou et al. 1999; Aschner et al. 2007; Yin et al. 2007) and reducing the cellular antioxidant defences (Shanker et al. 2005; Kaur et al. 2006). The present results confirm an imbalance in serum antioxidant activities, as shown by BAP and d-ROMs tests, especially in fish exposed to MeHg for 10 and 30 days. Furthermore, in the same fish, liver SOD and CAT activities were also lower than values observed in the control fish. Overall, the present results seem to suggest that the fish liver cells cannot eliminate all of the ROS produced as a consequence of the MeHg exposure. The liver of exposed fish suffers histopathological changes that are responsible for an increase in the hepato-somatic index. In the same way, in the liver of zebra seabream (*Diplodus cervinus*) exposed to waterborne 2 μg L^{-1} MeHg for 28 days, GR activity was higher and GPx activity lower than in control fish (Branco et al. 2012). Similar results were also recorded when MeHg was administered in the diet to trahira (*Hoplias malabaricus*) specimens; in this case, decreases in glutathione concentration, CAT, GST, and GPx activities, but increased SOD activity, were recorded suggesting also a possible accumulation of ROS in the liver as well as tissue damage (Mela et al. 2014). However, different results have been obtained for different fish species. For example, SOD and GSH-Px activities increased in liver and kidney of Atlantic salmon fed dietary MeHg for 4 months, whereas these activities decreased in the brain of the same fish (Berntssen et al. 2003). The role of other antioxidant systems merits investigation as does the connection between oxidative stress in liver and other tissues.

To date, very few studies have focused on the effect of MeHg on the fish immune system. In most of the studies

performed, MeHg increased the humoral parameters evaluated in exposed fish. For example, rainbow trout specimens exposed to Hg showed higher lysozyme activity than unexposed fish (Sanchez-Dardon et al. 1999); however, no significant effects were observed in the seric lysozyme activity in Japanese medaka exposed to Hg (Zelikoff 1998). Regarding complement proteins, most components are synthesized in the liver, although leukocytes can also take part in their production at a lower scale (Nakao et al. 2011). The fact that liver damage partly coincided with slight increases in serum complement and peroxidase activities in gilthead seabream exposed to waterborne MeHg suggests that hepatocytes, before being completely damaged under certain cell-stress situations, increase the production of important proteins involved in general homeostasis. Another hypothesis is that leukocytes could constitute, in this liver-damage scenario, the main source of such seric proteins. This last hypothesis would imply that exposure to MeHg activates fish leukocytes. Similar results were previously obtained in gilthead seabream specimens exposed to waterborne As (Guardiola et al. 2013a), although no such changes were recorded when fish were exposed to Cd or deltamethrin (Guardiola et al. 2014).

Seric peroxidase activity, which is related to the myeloperoxidase and/or eosinophil peroxidase activity, is considered a good marker of leukocyte activation (Rodríguez et al. 2003). As was in the case of MeHg in the present study, gilthead seabream serum peroxidase was also increased after exposure of fish to Cd or deltamethrin (Guardiola et al. 2013b, 2014) but not after As exposure (Guardiola et al. 2013a). All of these data strongly suggest that seric peroxidase activity may be considered a good toxicity biomarker, although further studies using different fish species will be needed to confirm this theory. In the case of IgM produced by B lymphocytes, its level in serum was not significantly affected in gilthead seabream specimens exposed to MeHg. However, IgM levels were decreased in rainbow trout exposed to Hg chloride and blue gurami exposed to MeHg (Roales and Perlmutter 1974; Sanchez-Dardon et al. 1999). New assays will be necessary to ascertain whether this is due to the decrease in circulating B lymphocytes or to a decrease in IgM production. Both situations were shown in other fish species after Hg exposure (Roales and Perlmutter 1974; Sanchez-Dardon et al. 1999; Oliveira Ribeiro et al. 2006). Regarding the cellular innate immune response, the HK (the main hematopoietic tissue in fish) is also a target tissue for Hg accumulation (Ciardullo et al. 2008), which would suggest that leukocyte functions might also be negatively affected by Hg. Strikingly, MeHg exposure increased the phagocytosis and peroxidase activities of HK leukocytes. Furthermore, similar results were obtained when gilthead seabream were exposed to other heavy metals such as As or Cd (Guardiola et al. 2013a, b). These data seem to support the idea that, although HK accumulates Hg, such accumulation is not correlated with an impairment of leukocyte function. However, it has also been shown that fish exposed to Hg naturally or through their diet showed decreased immune activities as determined by the reduced numbers of HK macrophages and inflammatory cells and/or altered inflammatory-related gene expression (Nøstbakken et al. 2012; Gehringer et al. 2013). In gilthead seabream, Hg accumulates in the kidney, which may alter the renal tubules and their excretion process but not the hematopoietic tissue as has been proposed for other fish species (Kirubagaran and Joy 1988; Mela et al. 2014).

To conclude, the present results showed that gilthead seabream exposed to sublethal concentrations of waterborne MeHg accumulated Hg in liver and muscle displayed acute toxicological effects. Histopathological alterations were observed in liver and skin and were more severe in fish exposed for lengthy times. Significant changes in the antioxidant status in serum and liver, as well as increased hepatosomatic indexes, were also detected in exposed fish. Finally, the presence of Hg in the water was capable of modulating the innate immune parameters of gilthead seabream. Further studies should elucidate the potential risks for general health and stress status of fish exposed to MeHg for longer times.

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