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Effect of sublethal concentrations of copper sulphate on seabream *Sparus aurata* fingerlings

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Abstract – The gilthead seabream is the most important Mediterranean aquacultured fish species. The main objective of this study was to investigate whether copper sulphate bath treatments used routinely in aquaculture have effects on important physiological functions of early life stages of the gilthead seabream (*Sparus aurata*). Fingerlings (80–90 days, 0.27 ± 0.06 g) were exposed to copper sulphate baths at 0, 0.25, 0.5 and 1.5 mg L^{-1} during 24 h. Effects on the central nervous function were evaluated analysing brain acetylcholinesterase activity (AChE). Oxidative stress was assessed by the quantification of lipid peroxidation (LP). Heat shock proteins (HSP70) were used as a general response to chemical stress, RNA/DNA ratio as an indicator of growth, and effects on detoxification function were estimated using glutathione–S-transferases activity (GST). Exposure of *S. aurata* fingerlings to copper sulphate induced increased lipid peroxidation. In contrast, there were no significant changes in AChE activity, HSP70 levels, RNA/DNA ratio or GST activity. Particularly, the absence of response in GST activity points towards a limited extent of the putative damage caused by the treatments at the highest doses (evidenced through the levels of peroxidation), since this enzyme is directly implicated in detoxification processes involving redox cyclic products. The results suggest that copper sulphate baths at the conditions tested are safe treatments for *S. aurata* fingerlings.

Key words: Copper sulphate / Biomarkers / Acetylcholinesterase / Lipid peroxidation / GST / RNA/DNA / Seabream

Résumé – Effets de concentrations sublétales de sulfate de cuivre sur les alevins de la daurade royale, *Sparus aurata*. La daurade royale est le poisson d'élevage le plus important en Méditerranée. Les objectifs principaux de cette étude sont d'analyser si les traitements par balnéation au sulfate de cuivre, effectués en routine en aquaculture, ont des effets sur les principales fonctions physiologiques aux premiers stades d'alevins chez la daurade royale (*Sparus aurata*). Les alevins (80–90 jours, 0,27 ± 0,06 g) sont exposés par balnéation au sulfate de cuivre à 0 ; 0,25 ; 0,5 et 1,5 mg L^{-1} durant 24 h. Les effets sur le système nerveux central sont évalués par l'analyse de l'activité de l'acétylcholinestérase du cerveau (AChE). Le stress oxydatif est établi en quantifiant la péroxidation des lipides (LP). Les protéines de choc thermique (HSP70) sont utilisées comme réponse générale au stress chimique, le rapport ARN/ADN comme indicateurs de croissance, et les effets sur la fonction de détoxification sont estimés par l'activité du glutathion–S-transférase (GST). L'exposition des alevins de *S. aurata* au sulfate de cuivre induit une augmentation de la péroxidation des lipides. En revanche, il n'y a pas de changement significatif ni dans l'activité de AChE, ni dans les niveaux de HSP70, ni dans le rapport ARN/ADN ni dans l'activité du GST. L'absence de réponse au niveau de l'activité du GST, en particulier, montre une étendue limitée du dommage supposé qui serait causé par les traitements aux plus hautes doses (mis en évidence par les niveaux de péroxidation) car cette enzyme est impliquée directement dans les processus de détoxification faisant intervenir des produits rédox cycliques. Les résultats suggèrent que les bains de sulfate de cuivre, aux conditions testées, sont des traitements sains pour les alevins de *S. aurata*.

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1 Introduction

The gilthead seabream is the most important Mediterranean aquacultured fish species. Greece, Turkey and Spain are the main producers of this fish species. Copper sulphate is commonly used under the form of baths as a chemotherapeutic in aquaculture (Schlotfeldt 1992; Carvalho and Fernandes 2006) to treat various external diseases (Rábago-Castro et al. 2006). For example, it can be used as a disinfectant treatment against protozoan ectoparasites of the genus *Cryptocarion* and *Amyloodinium*, as well as against bacteria (Rigos et al. 2001; Chen et al. 2006). Its use is widespread as a routine treatment in fish hatcheries and farms prior to the handling of fish stocks. Treatment conditions are very variable, copper concentrations may be as high as 1 mg L^{-1} and the copper sulphate bath may last from minutes to several days.

Copper is a trace metal essential for living organisms, but at high concentrations it becomes one of the most toxic heavy metals to fish. To name a few, effects on chemosensory function (Carreau and Pyle 2005), immunological parameters (Dethloff et al. 2001), antioxidant stress (Dautremepuits et al. 2004; Sanchez et al. 2005) and gill and intestine homeostasis (Kamunde et al. 2002; Grosell et al. 2004a,b; Monteiro et al. 2005; Carvalho and Fernandes 2006) of fish have been reported. Besides, toxicity can vary with the age of fish (Sellin et al. 2005). In seabream, Antognelli et al. (2003) reported (organ-specific) alterations of the metabolic pathway of glyoxalase of adult fish exposed to sublethal concentrations of copper sulphate. Therefore, it is important to evaluate the effects of copper sulphate, from the viewpoint of its toxicity to early life stages (potentially more sensitive) of fish, since the treatment can jeopardize the success of cultures by affecting survival, growth and development of the fingerlings. Recently, Chen et al. (2006) have pointed out that the literature on the toxicity of copper refers mainly to freshwater species. These authors recommend carrying out controlled studies on the use of copper sulphate in larval and juvenile of marine aquacultured species.

When deleterious effects are difficult to detect after exposure to low amounts of toxicants, or due to long-term mechanisms of action, the use of early-warning signals, or biomarkers, becomes mandatory. Biomarkers can be defined as the biological response (generally at sub-individual level) to a chemical that gives a measure of exposure and sometimes, also, of toxic effect (Walker et al. 2003).

Lipid peroxidation (LP) is an important result of oxidative damage. In an oxidative stress scenario, a depletion of the endogenous antioxidant defence mechanisms may occur, with the concomitant formation of reactive oxygen species (ROS). Due to their relative high reactivity and low capacity of diffusion across cellular membranes, these intermediates are likely to cause oxidative damage to biological structures in their immediacy, essentially membrane lipids. The radicals can then abstract a hydrogen atom from the double bonds present in the membrane molecules of fatty acids, giving rise to the lipid peroxidation cycle. Malondialdehyde (MDA), a main oxidation product of peroxidized polyunsaturated fatty acids, is used as an indicator of LP. Several studies have shown increases of LP in tissues from fish species *in vivo* exposed to various chemicals (Gabryelak and Klekot 1985; Ploch et al. 1999), including heavy metals (Roméo et al. 2000; Livingstone 2001; van der Oost et al. 2003).

Acetylcholinesterase (AChE) is a crucial enzyme in the nervous system of both vertebrates and invertebrates, where it is responsible for the degradation of the neurotransmitter acetylcholine in the synaptic cleft. AChE activity is inhibited by xenobiotics like organophosphate and carbamate pesticides (Key and Fulton 2002; Varó et al. 2002, 2003), but recent evidences also suggest AChE activity inhibition by metals including copper (Frasco et al. 2005).

Heat shock proteins (HSP), also known as stress proteins, are a group of proteins whose synthesis is increased in response to a variety of physical and chemical stressors, including temperature, salinity stress, metals and some xenobiotics (Sanders 1990, 1993; Varó et al. 2002; van der Oost et al. 2003). They are involved in the protection and repair of the cells in response to stress and harmful conditions.

RNA/DNA ratio gives a measure of the synthetic capacity of the cell. It has been used extensively in early life stages of fish to detect nutritional status and changes in growth rates (Clemmesen 1994). The amount of DNA per cell is constant for the individuals of the same species, whereas the RNA (mainly ribosomic) represents the degree of protein synthesis. RNA/DNA ratio can be envisaged as indicative of the amount of metabolic activity of the cells, and seems to be a valid biomarker in ecotoxicology (Miliou et al. 1998; Wo et al. 1999; Zhou et al. 2001; Ibiam and Grant. 2005) and environmental perturbation studies (Pottinger et al. 2002).

Glutathione S-transferases (GST) are a superfamily of primarily soluble enzymes with functions in cellular transport, synthesis of leukotrienes and prostaglandins and defence against oxidative damage and peroxidative products of DNA and lipids (George 1994). These enzymes are of paramount importance for major detoxification processes (van der Oost et al. 2003), since they conjugate electrophilic xenobiotics and endogenous substances with the nucleophilic tripeptide glutathione from the intracellular pool. This conjugation is likely to render the molecules more water soluble, therefore, favouring their elimination from the organism.

The objective of this work was to investigate the sublethal effects of copper sulphate baths as those commonly used in aquaculture routines, to seabream fingerlings by studying selected biomarkers related to important physiological functions. To achieve this goal, the in vivo effect of copper sulphate on lipid peroxidation (LP), AChE activity, unspecific stress induction (HSP70), RNA/DNA ratio, and detoxification-related enzymes (GST) were evaluated. To the best of our knowledge this is the first study of this kind carried out in early seabream fingerlings.

2 Materials and methods

Seabream fingerlings (80–90 days, 0.27 ± 0.06 g; 27 ± 2 mm) from a commercial hatchery (Piscimar, SL, Burriana, Spain), were transported to the laboratory and kept (3 fish L^{-1}) for at least one week in 70 L fiberglass tanks filled in open circuit with natural seawater (salinity: 38 g L^{-1}) with continuous aeration, at ambient temperature (24–28 °C) and natural photoperiod, before being used in experiments. They were fed

ad libitum with commercial food pellets (Pro-Aqua®, Spain). Exposure to copper sulphate was carried out in a static bath system for 24 h. The concentrations tested (0, 0.25, 0.5 and 1.5 mg L^{-1} of Cu $SO_4 \cdot 5H_2O$) were selected based on previous results and literature data (0.5–0.7 ppm for 1.1 g seabream, Rigos et al. 2001). For each concentration tested, 10 fishes were distributed in two 5 L aquaria filled with 3 L of filtered (0.2 μm of mean pore diameter) full-strength Mediterranean seawater (salinity 38 g L^{-1}) (20 fishes per treatment). Two additional aquaria without chemical were used as controls. Tests were performed in a temperature and photoperiod controlled room (20 ± 1 °C; 12hL: 12hD). Water quality was monitored before and at the end of the exposure period (oxygen concentration: 74–68%, conductivity: 57–58 mS cm, pH: 8.2–8, salinity: 38.1–38.2 g L^{-1}). Previous determinations indicated that under these experimental conditions actual concentrations never surpassed 50% of nominal values. Fish were not fed during test.

At the end of the exposure period, all fishes were anesthetised in ice-cold water, sacrificed by decapitation, dissected, and the tissues needed for the different analyses were stored at –80 °C. The small size of the animals precluded the use of specific target organs like liver or kidney, not to mention blood, for the assay of biomarkers. Instead, the determination of AChE activity and lipid peroxidation was performed on the whole head, the evaluation of HSP70 and RNA/DNA ratio was carried out in the muscle, and the analysis of GST activity was performed in the gills. All assays were performed in triplicate.

Homogenates of the tissues (1:10 w:v) were prepared in different ice-cold buffers using a tissue disrupter UltraTurrax (T-8, IKA, Germany), according to the biochemical analysis performed: phosphate buffer 0.1 M (pH = 7.2) was used for AChE and thiobarbituric acid reactive substances method (TBARS) determinations; phosphate buffer 0.1 M (pH = 6.5) was used for GST analysis; TE buffer (10 mM TRIS-HCl buffer containing 1mM EDTA, pH = 7.5) was used for RNA and DNA quantifications and calcium-magnesium free saline buffer containing 20 mM Hepes, 500 mM NaCl, 12.5 mM KCl (pH = 7.3), freshly complemented with 1mM dithiothreitol (DTT), 1mM phenymethysulfonyfluoride (PMSF), 0.1g ml^{-1} Tripsin inhibitor, and Igepal (1%) was used for stress proteins (HSP70).

Total protein content of the samples for all assays was determined by the Bradford method (Bradford 1976) adapted to microplate, using Bio-Rad microassay Kit and γ-globulin as standard. The absorbance was read at 595 nm.

LP in the head was determined by measuring the formation of TBARS and quantified as malondialdehyde (MDA) equivalents. MDA was measured after incubation of tissue homogenate (80 μl) at 95 °C with acetic acid (pH 3.4) and thiobarbituric acid following the method of Ohkawa et al. (1979). After cooling the samples, a mixture of n-butanol and pyridine (15:1, v/v) was used to separate the organic layer (supernatant). After centrifugation at 4000 g for 10 min, the fluorescence of the supernatant was measured at 530 nmEX/550 nmEM in a Hitachi F-2500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). MDA concentrations were derived from a standard curve using 1,1,3,3,-tetraethoxypropane as standard (in nmol of MDA equivalents per g wet weight tissue).

Total AChE activity was determined at room temperature (20–22 °C) by the Ellman method (Ellman et al. 1961) adapted to microplate (50 μl of homogenate at 0.3 mg ml^{-1} of protein) according to Guilhermino et al. (1996), using acetylthiocholine (ATC) as substrate. The absorbance increase at 405 nm was followed for 5 min using a Bio-Rad spectrophotometer microplate reader (Bio-Rad). The enzymatic activity was expressed as units (U) per mg of protein (1U = nmol of substrate hydrolysed per minute).

Stress proteins HSP70 in muscle were separated by 1D-SDS PAGE gel using a 5% stacking gel and a 12% separating gel and loading equal amount of protein (3 $\mu g\ \mu l^{-1}$) per lane. Gels were run following the procedure described in Varó et al. (2002), in a Bio-Rad Mini-PROTEAN III Electrophoresis system, and transferred to a nitrocellulose membrane according to Towbin et al. (1979) in a Bio-Rad Mini Trans-Blot electrophoretic system with cooling. Membranes were blocked 1 h at room temperature in 5% non-fat powdered milk. After blocking, membranes were probed 1 h at room temperature, with mouse monoclonal anti-heat shock protein 70 antibody (Sigma, H-5147) diluted in a 1:4000; and incubated 1 h at room temperature with an anti-mouse IgG secondary antibody conjugated with peroxidase (Sigma, A-4416) diluted 1:3000. HSP70 proteins were visualized on VERSADOC Imaging System (Bio-Rad) using the ECL Western Blotting Analysis System (Amersham). Quantification of HSP70 was done using the Quantity-One software of Bio-Rad (version 4.3.1). Stain densities of each blot were normalized to the density of the HSP70 band of a commercial standard (Sigma, H9776), which was given the value of 1, and relative values were calculated for all samples.

RNA and DNA in muscle were quantified following the procedure described in RiboGreen ™ RNA Quantitation Kit and PicoGreen™ DNA Quantitation Kit respectively. Briefly, for RNA measurements 50 μl of supernatant was transferred in triplicate into a 96-well black microplate containing 1 μl (10 U) of DNase I prepared in DNase reaction buffer (200 mM Tris-HCl, 100 mM $MgCl_2$, 20 mM $CaCl_2$, pH = 7.5). For DNA determinations 50 μl of supernatant was transferred in triplicate into a 96-well black microplate containing 10 μl of RNase A (4 mg ml^{-1}), diluted 1/400 using nuclease free water. After 1 h incubation at 37 °C, the volume was adjusted to 100 μl with TE buffer (10 mM Tris-HCl, 1mM EDTA, pH = 7.5), and 100 μl of RiboGreen or PicoGreen was added for RNA and DNA quantification, respectively, and allowed to stain for 5 min in darkness before reading in a TECAN Ultra Evolution microplate reader at 485 nmEX/535 nmEM (TECAN, Grödig, Austria). Concentrations were calculated from a standard curve of RNA or DNA (20 ng ml^{-1} to 1 $\mu g\ ml^{-1}$) prepared from standards supplied with Ribo-Green and Pico-Green reagents Kit.

Gill GST activity was measured at 340 nm by the Habig method (Habig et al. 1974) adapted to microplate as described by Frasco and Guilhermino (2002), using 0.1 ml of homogenate (0.3 mg ml^{-1} of protein) and 0.2 ml of the reaction solution (10 mM gluthathione (GSH) in phosphate buffer 0.1 M, pH = 6.5, and 60 mM 1-chloro-2,4 dinitrobenzene

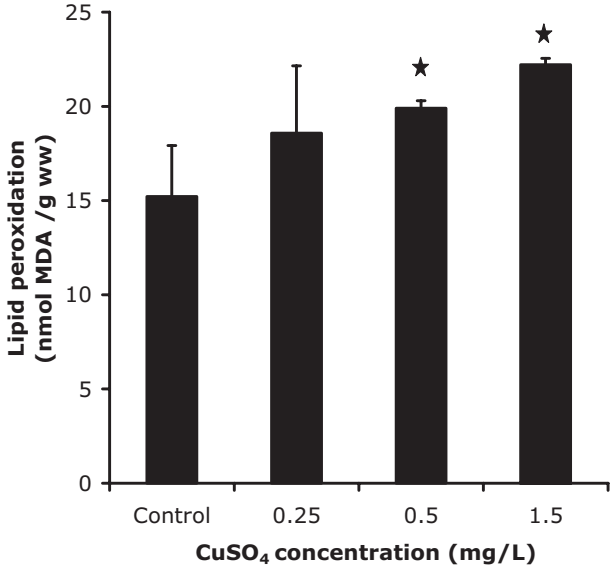

Fig. 1. Lipid peroxides in the heads of seabream fingerlings exposed to different copper sulphate concentrations. The asterisks show significant differences after ANOVA and Dunett test ($p < 0.05$). Error bars are 1 standard deviation.

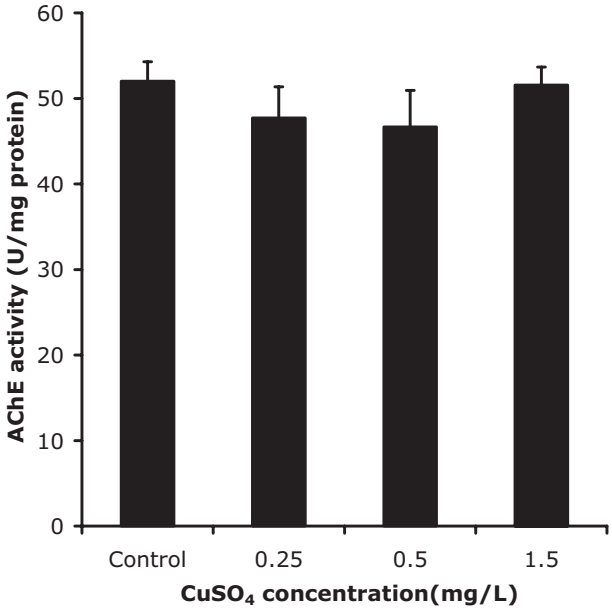

Fig. 2. Activity of acetylcholinesterase (AChE) in the head of seabream fingerlings exposed to different concentrations of copper sulphate. Error bars are 1 standard deviation.

(CDNB) in ethanol). GST activity was determined immediately at each 20 s during the first 5 min. The enzymatic activity was expressed as units (U) per mg of protein (1 U = nmol of substrate hydrolysed per minute).

One-way ANOVA and the Dunnett post hoc test were used to check differences among the means of treatments. All statistical analyses were carried out using SPSS 12.0 for Windows software (SPSS, 1989-2003). A significance level of 0.05 was used. Results are presented in the graphs as means + 1 standard deviation.

3 Results and discussion

The in vivo exposure of seabream fingerlings to copper sulphate produced oxidative stress. Lipid peroxidation increased with increased concentrations of copper sulfate. This increase was statistically significant at the two higher concentrations tested (0.5 and 1.25 mg L^{-1}, Fig. 1). No significant changes in the activity of AChE, in the levels of HSP70, in the RNA/DNA ratio or in the activity of the GST were found between the controls and the treated groups (Figs. 2, 3, 4 and 5).

A first point for discussion is whether the lack of response obtained for some biomarkers is due to relatively short time of exposure (24 h). Peyghan et al. (2003), in their work with common carp *Cyprinus carpio* exposed to copper in short-term and long-term experiments, showed accumulation in liver and muscle after only 1 day exposure to 1 mg L^{-1}. Accumulation in liver and muscle follow osmoregulatory imbalance (Hansen et al. 2006) by disturbance of the hydromineral equilibrium in gills (De Boeck et al. 2003). Wong et al. (1999) in their work with silver seabream (*Sparus sarba*) reported a 24 h LC50 value of 2.01 mg L^{-1} for 9.4 ± 2.1 g fingerlings, and showed that the order of concentration of copper in organs was intestine>liver>gonad>gills, skin and muscle. Besides,

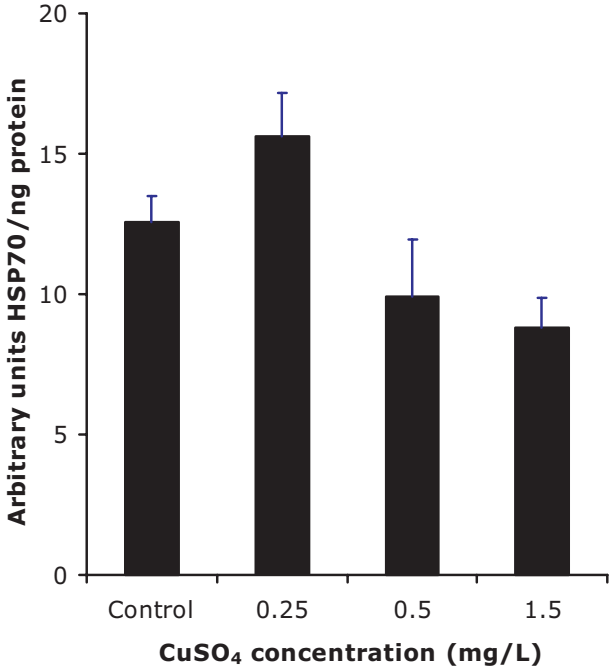

Fig. 3. Levels of heat shock proteins (HSP70) in the muscle of seabream fingerlings exposed to different concentrations of copper sulphate. Error bars are 1 standard deviation.

young marine life stages have been reported more sensitive than adults (Mance 1987), and the shorter diffusion distances in small fingerlings may anticipate faster toxicokinetics than in adults or larger fingerlings. Thus at the light of these data, it seems reasonable to conclude that our 24 h test period at least at some of the concentrations tested here seems adequate to produce biomarker response.

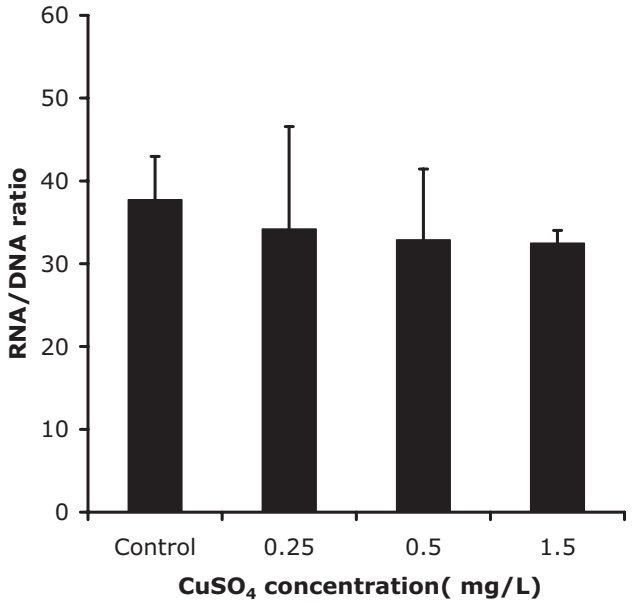

Fig. 4. RNA/DNA ratio in the muscle of of seabream fingerlings exposed to different concentrations of copper sulphate. Error bars are 1 standard deviation.

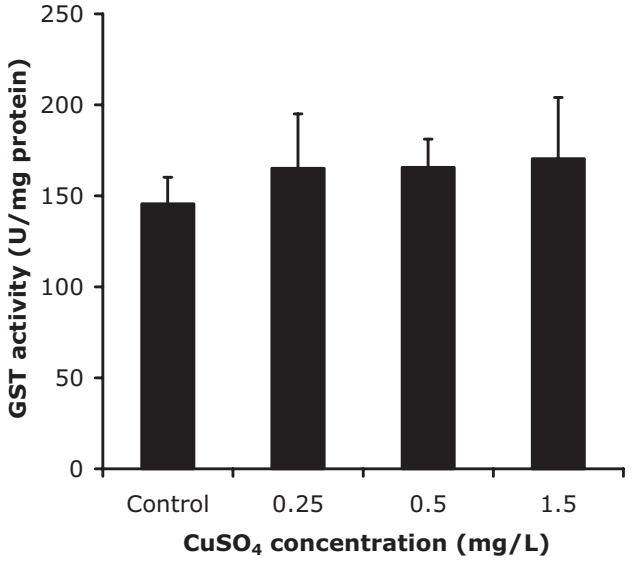

Fig. 5. Glutathione-S-transferase (GST) activity in the gills of seabream fingerlings exposed to different concentrations of copper sulphate. Error bars are 1 standard deviation.

AChE activity is strongly inhibited by several compounds: organophosphate pesticides, carbamates, pyrethroids etc. (van der Oost 2003). The response of this biomarker to heavy metals exposure has also been reported but is less clear (Frasco et al. 2005), and varies depending on the species, metal and conditions of the study. Recent evidence suggests that the metal ions mercury, cadmium, copper and zinc inhibit AChE activity, and that the confounding results found in the literature about the effects of metals on AChE inhibition may be attributable to methodological problems. These problems are linked with the interference of metal ions with thiol groups of thiocholine, a product of the hydrolysis of the substrate acethylcholine, and with certain buffers such as phosphate buffer (Frasco et al. 2005). Another valid explanation could be that metal ions cause conformational changes that result in loss of catalytic activity (Del Ramo et al. 1993).

HSP70 proteins are biomarkers of unspecific stress. The inducible form of HSP70 is synthesized in fish in response to acute stressors such as copper (Sanders et al. 1995; Boone and Vijayan 2002) as demonstrated in vitro. Feng et al. (2003) also reported HSP70 expression in rainbow trout hepatocytes during 24 and 48 h exposure to 25, 50, 100 and 200 μM copper sulphate. These doses are however, notably higher than those tested in the present work (1, 2 and 5 μM). In vivo, copper sulphate produces ionoregulatory or respiratory disturbances that imply an increase in energy consumption to restore homeostasis (Carvalho and Fernandes 2006). In rainbow trout (*Oncorhynchus mykiss*), exposures of only 4 h to 1.65 μM of waterborne copper caused hypoxia in the gill epithelium, the activation of hypoxia-inducible factor-1α as well as an increase in the metallothionein mRNA levels (van Heerden et al. 2004). Caution should be exerted when discussing in vivo and in vitro results since comparisons are not always straightforward especially for biomarkers of general stress. To complicate the picture, De Boeck et al. (2003) in a study with common carp exposed in vivo to copper, concluded tissue specific HSP70 levels and cortisol-related HSP response. Moreover, in the same work, these authors suggested species -and life history-dependent HSP responses. Thus, the lack of response obtained in the present study may be considered just an indication that the stress induced by exposure to copper sulphate in the conditions tested here does not reach the level required to trigger a detectable response.

RNA/DNA ratio indicates the degree of metabolic (protein) synthesis. It is based on the fact that the DNA content per cell is constant within the same species, and the RNA is mainly ribosomic and varies with the rate of protein synthesis. It has been traditionally used as a growth index in ecological studies, in aquaculture, and as a biomarker in long-term exposure experiments in ecotoxicology (Miliou et al. 1998). The sensitivity of the ratio as a biomarker has been questioned based on its lack of response in some experiments (Benton et al. 1994; Kim and Kang 2004). In a recent study, Vidal et al. (2006) were able to associate RNA/DNA variations in 24 h periods and at different hours of the day in starved paralarvae of the loliginid squid. The larval stages of cephalopods are very fast growing forms, metabolically very active. The sensitivity of the ratio is perhaps associated to the rate of metabolic activity and thus, early forms and fast-growing stages are more sensitive. For example, Barber et al. (1994) used successfully the ratio in 24 h exposure of the crustacean *Daphnia magna* to cadmium, and Ibam and Grant (2005) were also able to use the ratio as a sublethal endpoint of copper exposure working with a nematode. In this sense, the fish used in the present study are still young fast-growing animals of only 80 days of age. Sellin et al. (2005) have postulated that age might be a key factor in the time course and possibly the mechanisms of acclimation of fathead minnows exposed to copper. Our results point out to a reduction in the RNA/DNA ratio of the animals exposed

to increasing copper concentrations, that was not statistically significant due to the variability of certain treatments, a problem encountered by others (Clemmesen 1994; Narvaez et al. 2005). The lack of significant response however, matches the results obtained with other biomarkers (AChE, HSP70, GST) and can be considered further evidence that the treatment concentration and exposure did not induce significant metabolic changes to the fish.

The significant levels of TBARS in the treated groups clearly indicate copper induced lipid peroxidation in the two higher concentrations tested. There is compelling evidence in the literature about the pro-oxidant activity of copper (Roméo et al. 2000; Dautremepuits et al. 2004). Copper can act as a catalyser of the Fenton reaction helping the conversion of the superoxide anion and of the hydrogen peroxide to a hydroxil radical, which is a molecular species often proposed as a trigger of lipid peroxidation (Stohs and Bagchi 1995).

On the other hand, the absence of response in GST activity confirms again that the extent of the putative damage caused by the treatments at the highest doses is moderate since this enzyme is directly implicated in detoxification processes involving redox cyclic products. If we consider that the response of the GST is transient (Barata et al. 2005), we could be facing an advanced stage of oxidation. This does not seem to be the case, since Paris-Palacios et al. (2000) found increasing GST activity in the liver of the cyprinid *Brachydanio rerio* exposed during 14 days to sublethal concentrations of copper sulphate (40 and 140 μg L^{-1}). Dautremepuits et al. (2004) found no effect in GST activity in the head kidney and liver of carps exposed to 0.25 mg L^{-1} of copper regardless the length of exposure (4 or 8 days). However, Sanchez et al. (2005) working with three-spined stickleback, showed that copper induced oxidative stress in fish liver before significant metal accumulation could be detected and suggested the existence of homeostatic regulation mechanisms. Other explanations for the lack of GST activity include seasonal (Geracitano et al. 2004), and species-specific ecophysiological differences (Livingstone 2001). In general, antioxidant defences in fish are very dependent on a combination of biotic and abiotic factors (Martínez-Álvarez et al. 2005) which make very difficult direct inter-study comparisons. Within the framework of our results, the lack of activity obtained with HSP70 and the other biomarkers seems to substantiate the hypothesis of a rather incipient degree of oxidative damage. AChE inhibition has been correlated with peroxidation in a number of studies (Yang and Dettbarn 1996; Yang et al. 1996; Üner et al. 2006). More specifically, Song et al. (2006) have described AChE deactivation in carp brain as a result of oxidative damage caused by hexachlorobenzene exposure. Under this point of view, the lack of AChE inhibition found in the present results points out once again to a very early stage of oxidative damage.

Chen et al. (2006) found that copper sulphate baths (1–2 h, 0.1–1 mg L^{-1}) reduced bacterial pathologies on white seabass *Atractoscion nobilis* and increased larval and juvenile survival, without microscopic evidences of toxicity due to the treatments. Our results are more conservative given that the exposure is lengthier and the concentrations higher. The safest approach for the use of these prophylactic and disinfectant treatments would be to characterise the particular toxicity response of developmental stages in the different species.

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