J. Elem. s. 255–267 DOI: 10.5601/jelem.2012.17.2.08

EFFECTS OF WATERBORNE COPPER ON GILLS CATALASE AND BLOOD BIOCHEMISTRY IN GILTHEAD SEABREAM (*SPARUS AURATA* L.)

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Abstract

Cu is a transition metal essential for every organism, but an excess can lead to toxicity caused by oxidative stress, which can disturb the natural antioxidant defence systems. Since catalase (CAT) is a major enzyme involved in the decomposition of oxygen peroxide, the main goal of this study was to evaluate changes in the concentration and activity of CAT by means of enzymatic and immunohistochemical methods. Modifications in the blood biochemistry profile of Cu-exposed gilthead sea bream (*Sparus aurata* L.) were also studied. In gills of the exposed fish, Cu significantly increased throughout the study to a maximum of 6.9±4.7 μg g^{-1} wet weight at the end of the treatment. Immunohistochemistry (IHC) evidenced a brown cytoplasmic stain in the epithelial cells of the primary and secondary lamellae and in pillar cells, while enzyme activity was localized in the epithelium and pillar cells of both primary and secondary lamellae and appeared as a strong cytoplasmic stain particularly at the base of the primary lamellae. IHC and enzymohistochemistry (EHC) quantitative analysis suggested that the main variations in amount and activity of the enzyme were recorded 15 days after exposure (both IHC and EHC expressed a decrease in CAT in exposed fish compared to controls, $P<0.0001$) and 28 days after exposure (IHC recorded increased CAT in exposed specimens compared to controls, $P<0.0001$; EHC evidenced a decrease in CAT in exposed subjects compared to controls, $P<0.0001$). The biochemical blood profile was monitored with a standard blood biochemistry panel. An increase in plasma urea was evident only in exposed fish, while – as a function of time – a decrease of glucose in both exposed and control fish was apparent. The three investigation methods evidenced that CAT was effective against Cu toxicity, and the increase in plasma urea could be considered a suitable metabolic marker of Cu exposure in fish.

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Key words: copper, *Sparus aurata*, catalase, gills, blood biochemistry, immunohistochemistry, enzymohistochemistry.

WPŁYW MIEDZI W WODZIE NA KATALAZĘ W SKRZELACH I BIOCHEMIĘ KRWI U DORADY (*SPARUS AURATA* L.)

Abstrakt

Miedź jest metalem przejściowym niezbędnym w każdym organizmie, lecz jej nadmiar może prowadzić do skutków toksycznych powodowanych przez stres oksydacyjny, który zakłóca naturalne systemy obrony antyoksydacyjnej. Ponieważ katalaza (CAT) jest jednym z głównych enzymów uczestniczących w rozkładzie nadtlenku tlenu, głównym celem badań była ocena zmian zawartości i aktywności CAT za pomocą metod enzymatycznych i immunohistochemicznych. Badano również zmiany w biochemicznym profilu krwi u dorady (*Sparus aurata* L.) poddanej działaniu Cu. Stwierdzono znacząco wyższe stężenie tego metalu w skrzelach ryb, na które oddziaływała miedź, aż do maksymalnej zawartości 6,9±4,7 μg g^{-1} mokrej masy pod koniec doświadczenia. Badania immunohistochemiczne (IHC) ujawniły brązową plamkę cytoplazmatyczną w komórkach nabłonka blaszek pierwszego i drugiego rzędu oraz w komórkach filarowych, podczas gdy aktywność enzymatyczna została zlokalizowana w nabłonku oraz w blaszkach pierwszego i drugiego rzędu, i ujawniła się jako silna plamka cytoplazmatyczna, szczególnie u podstawy blaszek pierwszego rzędu. Analiza ilościowa wyników badań IHC oraz enzymohistochemicznych (EHC) sugeruje, iż główna zmienność w ilości oraz aktywności enzymu nastąpiła w 15. dniu po ekspozycji (zarówno IHC, jak i EHC wykazały spadek zawartości CAT u ryb wystawionych na działanie Cu w porównaniu z kontrolą, $P<0,0001$) oraz w 28. dniu po ekspozycji (IHC wykazało wzrost CAT u ryb wystawionych na Cu w porównaniu z kontrolą, $P<0,0001$; EHC ujawniło spadek CAT u ryb doświadczalnych w porównaniu z kontrolą, $P<0,0001$). Biochemiczny profil krwi monitorowano za pomocą standardowego zestawu biochemicznego krwi. Wzrost zawartości mocznika we krwi ujawnił się jedynie u ryb poddanych działaniu Cu, natomiast spadek zawartości glukozy – z upływem czasu – wystąpił u ryb doświadczalnych i kontrolnych. Trzy zastosowane metody badawcze udowodniły, iż CAT skutecznie przeciwstawia się toksyczności Cu, a wzrost zawartości mocznika we krwi można uznać za odpowiedni marker metaboliczny oznaczający wystawienie ryb na działanie miedzi.

Słowa kluczowe: miedź, *Sparus aurata*, katalaza, skrzela, biochemia krwi, immunohistochemia, enzymohistochemia.

INTRODUCTION

Cu is an essential trace element for all organisms, but an excess can result in reactive oxygen species (ROS) production (Gaetke, Chow 2003) and lipid peroxidation (Halliwell, Gutteridge 1992). Recently, changes have been reported in *Sparus aurata* in the expression of proteins involved in the inflammation/immunity network (Isani et al. 2011).

Defense systems against oxyradicals include catalase (CAT) among other enzymes. CAT is a Fe^{3+} dependent metalloenzyme fundamental to all living organisms exposed to oxygen and is usually located in the matrix of cellular peroxisomes (Novikoff, Novikoff 1973), where CAT has been immunohisto-

chemically evidenced in primary cell cultures from fish (*Danio rerio) (*KRYSKO et al. 2010). CAT is present in nearly all cells exposed to oxygen, but its distribution in gilthead sea bream *Sparus aurata* (L.) gills is still unknown. Gilthead sea bream was chosen as a model for our study due to the available knowledge of its basic biochemical response to Cu (ISANI et al. 2003, 2011) and economic importance.

Since studies concerning blood biochemistry, immunohistochemistry (IHC) and enzymohistochemistry (EHC) for CAT on fish tissues exposed to a toxic amount of Cu are lacking, the aims of this work are: 1) to evaluate the effects of Cu exposure on blood biochemistry; 2) to test the methods for CAT location; 3) to show any variation in quantity and activity of the enzyme in *S. aurata* gills after Cu exposure.

MATERIALS AND METHODS

All experimental procedures were approved by the Ethics and Scientific Committee of the University of Bologna and were carried out in accordance with European legislation regarding the protection of animals used for experimental and other scientific purposes (Council Directive 86/609/EEC).

Experimental design

Eighty juvenile gilthead sea bream (*S. aurata*), average wt 65±5 g, were obtained from farmed stock. The fish were maintained in ten 500L tanks (at a density of eight fish/tank) filled with natural sea water taken from 300 m offshore Cesenatico (FC, Italy). Before the experimental exposure, fish were acclimatised for 15 days at 13°C and at a salinity of 32.0±2.6‰.

The work was performed during January-February 2009 when water temperature drops below 13°C and *S. aurata* experience the natural period of starvation (IBARZ et al. 2007). Therefore, fish were not fed throughout the Cu exposure time avoiding water contamination and additional Cu intake with the diet.

$CuSO_4$ was added to 5 tanks (40 specimens) to give a nominal Cu concentration of 0.5 mg dm^{-3}, while the fish in the other 5 tanks (40 specimens) served as controls. The waterborne concentration of Cu was chosen based on the evidence of previous studies (ISANI et al. 2003). Cu concentrations in water were verified by atomic absorption spectrometry (AAS). On day 0 (T0) prior to Cu exposure and after 15 (T1), 28 (T2) and 50 (T3) days fish were randomly chosen from each tank, sacrificed and tissues immediately sampled for metal, IHC and EHC analysis.

Sample collection

Gills were sampled from 32 control and 24 exposed fish and divided into two aliquots: 1) one for metal analysis was stored at -20ºC; 2) the other one was cut into halves, one for routine histology and IHC and the other for EHC: the samples for histology and IHC were formalin-fixed and paraffin-embedded, then processed according to routine procedures and cut at 4 µm; one section was stained with Haematoxylin and Eosin (H&E) and a further serial section was used for CAT IHC; for EHC, fish gill specimens were frozen in liquid nitrogen and stored at -80ºC for 7 days before processing and 4 µm-thick sections were cut with a cryostat.

Cu determination

Cu determination in gill samples and sea water were determined as reported by ISANI et al. 2011. The accuracy of the method was evaluated by calibration to an international standard (CRM 278).

Blood collection and biochemistry analyses

Blood samples from 21 control and 16 exposed specimens were collected and treated as previously reported (ISANI et al. 2011). Plasma samples were immediately analysed with an automated biochemical analyzer (Olympus AU400). A standard biochemical profile was chosen, including: glucose, urea, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatine kinase (CK), gamma glutamyl transferase (GGT), Ca, P, Mg, Na, Cl and K.

Immunohistochemistry

Four-m-thick paraffin-embedded tissue sections were dewaxed and rehydrated in decreasing ethanol solutions. Endogenous peroxidase was blocked by immersion in 0.3% hydrogen peroxide in methanol. Sections were then rinsed in Tris buffered saline (TBS) and antigen was retrieved with citrate buffer (2.1 g citric acid monohydrate/litre distilled water), pH 6.0, and heating for two 5 min periods in a microwave oven at 750 W, followed by cooling at room temperature for 20 min. A reduction of background staining was achieved with Protein Block Serum Free (cod. X0909 DAKO, Milan, Italy) for 10 min. The primary antibody (cod. C0979 Sigma, St. Louis, Missouri, USA), diluted (1:300) in PBS containing bovine serum albumin, was incubated with the tissue sections overnight at 4ºC. Sites of antibody binding were identified with Dako LSAB+ System-HRP code K0690. 3,3'-Diaminobenzidine (DAB) (cod. D5637 Sigma) was used as chromogen (0.05% in TBS 0.05 M pH 7.2 and H_2O_2: concentration 0.05% for 10 min at room temperature). The sections were then counterstained with Papanicolaou hematoxylin. In negative control sections, the primary antibody was replaced with phosphate buffered saline (PBS) containing bovine serum albumin.

Enzymohistochemistry

EHC CAT was performed according to CAJARAVILLE et al. (1993) on gill frozen specimens.

Control reactions were carried out with incubation media lacking H_2O_2, DAB or containing only imidazole (0.01 M) (CAJARAVILLE et al. 1993).

Quantitative analysis of CAT

CAT IHC: ten fields per each gill section, randomly chosen, were analysed to obtain a total number of 260 fields. Digital images of the selected fields have been achieved with a digital camera (Leica DFC320, Solms, Germany) mounted on a Leica DMLB (Solms, Germany) microscope and connected with a PC. All images were obtained using a 63x objective and a 10x eyepiece, yielding a final magnification of 630x. Images were acquired with an image analysis software Leica QWIN at 24-bit, saved as JPEG format. Each selected field had an area of $2.1x10^{-2}$ mm^2. For image analysis a standard grid with sixteen horizontal-lines was set up and superimposed to each image. Only the positive cells with nucleus adjacent to or cutting one of the lines were counted and the data expressed as the mean number of positive cells assessed irrespectively of the location (stroma or epithelium of the lamellae).

CAT EHC: images (400x magnification, $5.4x10^{-2}$ mm^2 per field) were acquired using the same tools as for IHC. Intensity of cytoplasmic staining was estimated in each of the 260 images, automatically measuring the integrated optical density with the image analysis software Leica QWIN.

Statistical analysis

The recorded values of IHC and EHC quantification were expressed as means ±SE (Standard Error) of the ten measured fields. For each case, CAT EHC and IHC quantitative data were tested for normality using the Shapiro-Wilk W-test. As the data distribution was not normal, the non-parametric Spearman rank order correlation test was utilized. Values of $P<0.05$ were considered significant. Blood biochemistry parameters were analysed by a two ways full factorial design time exposure with the R-statistics program.

RESULTS

Cu concentration

Cu analysis in the sea water samples after 50 days of exposure resulted in 0.35±0.05 mg dm^{-3}, indicating that 70-80% of the nominal dose of Cu was present in the exposure tanks at the end of the experiment. Cu concentrations in gills of controls at T0, T1, T2 and T3 did not change significantly;

while in exposed fish, metal concentrations increased significantly reaching a maximum of 6.9±4.7 μg g^{-1} wet weight at the end of the treatment (P<0.05) – Figure 1.

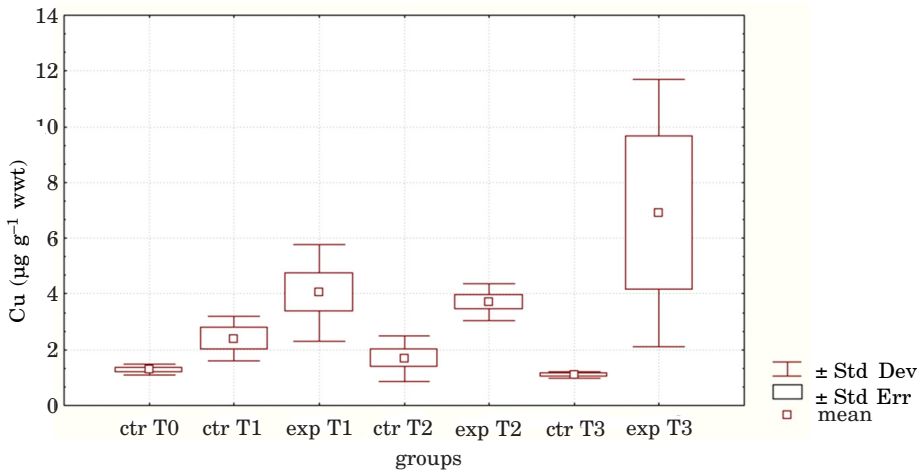

Fig. 1. Cu concentrations in gills of control (N=32) and exposed (N=24) fish. Significant increases were noted in treated fish (P<0.05)

Blood biochemistry

Only plasma urea significantly increased in Cu exposed fish (P<0.01). A significant decrease in glucose (P<0.01) and a significant increase for CK and ALP (P<0.05) were observed in control and exposed fish, as a function of time. The free ions did not show significant variations with the exception of a significant increase (P<0.05) of P both in control and exposed fish, as a function of time (Table 1).

Macro- and microscopic aspects

No mortality and no gross lesions were detected in the gills of exposed and control subjects. On histological ground no lesions were recorded.

CAT IHC and quantitative analysis

Brown intracytoplasmic positivity was evident in both exposed and control fish only in the epithelial cells of the primary and secondary lamellae and in pillar cells of the latter (Figure 2a). Scant positivity was noted in stromal cells. Quantitative analysis for CAT presence (Figure 3a), expressed by IHC, demonstrated a significant decrease in exposed subjects compared to controls at T1 (P<0.0001), an increase in positivity in exposed fish compared to controls (P<0.0001) at T2; similar values in positivity were obtained in exposed and control fish (P=0.09) at T3.

Table 1

Plasma biochemical parameters in control and treated specimens of gilthead seabream. Values are expressed as mean±standard error (SE)

Item	Glu* (mg dl^{-1})	Urea** (mg dl^{-1})	Ca (mg dl^{-1})	P* (mg dl^{-1})	Na (mg dl^{-1})	K (mg dl^{-1})	Cl (mg dl^{-1})	Mg (mg dl^{-1})	AST (U l^{-1})	ALT (U l^{-1})	ALP* (U l^{-1})	CK* (U l^{-1})	GGT (U l^{-1})
1	2	3	4	5	6	7	8	9	10	11	12	13	14
T0 (*N*=6)													
Mean	104	4.9	8.7	6.2	218	5.2	140	3.5	120	5.1	108	Nd	Nd
SE	14	0.8	0.6	0.7	4	0.5	3	0.2	29	0.1	18		
Min-Max	46-142	2.7-8.5	7.1-11.4	3.9-9.4	204-229	3.8-6.8	134-152	3-4.4	36-267	5-5.2	54-187		
T1 C (*N*=6)													
Mean	80	5.2	6.9	6.4	250	4.6	125	2.9	48	4.6	108	1274	9.7
SE	12	0.7	0.7	0.4	8	0.3	7	0.2	10	0.9	12	561	0.7
Min-Max	50-121	3.2-7.8	4.8-8.5	5.2-7.5	226-274	3.7-5.9	103-146	2.5-3.7	29-62	3-8	58-137	137-3475	7.7-11.4
T1 E (*N*=6)													
Mean	72	11.9	6.6	5.9	236	4.3	135	3.3	50	5.2	108	2685	8.9
SE	2	3.7	0.8	0.8	8	0.4	11	0.1	18	2	9	1035	0.8
Min-Max	67-76	5.5-26.4	4.8-8.5	3.1-7.4	224-260	3.5-5.3	105-155	2.9-3.6	14-114	1-12	92-136	710-5887	6.1-11.2

cont. Table 1

1	2	3	4	5	6	7	8	9	10	11	12	13	14
T2 C (*N*=5)													
Mean	81	6.0	7.8	7.0	248	4.6	131	2.9	78	5.8	119	5461	10.7
SE	6	0.2	0.1	0.6	5	0.4	2	0.2	19	0.8	3	1430	0.1
Min-Max	67-98	5.3-6.5	7.5-8.4	5-8.2	233-260	3.9-5.3	127-135	2.6-3.2	21-104	4-7	112-126	1448-6217	10.4-11.1
T2 E (*N*=6)													
Mean	53	7.7	7.2	6.2	241	3.9	125	3.5	60	5	76	4184	10.2
SE	4	0.8	0.9	0.5	4	0.1	6	0.5	9	0.5	9	942	0.8
Min-Max	35-67	5.0-10.6	4.3-10.6	4.4-7.4	233-261	3.4-4.1	103-145	2.6-6.1	30-80	3-6	48-112	1080-7377	7.3-13.4
T3 C (*N*=4)													
Mean	61	5.6	9.5	7.7	221	4.4	148	3.4	36	6.5	169	2755	12.3
SE	5	0.2	0.3	0.4	3	0.2	3	0.2	9	0.7	18	772	0.3
Min-Max	50-71	5.1-5.9	9.0-10.5	6.7-8.7	215-230	4-4.7	143-154	3.0-3.6	19-58	5-8	136-206	1282-4873	11.8-13.1
T3 E (*N*=4)													
Mean	57	7.5	7.9	9.1	225	4.6	142	4.1	75	9.5	141	4927	10.7
SE	5	0.2	0.6	1.6	2	0.2	6	0.5	23	2.3	35	1227	0.6
Min-Max	46-68	6.9-8.0	6.9-9.4	5.6-12.9	218-229	4.2-4.7	128-156	3.1-5.4	33-142	6-16	69-234	2529-6321	9.7-12.4

* Values presented significant variations as a function of time: glucose $P<0.01$; phosphorus $P<0.05$; alkaline phosphatase $P<0.01$ and creatine kinase $P<0.05$.
** Values presented significant variations as a function of Cu exposure: urea $P<0.01$.

CAT EHC and quantitative analysis

Enzyme activity was localized in the epithelium and pillar cells of both primary and secondary lamellae and appeared as a strong cytoplasmic stain particularly at the base of the primary lamellae (Figure 2b). Low positivity was observed in the connective tissue of the blood vessel walls of primary lamellae. Some positive spots were recorded within the branchial arch stroma. Quantitative analysis of enzyme activity in positive areas, evaluated as integrated optical activity, showed a significant decrease in exposed subjects

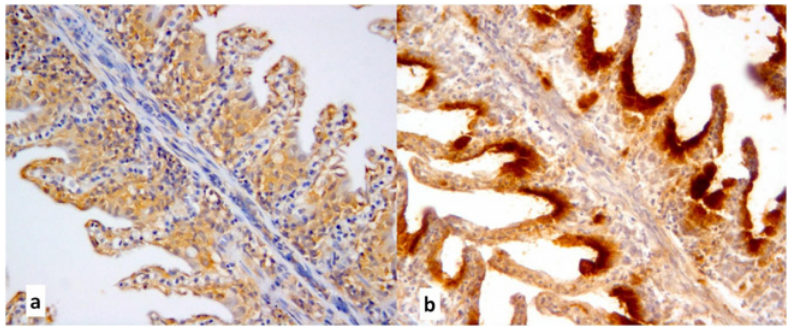

Fig. 2. Gills of the same control specimen: *a* – CAT IHC: pulverulent brown positivity in the cytoplasm at the base of secondary lamellae 40x; *b* – CAT EHC: enzyme activity is evidenced by brown colour in the epithelial cells of secondary lamellae 40x

compared to controls at T1 and T2 ($P<0.0001$) – Figure 3b. At T3 the activity of the enzyme in the exposed and control fish reached equal values ($P=0.68$).

Correlation analysis of CAT data

No evident correlation ($R=0.3$; $P=0.12$) was found between CAT IHC and EHC by linear regression analysis.

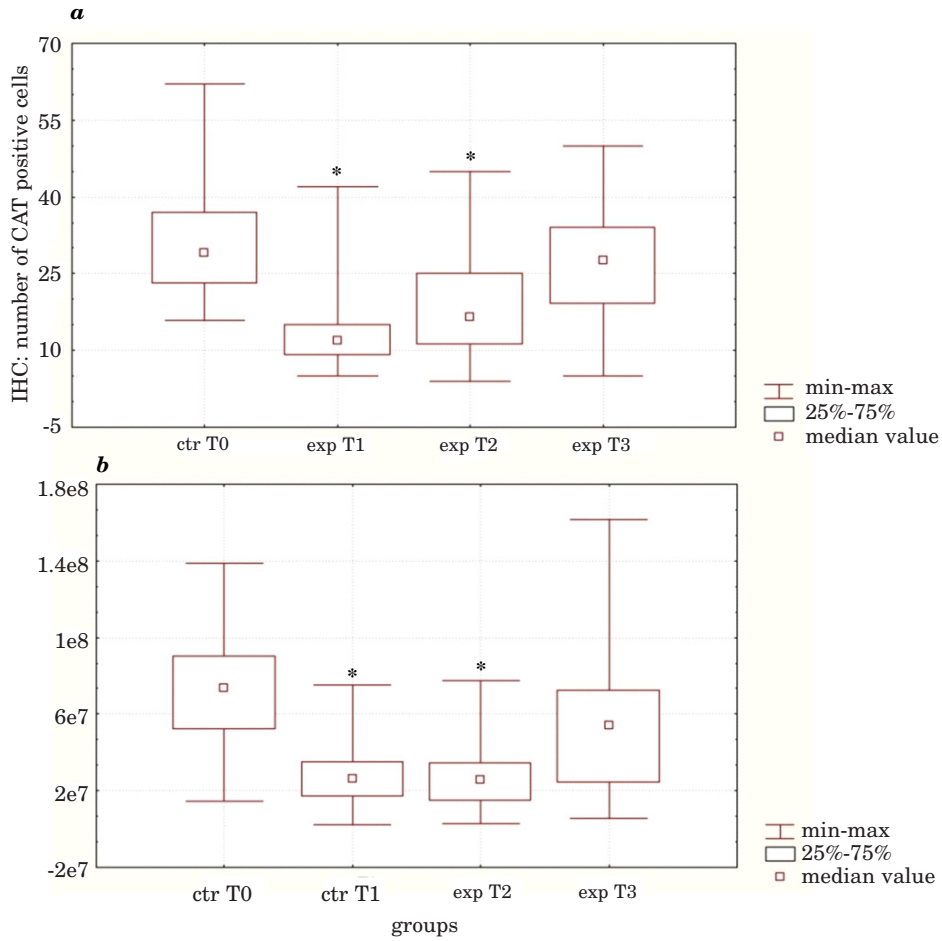

Fig. 3. CAT IHC (*a*) and CAT EHC (*b*) quantitative analysis.
*Indicates significant differences with respect to T0 (P<0.0001).

DISCUSSION

Cu accumulation in gills

Cu exposure has resulted in a significant increase in metal (5.3 folds) concentration in gills of exposed fish. Similar accumulation rates were found in *S. aurata* exposed for 20 days to 0.1 mg dm^{-3} and 0.5 mg dm^{-3} (Isani et al. 2003) and in black sea breams exposed to 0.05 mg dm^{-3} for 20 days (Dang et al. 2009). Cu concentrations in gills originate from mechanisms that regulate uptake, sequestration and export; as concerns membrane transport, also in gilthead sea bream the presence of the Ctr1 was demonstrated

(Minghetti et al. 2008). Moreover, Cu can be bound to mucus glycoproteins, which are present on gills surface and effectively bind diffusible cations such as Cu (Speare, Ferguson 2006), hence representing the first defensive line against waterborne Cu.

Blood biochemistry

Measurements of blood parameters are commonly used as a diagnostic tool in mammals, while they are not extensively applied to fish. The basal values of plasma parameters measured in T0 fish were in the range of those reported for other control or unexposed marine teleosts (Folmar 1993) and were indicative of a healthy status of fish used in the present work. Changes in blood biochemistry could be related to unsuitable environmental conditions (Barcellos et al. 2004), including chronic exposure to chemical pollutants (Folmar 1993). In the present experiment, Cu exposure determined a significant increase in plasma urea. In fish, urea is mostly used as an osmolyte or is excreted as a waste of ammonia detoxification after its biosynthesis in the liver (Grosell et al. 2004). The gills are the major site of urea excretion, through an urea transporter protein, which was indentified in several marine species (Walsh et al. 2001). The higher plasma urea levels in Cu-treated fish could be due to the impairment of gills nitrogen excretory function. The plasma urea increase could derive directly from the inhibition of the urea transporter, or indirectly from the inhibition of ammonia excretion; this metabolite in turn should be transformed in urea by the liver in order to avoid systemic toxicity. In both cases, the higher urea plasma concentration could derive from a molecular damage of gill transport systems. Accordingly, the measure of blood urea nitrogen, routinely determined in mammals as an index of renal function, could represent in fish a simple and useful biomarker of gill function. We also found a significant decrease of glucose as a function of time in both control and exposed fish. These changes could be due to starvation and are in agreement with data reported in river lamprey (Emelyanova et al. 2004). The observed increase in ALP may be linked to alteration of P metabolism (Whyte 2010) and together with changes of CK activity could be related to muscle proteolysis that naturally occurs during food deprivation (Seiliez et al. 2008).

CAT IHC and EHC

The IHC findings assessed CAT presence located mostly in the cytoplasm of epithelial cells at the base of the primary lamellae; to a lesser extent CAT was found in the epithelium of the secondary lamellae, where Cu is absorbed through the Ctr1 mediated transport (Minghetti et al. 2008). The role of CAT at these sites was to prevent the damaging of the epithelium in the form of membrane lipid peroxidation operated by Cu redox activity (Craig et al. 2007). The granular pattern found in the present paper confirmed CAT presence in peroxisomes.

In accordance with Guderley et al. (2003), starvation induced an increase in CAT presence. The significant decrease in CAT presence noted in our cases at T1 compared to T0 was consequent to Cu toxicity. The following increase of CAT concentration from T1 to T3 was partially due to starvation and in part to the protective role of CAT against metal-induced oxidative stress (FIRAT, KARGIN 2010).

CAT presence disclosed by IHC was integrated by EHC results indicating enzyme activity particularly at the base of the primary lamellae. The significant decrease in CAT activity at T1 and T2 in the gills of treated fish could be related to Cu exposure either by direct binding of the metal to the enzyme or by ROS-induced inhibition as reported by CRAIG et al. (2007) in *Danio rerio*. At T3, the increase in enzyme activity up to control levels may indicate an acclimation to waterborne Cu exposure or, more likely, the activation of protection mechanisms, such as an increase in metallothionein (MT) (ISANI et al. 2003) and/or CAT expression (CRAIG et al. 2007) in heavy metal exposed fish. CRAIG et al. (2007) examined the genes encoding for major proteins (including CAT) involved in combating oxidative stress in *D. rerio* and found no significant changes in the expression of CAT in the gills. However, similarly to our T1 data, they noted a decrease in CAT activity within 48 hours post exposure, which could suggest a rapid rise in oxidative stress due to sub-lethal doses of Cu and a comparably rapid response of defense mechanisms. The biphasic responses of CAT expression and its enzymatic activities were consistent with an increase of CAT gene transcription and a detoxifying activity of MT. At T3, Cu toxicity may be neutralized by the increase in CAT expression, which in turn appeared as a rise in enzymatic activity.

IHC positivity did not necessarily coincided with EHC positivity. As two CAT isoforms were recognized (BAILLY et al. 2004), one active (55 kDa) and one inactive (59 kDa), we assumed that when the inactive isoform is present, only IHC results positive, whereas both methods reveal the active isoform.

All the three main investigation methods, i.e. analytical chemistry, IHC-EHC and blood biochemistry, have proven effective in highlighting responses to Cu exposure: increased Cu levels were associated with an initial decrease in CAT expression and activity and with increased plasma urea that could be considered a metabolic marker of gills function in exposed fish.

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