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Effects on Metallothionein Levels and Other Stress Defences in Senegal Sole Larvae Exposed to Cadmium

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Cadmium (Cd^{2+}) is a toxic metal because it interferes with essential metals such as Ca, Zn and Fe that may distort many physiological processes regulated by metallic enzymes (Wright and Welbourn, 1994). Deleterious effects caused by Cd have been observed in aquatic organisms *e.g.* adult fish and larvae (Hwang et al., 1995; Ricard et al., 1999; Vaglio and Landriscina, 1999; de la Torre et al., 2002). Cd is a transition metal that doesn't undergo redox cycling, however, some studies have shown its capacity for the formation of reactive oxygen species (ROS) (Viarengo et al., 1990; Stohs et al., 2000). In order to maintain cell integrity, aquatic organisms possess a suite of enzymes and proteins that enable them to cope with environmental stressors. Metallothionein (MT) plays an important role in metal metabolism and also acts as a detoxifying system for excess metals within the cell (Roesijadi and Robinson, 1994). MT is induced by metal exposure but it is also affected by oxidants (Viarengo et al., 2000). Aerobic organisms also possess antioxidant enzymes to enable them to counteract ROS formation. Catalase detoxifies H_2O_2 to H_2O and O_2; total glutathione peroxidase (t-GPX) (sum of Selenium dependent and independent forms) detoxifies organic peroxides, some glutathione *S*-trasferase (GST) forms detoxify H_2O_2 and organic peroxides and DT-diaphorase catalyses a 2 electron reduction of quinones to hidroquinones, thus preventing the formation of reactive quinone intermediates (Halliwell and Guteridge, 2000). The cytochrome P4501A dependent ethoxyresorufin *O*-deethylase (EROD) activity was also included as an indicator of possible effects of metal exposure on the xenobiotic metabolism system.

The imbalance between the formation of ROS and the protective systems repairing action is responsible for damage to key molecules such as DNA, proteins or lipids. Lipid peroxidation (LP) is indicative of ROS exposure (Livingstone, 2001). Cd^{2+} can also have a neurotoxic effect on the enzyme AChE which is the main neurotransmitter in vertebrates, including fish (Donald, 1998). Its neutotoxic action occurs by interfering with Ca^{2+}-mediated neurotransmitter release at neuromuscular junctions. Stress proteins, also termed as heat shock proteins, act as a repair mechanism for damaged proteins (Iwama et al., 1998). In short, whereas LP indicates damage to lipids, expression of stress proteins indicates proteolysis.

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Due to industrial and/or agricultural applications, Cd in the aquatic environment is likely to be enhanced in proximity to urban areas and is typically associated with the sediment, which acts as sink for non-degradable pollutants. We selected the Senegal sole, *Solea senegalensis* Kaup, as a native sentinel species. As a benthic organism it is more likely to be exposed to sediment-bound pollutants. Also, it is a warm-water flatfish that is cultured in countries such as Spain and Portugal where it is more abundant that *S. solea*. Larval stages of fish are considered to be very sensitive periods during the animal's growth and development. Therefore, measuring the negative effects of man-made chemicals and/or metals, even at sublethal doses, at these stages may highlight any potential for future harm in these animals. The selected biomarkers of exposure (MT, AChE, EROD, GST), effect (LP) or general stress (antioxidant enzymes and stress proteins), were evaluated following metal exposure.

Before the application of biomarkers in field studies, laboratory experiments are necessary to establish cause-effect relationships. Therefore, the aim of our study was to apply a suite of stress biomarkers in 21-day old larvae of the benthic flat fish, *S. senegalensis*, following exposure to a sublethal dose of 100 μg/L Cd for 48 hours in order to establish their suitability for further application to areas chronically or acutely exposed to metals. The age of the larvae was based on previous observations of our group, which pointed to the completion of metamorphosis as a potentially sensitive period to evaluate environmental stressors.

MATERIALS AND METHODS

Solea senegalensis were reared at the IFAPA-CIFPA facilities, Junta de Andalucía, Cádiz (Spain) in rearing conditions established to be optimal for this species. 250 larvae of 21 days post hatch (dph) were maintained in two replicate 35-L tanks at 20°C temperature with constant illumination, 6.8-mg/L oxygen concentration, pH 8.26 and 37 PSU salinity. Larvae without or with exposition to Cd (100 μg/L $CdCl_2$) were sampled after 48 h starvation to avoid interference of gut contents in the analysis. All measurements and determinations were performed in quadruplicate. About 20 individuals were selected to measure mean total length (11.3 ± 0.87cm) and dry weight (1813.3 ± 0.37 μg).

Metal analysis was undertaken in digested simples according the procedure described by Amiard et al (1987), and the results were validated using reference material (DORM1 and TORT1 of NRC Canada). The certified values for DORM1 were: Cd (0.086 ± 0.012), Cu (5.22 ± 0.33) and Zn (21.3 ± 1.0); for TORT1: Cd (26.3 ± 2.1), Cu (439 ± 22) and Zn (177 ± 10). Our values were for DORM1: Cd (0.082 ± 0.010), Cu (5.32 ± 0.29), Zn (20.3 ± 0.7); for TORT1: Cd (26.2 ± 1.5), Cu (417 ± 13), Zn (171 ± 7). These values correspond to the mean and the 95% confidence intervals. Certified and our values are very close for all analysed metals. The detection limits were: Cd (0.002 μg/g d.w.); Cu (0.05 μg/g d.w.) and Zn (0.02 μg/g. d.w.). Metal concentration were analysed by FAAS for Cu and Zn and by GFAAS with Zeeman background correction for Cd. The results are expressed as μg/g dry weight.

Biochemical determinations were carried out on the whole body homogenate after centrifugation at 13,500 g x 5' at 4°C. Catalase, t-GPX, GST, DT-diaphorase and AChE were measured spectrophotometrically, EROD measured fluorometrically and stress proteins were separated by SDS-PAGE and further immunodetected using commercial antibodies: HSP 70 (H5147 Sigma) and HSP 60 (H3524 Sigma) at the dilutions 1:2500 and 1:500, respectively. MT levels were determined by differential pulse polarography and lipid peroxidation as the formation of MDA. Activities were referred to total protein content. A more detailed description of the methods in Peters and Livingstone (1996) and Solé et al., (2000).

Student's *t*-test was applied to compare both groups. Biochemical data is presented as mean ± SEM (n=4). Significance was considered when * $p<0.05$.

RESULTS AND DISCUSSION

Metal analysis confirmed a clear increase in Cd, but not for Cu and Zn, after 48-h exposure to 100 μg/L Cd. Levels of Zn were also analysed as changes in Zn homeostasis can affect MT expression. This period (48-h) was enough to cause a 55% induction of MT in the exposed larvae with respect to controls, whereas total protein yield –PY- remained unaffected (Table 1). An early appearance of MT mRNA in oocytes and newly hatched larvae has been described in Cd-treated tilapia (Lin et al., 2000) and in turbot (*Scophthalmus maximus*), at various stages of development, after 48-h acute waterborne Cd exposure (George et al, 1996). MT protein expression was seen in *S. senegalensis* from 0 to 28 dph (unpublished data from our group), with comparable levels in MT for the same age group in both studies. Also a quick maximal response in MT mRNA expression was found in adult channel catfish, *Ictalurus punctatus*, after 48 h Cd exposure at the dose of 100 μg/L (Zhang and Schlenk, 1995). In contrast, 7 days were necessary to elevate MT response in gills of *Ruditapes decussata* exposed to the same Cd dose (Geret et al., 2002). It is reasonable to expect the response in fish and invertebrates to be different, in the same way that fish adults and larvae can also respond differently to metal insults.

Table 1. Metals and MT content in control and Cd (100 μg/L) exposed *S. senegalensis* larvae.

	Cd[a]	Cu[a]	Zn[a]	MT[b]	PY[c]
Control	0.009 ± 0.008	2.67 ± 0.25	32.21 ± 4.03	4.93 ± 0.13	40.1 ± 3.7
Cd	1.62 ± 0.13*	3.10 ± 0.41	23.20 ± 2.46	7.68 ± 0.05*	48.1 ± 6.7

[a] μg/g d.w. [b] μg/mg prot. [c] mg prot/mg w.w

The antioxidant enzyme catalase and NADH DT-diaphorase were significantly inhibited in the Cd exposed group (Table 2). t-GPX activity in the Cd-group did not significantly differ from the control ($p>0.05$). Similarly, catalase and other GSH-dependent enzymes were depleted in liver and blood of the adult fish *Sparus aurata*, after 3 and 6 days Cd administration (Vaglio and Landriscina, 1999). Dietary exposure to increasing doses of Cd for 1 month in Atlantic salmon *Salmo salar* caused a significant depletion of Se-GPX but LP was only enhanced at the highest dose (204 mg Cd/Kg) (Berntssen et al., 2000). *In vitro* kidney subcellular

fractions from sea bass, *Dicentrarchus labrax* exposed to several Cd concentrations depleted catalase but had no effect on LP (Romeo et al., 2000). Similar observations were encountered in invertebrates. In the clam *R. decussatus* exposed to 100 μg/L Cd for similar duration depleted catalase, t-GPX, Se-GPX, but elevated SOD (Geret et al., 2002). In some studies, depletion of GSH-dependent enzymes has been attributed to GSH decrease, but not consistently (Thomas and Wofford, 1993). Cd interaction with metalic enzymes altering their structure and functioning has been proposed as another plausible mechanism for the reduced activity of antioxidant enzymes (Vaglio and Landriscina, 1999).

Table 2. Antioxidant enzymes in control and Cd (100μg/L) exposed *S. senegalensis* larvae.

	Catalase [a]	t-GPX [b]	NADH DCPIP red.[b]	NADH DT-diaphorase [b]	NADPH DCPIP red.[b]	NADPH DT-diaphorase [b]
Control	15.1 ± 1.9	50.1 ± 2.0	15.5 ± 2.1	3.0 ± 0.5	12.4 ± 0.7	2.2 ± 0.5
Cd	8.5 ± .4*	35.4 ± 6.3	13.1 ± 0.7	1.5 ± 0.4 *	11.3 ± 0.6	2.4 ± 0.4

[a] μmol/min/mg prot. [b] nmol/min/mg prot.

Phase I (EROD) and phase II (GST) enzymes were also measured as a possible indication of metal effects on xenobiotic metabolism. Neither were affected by Cd. Indication of liver damage, measured as an alteration of cytochrome P450 reductases, was seen in Cd fed fish (Vaglio and Landriscina, 1999). AChE, indicative of neurotoxicity was also slightly depleted in the Cd-exposed larvae (Table 3). Despite a 31% inhibition of AChE in the exposed group statistical significance was not reached due to high variability in the control animals. AChE was not affected in rainbow trout (*Oncorhynchus mykiss*) larvae after exposure to lower doses of Cd (Beauvais et al., 2001), or juvenile carp *Cyprinus carpio* exposed to a higher Cd dose (1,6 mg/L) for 14 days (de la Torre et al., 2002). Nonetheless, AChE was enhanced in other fish species (Christiensen, 1975; Gill et al., 1991). However, caution should be taken when contrasting these last reported observations with our results. In the first study (Christiensen, 1975), although the age of fish was the same (21 days), the species was different (brook trout *Salvelinus fontanalis*), the doses lower (3.43 μg/L maximal dose) and the length of exposure longer (21 days). In the second study (Gill et al., 1991), the time of exposure was the same (48 hours), but the fish were adults of the freshwater species *Barbus conchius,* and the dose was much higher (12.6 mg/L). A moderate inhibition in AChE could affect swimming speed, which in natural waters may imply reduced survival (Beauvois et al., 2001).

Oxidative damage, measured as lipid peroxidation, was evaluated by the formation of malondialdehyde (MDA). Some studies with fish have indicated enhanced lipid peroxidation responsible for tissue damage following Cd exposure (Thomas and Wofford, 1993). Despite the depletion of certain antioxidant defences, LP in Cd treated fish larvae was not different from control, neither was it in other short-term exposure studies with molluscs at comparable doses (Arasu and Reddy 1995; Viarengo et al., 1990). LP occurred after 21 days exposure in *R.*

decussata using 100μg Cd/L (Geret et al., 2002). The hypothesized reason for not altering MDA levels is that Cd does not undergo redox cycling, which clearly stimulates ROS production and LP. Therefore other indirect mechanism such as stress defence depletion could be responsible for the enhanced LP observed in other studies.

Table 3. Biomarkers of exposure (AChE and GST), of general stress (HSPs) and effect (LP) in control and cadmium (100μg/L) exposed *S. senegalensis* larvae.

	AChE [a]	GST [a]	EROD [b]	HSP70 [c]	HSP60 [c]	LP [d]
Control	147.8 ± 26.4	67.0 ± 7.3	16.6 ± 1.0	8.4 ± 0.3	17.5 ± 0.8	146.5 ±16.5
Cd	101.8 ± 3.0	53.9 ± 4.7	15.0 ± 1.8	7.2 ± .4*	14.1 ± .7*	123.2 ± 8.5

[a] nmol/min/mg prot. [b] nmol/min/mg prot. [c] a.u./mg prot. [d] nmol MDA/g w.w.

A short-term reduction in antioxidant defences in fish larvae contrasts observations in adult fish, which required much higher doses of $CdCl_2$ (1500-3000 μg/L Cd) and 60 days exposure to cause an effect on the antioxidant defences SOD and GPX. Despite these high doses LP did not occur in the resistant fish (Almeida et al., 2002). The reason for this difference could be the lower MT induction and less cellular protection during larval stages in contrast to adult fish (George et al., 1996).

Stress protein (HSP 70 and HSP 60) levels measured by inmunoblotting were also significantly lower ($p<0.05$) in Cd exposed individuals (Table 3). Although some studies have reported an increase in this response after Cd exposure (Gaubin et al., 2000), the mechanism responsible for this is not yet clear. In fish, a tissue- and seasonal-dependence was seen in HSP70 response to Cd polluted sites (Yoo and Janz 2003). Studies with a human lung cell line indicated an increase in the stress response (HSP90, HSP 72 and HSP 27) after Cd exposure, in this case it was more related to ROS formation and potential damage to proteins than to GSH depletion (Gaubin et al., 2000).

Our observations, in fish larvae, together with the information from the available literature on Cd toxicity, seem to point out that acute short-term exposure to sublethal doses of Cd has an immediate effect on depletion of antioxidant defences (mainly catalase and GPX) and stress proteins, whereas MT increases. Chronic longer-term exposure to sublethal doses implies an increase in antioxidant defences and LP prevention. All enzymatic activities and protein levels determined in the control animals in this study are in agreement with levels found in *S. senegalensis* larvae after metamorphosis completion in a study lasting 28 days from hatch (unpublished data).

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