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Impact of cadmium on aquatic bird *Cairina moschata*

Magali Lucia · Jean-Marc Andre · Patrice Gonzalez · Magalie Baudrimont · Karine Gontier · Regine Maury-Brachet · Stephane Davail

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Abstract The impact on palmiped *Cairina moschata* of two levels of dietary cadmium (Cd) contamination (C1: 1 mg kg^{-1} and C10: 10 mg kg^{-1}) was investigated on liver gene expression by real-time PCR. Genes involved in mitochondrial metabolism, in antioxidant defences, detoxification and in DNA damage repair were studied. Metallothionein (MT) protein levels and Cd bioaccumulation were also investigated in liver, kidneys and muscle. Male ducks were subjected to three periods of exposure: 10, 20 and 40 days. Cd was mainly bioaccumulated in kidneys first and in liver. The concentrations in liver and kidneys appeared to reach a stable level at 20 days of contamination even if the concentrations in muscle still increased. Cd triggered the enhancement of mitochondrial metabolism, the establishment of antioxidant defences (superoxide dismutase Mn and Cu/Zn; catalase) and of DNA repair from 20 days of contamination. Discrepancies were observed in liver between MT protein levels and MT gene up-regulation. MT gene expression appeared to be a late hour biomarker.

Keywords Cadmium · Palmiped · *Cairina moschata* · Gene expression · Metallothionein

M. Lucia (✉) · J.-M. Andre · K. Gontier · S. Davail
IPREM-EEM (Institut Pluridisciplinaire de Recherche sur l'Environnement et les Matériaux, Equipe Environnement et Microbiologie), UMR 5254, IUT des Pays de l'Adour, 371 rue du Ruisseau, BP 201, 40004 Mont de Marsan Cedex, France
e-mail: magali.lucia@etud.univ-pau.fr

P. Gonzalez · M. Baudrimont · R. Maury-Brachet
UMR CNRS 5805 EPOC, Team GEMA, Université Bordeaux 1, Place du Dr Peyneau, 33120 Arcachon, France

Introduction

In environment, palmipeds and birds in general, appear to be highly contaminated by pollutants and especially by metals such as cadmium (Cd) (Elliott and Scheuhammer 1997; Lock et al. 1992). These species are considered as good integrators of environmental contaminations and good subjects for examination of pollution and its impact on populations. Indeed, birds feed at different trophic levels, with piscivores species representing the most exposed to a great pressure of contamination (Boening 2000; Hernández et al. 1999). They can be long-lived, and many are both abundant and widely distributed (Rothschild and Duffy 2005).

Cadmium is a widely distributed metal. Its presence in numerous environments is due to its widespread industrial use. For instance, in Alaska, very high levels of cadmium were reported in kidneys of Spectacled eiders (*Somateria fischeri*) (Trust et al.

2000). These levels can lead to various effects on organisms such as the induction of inflammation of renal interstitium and marked degenerative changes in testes (Hughes et al. 2000). However, few studies have focused on the possible impact of this non essential element on cellular metabolism of aquatic birds. Moreover, these contamination studies are often realized with very high Cd concentrations which can hide the real bioaccumulation and impact of the metal as previously described on the fish *Anguilla anguilla* (Pierron et al. 2008).

The aim of this study was to investigate, through experiment, the effects of two Cd contamination levels in birds (1 and 10 mg kg^{-1}) using a breeding palmiped as model: C*airina moschata*. This biological model was chosen according to its facility of access and its stable evolution in a controlled system. The lowest concentration used here permitted us to investigate Cd effects at field contamination level. Indeed, Cd concentration remains similar to level that can be observed in environment such as in the Gironde estuary which is characterized by its contamination by Cd along the estuarine gradient (Baudrimont et al. 2005). Many studies were done on molluscs and fishes of the Gironde estuary, the potential food of birds. For example, studies have reported around 2 mg kg^{-1} of Cd in liver of European eels (Pierron et al. 2008) and around 2 mg kg^{-1} of Cd in kidneys of Siberian sturgeon (Maury-Brachet et al. 2008). The levels recorded in molluscs are important: 24 mg kg^{-1} in oysters, 1.4 mg kg^{-1} in cockles and in clams (Baudrimont et al. 2005). For now, no studies were done on the bird population of the Gironde estuary and their metal levels even if this ecosystem represents an important transition along the migratory way of numerous bird species and a habitat for sedentary palmiped species such as *Anas platyrhynchos*. In addition, the highest level was used to study acute pollution and to evaluate possible damages caused by high Cd level observed in liver and kidneys.

The effect of Cd was investigated through three kinds of responses. This study describes (1) Cd bioaccumulation at the organ level, (2) MT protein response levels and (3) genes expressions measured by real-time PCR and normalized according to expression of the reference gene: β-actin. Gene expressions were investigated in liver because this organ is the main site of detoxification in ducks.

In order to study Cd effects, nine genes involved in detoxification processes, mitochondrial metabolism, lipogenesis, responses to oxidative stress and DNA repair were chosen. Toxicological studies at cellular scale have shown that Cd inhibits the mitochondrial electron transfer chain and induces reactive oxygen species (ROS) production (Wang et al. 2004) leading to DNA strand damage. Mitochondrial metabolism was investigated using the cytochrome *c* oxidase subunit 1 (*cox1*), NADH dehydrogenase subunit 5 (*nad5*) and lipogenesis was investigated through the expression of acetyl-CoA carboxylase (*acc*). Moreover, organisms display different mechanisms to counter Cd toxicity. Among them, metallothioneins (MT), low molecular weight and cysteine-rich proteins, are known to be involved in homeostasis of essential metals such as zinc and in protection against toxic metals such as Cd. Another way employed to counter Cd toxicity, is the increase of antioxidant defences as cytoplasmic and mitochondrial superoxide dismutase (*sod1* and *sod2*, respectively) and catalase (*cat*). Moreover, a gene coding for a protein involved in the repair system of the DNA has been investigated: the growth-arrest-DNA-damage (*gadd*).

Materials and methods

Experimental design

Male ducks were raised in a breeding structure belonging to the French National Research Institute for Agronomy (INRA) at the "domaine d'Artiguères", Benquet, Landes, France. From the first day of life to 4 weeks of age, males were fed ad libitum with small granules (length of 2.5 mm) enriched in protein. Between 4 and 10 weeks of age, ducks were fed ad libitum with growth food (granule with length of 4 mm) less enriched in protein.

Animals were placed in individual cages and three conditions were studied: uncontaminated ducks which constituted control animals, and two levels of contamination: 1 mg kg^{-1} of Cd (C1) and 10 mg kg^{-1} of Cd (C10). To contaminate animals during experimental design, capsules of Cd were made by adding Cd to crushed granules in the form of $CdCl_2$. One capsule per day was added to 200 g of granule which contained 14% water, 15.7% protein, and 2.3% fat and were given to animals. The feeding of ducks was carefully

monitored to make sure that animals eat all the granules. The Cd concentrations in food were checked and the averages represented 0.05 ± 0.01, 1.02 ± 0.01 and 10.03 ± 0.04 mg kg^{-1} for control ducks, 1 and 10 mg kg^{-1} conditions, respectively. The lowest level of contamination was chosen in relation to Cd levels encountered in Gironde estuary (C1: 1 mg kg^{-1} of Cd). The upper level was chosen to compare the impact of chronic environmental level and acute concentrations of metal (C10: 10 mg kg^{-1} of Cd).

At the beginning of the experiment (before addition of Cd), five ducks were removed to study the level of metal accumulated prior to the experiment. Animals were sampled at three others time of Cd exposure: 10, 20 and 40 days of contamination. A total of 50 animals were sampled for this experiment. At each time, five ducks per experimental condition were removed. The total weight of the body, the liver and the kidneys were recorded. The liver, kidneys and muscle were dissected and divided in two parts. The first part was stored at −80°C for metallothioneins quantification (muscle, kidneys, liver) and for genetics analyses (liver only after immediate freezing with liquid nitrogen). The second part was stored at −20°C before metal determination.

Metal determination

Metal determinations were made on five replicates per experimental condition. Biological samples (liver, muscle and kidneys) were dried (45°C, 48 h) and digested with 3 ml of pure nitric acid (Fluka; Buchs, Switzerland) added in tubes at 100°C for 3 h. After a six-fold dilution of the digestates with ultrapure water (MilliQ, Bedford; MA, USA), Cd concentrations were measured by electrothermic atomic absorption spectrophotometry with Zeeman correction, using a graphite tube atomizer (EAAS Thermoptec M6Solaar). Samples of 20 µl were mixed before atomisation with 4 µg of Pd (analyte modifier) and 3 µg of $Mg(NO_3)_2$ (matrix modifier). The detection limit was 0.1 µg Cd l^{-1} (3 × standard deviation of the reagent blanks). The analytical methods were simultaneously validated for each sample series by the analysis of standard biological reference materials (Tort-2: lobster hepatopancreas and Dolt-2: dogfish liver from NRCCC-CNRC; Ottawa, Canada). Values were consistently within the certified ranges (data not shown).

Metallothionein determination

Total MT proteins were determined in liver, kidneys and muscle by mercury-saturation assay using cold inorganic mercury (Hg) as previously described (Baudrimont et al. 2003; Dutton et al. 1993). This technique is based on the quantification of Hg bound to the saturated MT. MT analysis was performed on five replicates per experimental condition. The different tissues were homogenized under nitrogen atmosphere to avoid denaturation of MT proteins and kept on ice to inhibit protease activity. The samples were centrifuged at 20,000×*g*, for 60 min, at 4°C (Sigma 3K12, rotor 12154). The saturation assay was repeated twice on each sample with the $HgCl_2$ solution (Merck) at 50 mg l^{-1} in trichloroacetic acid 10%. The excess of Hg was eliminated with addition of bovine or porcine hemoglobin (Sigma) prepared in 30 mM Tris–HCl buffer (pH 8.2 at 20°C) and a rapid centrifugation (20,000×*g*, 20 min). At the same time, three blanks (Tris–HCl 25 mM) were used to control the Hg complexation efficiency of the hemoglobin and three standards (purified rabbit liver MT, Alexis Biochemicals) were employed to determine a recovery percentage.

The quantification of Hg bounded to MT was determined using flameless atomic absorption spectrometry (Leco Ama 254, Altec, Prague, Czech Republic) with a detection limit of 0.01 ng Hg. The results were expressed in nmol Hg g^{-1} (wet weight), because of the unknown number of Hg binding sites per MT.

Sequencing of genes

Three genes DNA fragments were searched for: mitochondrial superoxide dismutase (*sod2*), catalase (*cat*) and growth arrest DNA damage protein (*gadd*).

A quantity of 40 mg of fresh liver was homogenized to extract total RNAs using Absolutely Total RNA Miniprep kit (Stratagene, Netherlands), according to the manufacturer's instructions. The quality of all RNAs extracted was evaluated by electrophoresis on a 1% agarose-formaldehyde gel, and their concentration determined by spectrophotometry. First-strand cDNA was synthesized from 5 µg of previously extracted total RNA with AffinityScript Multiple temperature cDNA synthesis kit (Stratagene, Netherlands), according to manufacturer's instructions.

The unspecific primers, used for PCR to obtain amplified cDNA fragment of the different genes, were determined after multiple sequence alignment of birds or mammalian species using Clustal W software (Infobiogen) (Table 1). Amplified products were cloned into pGEM-T vector (Promega) and sequenced (Millegen, France).

Table 1 Primer pairs used to clone partial mRNA sequences of the mitochondrial superoxide dismutase (*sod2*), catalase (*cat*) and growth arrest DNA damage (*gadd*) genes

Gene name	Primers (5′–3′)
sod2	CGACTATGGCGCGCTGGAGCC[a]
	CCCAAGCCACCCCCAGCCTGA[b]
cat	GTTTTCACTGATGAGATGGC[a]
	GGGTTCCTCTTCTGGCTATGGAT[b]
gadd	GAGGCGGCCAAGCTGCTCAACGT[a]
	GTTGATCACCGGCACCCACTG[b]

[a] Forward primers

[b] Reverse primers

For actin, acetyl-CoA carboxylase and superoxide dismutase 1, multiple sequence alignments of corresponding sequences from birds were used to determine primer pairs usable to amplify these three genes in *C. moschata*. Then, alignments between the resulting sequences of *C. moschata* and those of another duck *Anas platyrhynchos* have demonstrated that primer pairs chosen in conserved regions could be used for both species during qPCR analysis (Table 2).

Real-time PCR

Real-time PCR reactions were performed in a MxP3000 (Stratagene) following the manufacturer's instructions (one cycle at 95°C for 10 min, and 40 amplification cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s). Each 25 µl reaction contained 1 µl of

Table 2 Specific primers and accession numbers or reference of genes used for qPCR

Gene name	Accession number/reference	Specific primers (5′–3′)
act	EF667345[a]	ACAGGAAGTTACTCGCCTCT[b]
		TGGGGAACACAGCCCGC[c]
cox1	EU755254	GCACAGCACTCAGCCTAC[b]
		AATGCCATGTCGGGGGC[c]
nad5	EU755254	ATTCGCCGCCACATGC[b]
		ATGGACCCGGAGCATAGGAA[c]
acc	EF990143[a]	GTCCTCCAAGCCAAGCAATGTG[b]
		GGCCTTGATCATGACAGGGTAGCC[c]
12s rRNA	AM902523	CAACCCAACCACCCCT[b]
		TCCTCCTTCCAGAAGCAGT[c]
sod1	Liu et al. 2002[a]	GCGCACCATGGTGGTCCATG[b]
		GTCTTCACCAGTTTAACTGATACTCA[c]
sod2	EU598450	ACGCCGAGATCATGCAG[b]
		CGAAAGATTTGTCCAGAAGATGGT[c]
cat	EU598451	GTTTGAACACATTGGAAAAAGAACACC[b]
		GGCTATGGATGAAGGATGGAAACA[c]
mt	U34230	TGGACCCCCAGGACTGC[b]
		CCGGCTATTTACAGGCGGA[c]
gadd	EU598452	GGACGAGGAGGAAGCGG[b]
		GGGGTTCGTGACCAGG[c]

act Actin, *cox1* cytochrome *c* oxidase subunit 1, *nad5* NADH dehydrogenase subunit 5, *acc* acetyl-CoA carboxylase, *sod1* superoxide dismutase (Cu/Zn), *sod2* mitochondrial superoxide dismutase (Mn), *cat* catalase, *mt* metallothionein, *gadd* growth-arrest-DNA-damage

[a] Accession number/reference of *Anas platyrhynchos*

[b] Forward primers

[c] Reverse primers

reverse transcribed product template, 12.5 µl of Brilliant master mix including the SyberGreen I fluorescent dye (Stratagene), enabling the monitoring of the PCR amplification, and the gene-specific primer pair at a final concentration of 200 nM for each primer.

Gene-specific primer pairs were determined using the LightCycler probe design software (version 1.0, Roche) (Table 2).

Reaction specificity was determined for each reaction from the dissociation curve of the PCR product. This dissociation curve was obtained by following the SyberGreen fluorescence level during a gradual heating of the PCR products from 60 to 95°C. Relative gene expression level was normalized according to the β-actin gene expression. For each gene expression level, mean value and the associated standard deviation ($n = 5$) were determined. Induction or repression factors were obtained with the comparison of each mean value between contaminated ducks and control ducks.

Statistical analysis

One-way analysis of variance was applied to assess differences in Cd accumulation, MT concentrations and gene expression levels between control and contaminated ducks. The effect of exposure time and Cd level was also determined. Post-hoc test was used if significant differences ($P < 0.05$) appeared between data to identify which group differed (Tukey HSD test). Normality and homogeneity of variance, necessary for the use of this parametric test, were verified. If these assumptions were not achieved despite $\log_{10}(x + 1)$ transformation (Cochran C test), non-parametric analysis of variance (Kruskal–Wallis test, Statistica 7.1) was applied. Two by two comparisons were performed with the non-parametric Mann–Whitney U-test (Statistica 7.1). The Spearman correlation test was applied to evaluate the relationship between the concentrations of Cd and metallothionein.

Results and discussion

Cd bioaccumulation

There were no differences of total weight, liver and kidneys weight between control and contaminated ducks during the experiment.

At each exposure time (10, 20 and 40 days) and for all organs, Cd concentrations of control ducks were lower than those of contaminated ducks of the conditions C1 (1 mg kg^{-1}) and C10 (10 mg kg^{-1}). Cd bioaccumulation in liver and kidneys was also significantly different following levels of metal (C1 or C10) given to ducks (Fig. 1a, b). Indeed, Cd concentrations of ducks subjected to C1 contamination was significantly lower than at C10 contamination level ($P < 0.05$). In this study, kidneys displayed the highest Cd concentrations (C1: 10.7 ± 0.4 mg kg^{-1} dw; C10: 83.4 ± 11.2 mg kg^{-1} dw at 40 days).

Previous studies have shown an impact of cadmium on organs such as kidneys (Hughes et al. 2000). These impairments have often been observed on very high contamination levels which are not relevant with concentrations found in natural environments or with Cd levels recorded in wild bird species. However, in some field studies, very high concentrations were reported in birds' kidneys which represent the main internal organ for the accumulation of this metal (Barjaktarovic et al. 2002; Trust et al. 2000). During this study, Cd showed the same pattern of accumulation in tissues as those previously established for bird tissues and organs (Gómez et al. 2004; Kalisinska et al. 2004; Nam et al. 2005). Indeed, kidney was the main site of Cd accumulation demonstrating the role of this organ in detoxification and storage of non essential elements. In contrast, muscle was often described as a minor site of accumulation (Nam et al. 2005). In our study, concentrations in this tissue were lower than in other organs studied (Fig. 1c). Moreover, no relation was observed between the level of contamination and the concentrations of Cd in this tissue. However, an increase of concentrations in muscle was recorded between 10 and 20 days for all contaminated groups ($P < 0.05$) and between 20 and 40 days of exposure time for C1 condition only. In contrast, this increase of Cd levels was not observed during the last 20 days of the experiment in liver and kidneys even if bioaccumulation in these organs increased significantly during the first 20 days. White and Finley (1978) have previously observed on Mallard ducks fed with Cd in their diet that accumulation of the metal reached equilibrium in most tissues around 60 days. In this study, between 20 and 40 days of contamination, the metal appeared to be unable to

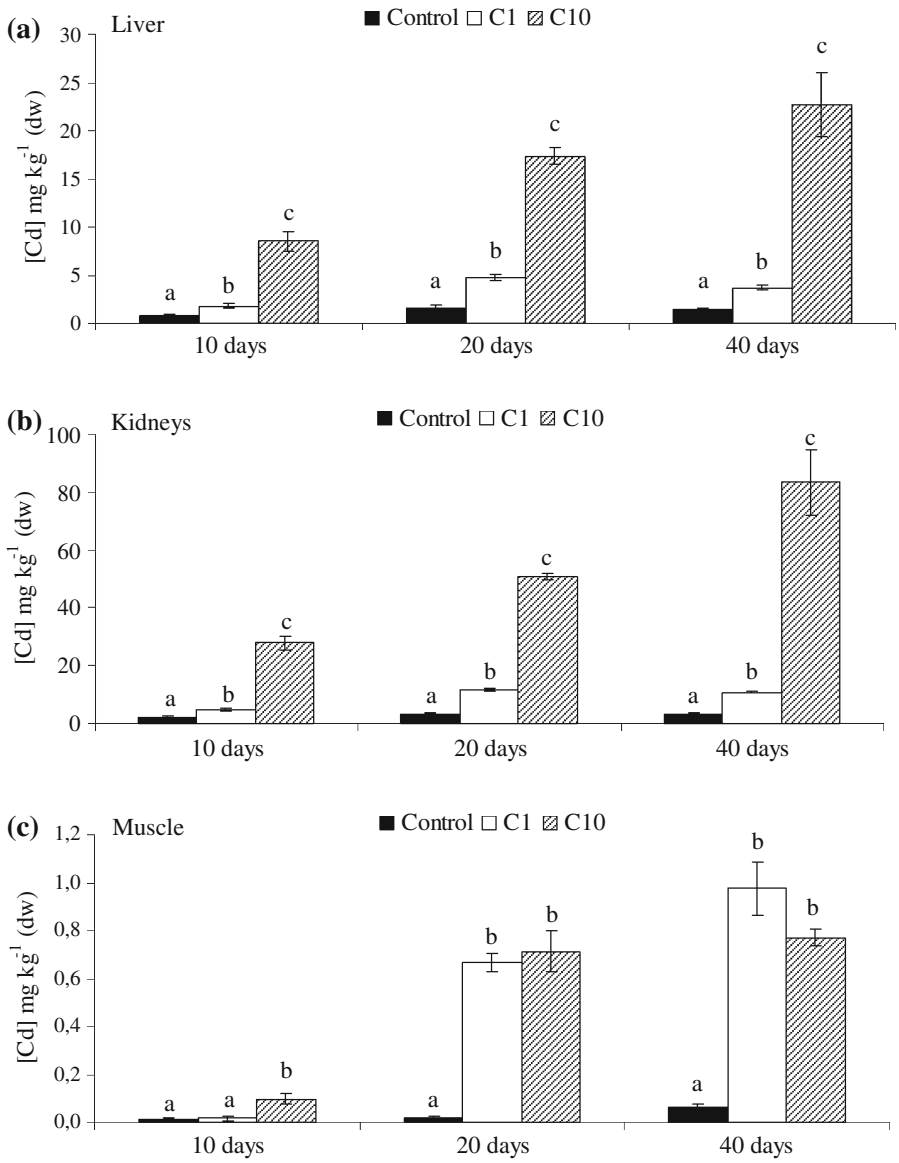

Fig. 1 **a** Cd concentrations (mean ± standard error of mean (SE), $n = 5$) in liver of control and contaminated ducks (C1: 1 mg kg^{-1}; and C10: 10 mg kg^{-1}) exposed for 10, 20 and 40 days; **b** Cd concentrations (mean ± SE, $n = 5$) in kidneys of control and contaminated ducks (C1: 1 mg kg^{-1}; and C10: 10 mg kg^{-1}) exposed for 10, 20 and 40 days; **c** Cd concentrations (mean ± SE, $n = 5$) in muscle of control and contaminated ducks (C1: 1 mg kg^{-1}; and C10: 10 mg kg^{-1}) exposed for 10, 20 and 40 days. Letters (a b c) represents the significant differences between contamination level independently for 10, 20 and 40 days of exposure time at the level $\alpha = 0.05$

efficiently penetrate the intestinal epithelium to reach liver but was still accumulated in muscle. This can be relevant with previous studies that have shown that Cd uptake triggers an inflammatory and secretory response in the small intestines of chickens (Berzina et al. 2007). Moreover, Cd dietary contamination with enriched food can impair the efficiency of the metal to penetrate the intestinal epithelium of fish species (Pierron et al. 2008). Indeed, a decrease in protein digestibility and an inhibition of intestinal enzymes due to Cd was observed in different phyla (Eriyamremu et al. 2005; Moza et al. 1995).

Metallothionein gene expression and protein quantification

The MT protein level was significantly correlated to the concentration of metal at all time of exposure and for all organs ($P < 0.05$; liver: 10 days—$r = 0.61$, 20 days—$r = 0.69$, 40 days—$r = 0.79$; kidneys: 10 days—$r = 0.71$, 20 days—$r = 0.80$, 40 days—$r = 0.80$; muscle: 10 days—$r = 0.70$, 20 days—$r = 0.81$, 40 days—$r = 0.83$) as previously described in various bird species (Barjaktarovic et al. 2002; Carpenè et al. 2006; Elliott and Scheuhammer 1997).

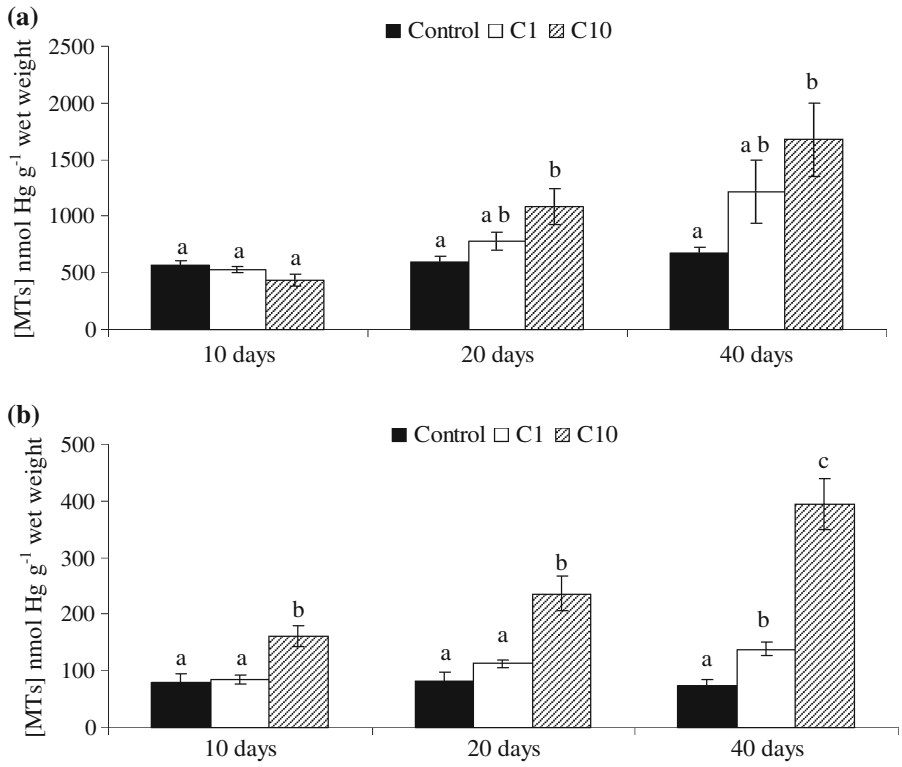

Fig. 2 **a** Metallothionein concentrations (mean ± standard error of mean (SE), $n = 5$) in liver of control and contaminated ducks (C1: 1 mg kg^{-1}; and C10: 10 mg kg^{-1}) exposed during 10, 20 and 40 days; **b** Metallothionein concentrations (mean ± SE, $n = 5$) in kidneys of control and contaminated ducks (C1: 1 mg kg^{-1}; and C10: 10 mg kg^{-1}) exposed during 10, 20 and 40 days. Letters (a b c) represents the significant differences between contamination level independently for 10, 20 and 40 days of exposure time at the level $\alpha = 0.05$

Contamination of animals resulted in an increase of MT production, which represents the main pathway of detoxification of Cd. Indeed, ducks exposed to C10 condition significantly displayed higher MT protein levels than control in liver (Fig. 2a). MT protein synthesis was thus induced in this organ by Cd after 20 days of exposure. In parallel, MT gene transcription was often shown to be induced by this metal and the oxidative stress related to the contamination (Bertin and Averbeck 2006). However, in our study, Cd induced a significant increase of *mt* gene expression in liver only after 40 days of exposure for the two contamination conditions with a similar 7.5-fold up-regulation (Mann–Whitney U test, $P < 0.05$) (Table 3). MT gene expression appeared to be a late hour biomarker suggesting that MT protein synthesis in *C. moschata* is regulated at the transcriptional and the transductional level. This delay was reported in various phyla such as in the zebrafish *Danio rerio* and rats (Gonzalez et al. 2006; Vasconcelos et al. 2002). Moreover, in this study, MT protein levels were not directly correlated with the mRNA levels in liver, demonstrating the importance of measuring both levels in studies.

After 40 days of contamination, the MT protein level in liver was still significantly higher for C10 than for controls (contaminated/control ratio = 2.5) in contrast with C1 condition. However, MT protein level in this organ did not increase between 20 and 40 days for C10 and C1 conditions. The stability of MT protein level in liver during the last 20 days of Cd exposure could have several explanations. First, the very high basal levels reported in this organ could be sufficient to counter the toxicity of Cd present in liver. In this way, the metal did not significantly trigger the synthesis of new MT. Secondly, these data only represent the quantification of MT proteins at a given moment. It is not incompatible with a possible increase of proteins hidden by the rapid rates of MT synthesis and degradation as observed in chickens (Laurin and Klasing 1990) and mammals (Held and Hoekstra 1984).

In contrast with liver, discrepancies in kidneys between MT protein level of the most contaminated group and MT protein level of control group were observed after 10 days of contamination (Fig. 2b). Indeed, after this period, ducks exposed to C10 condition significantly showed higher MT protein levels than control and C1 condition. Moreover,

Table 3 Differential gene expressions (mean ± SD, $n = 5$) compared to β-actin in liver from *C. moschata* after 10, 20 and 40 days of contamination to Cd: control, C1 (1 mg kg^{-1} dw) and C10 (10 mg kg^{-1} dw)

Function	Genes	10			20				
		Control	C1	C10	Control	C1	Cd effect	C10	Cd effect
Mitochondrial metabolism	*cox1*	3.7 ± 1.3	2.5 ± 0.6	5.1 ± 1.4	0.3 ± 0.1	0.8 ± 0.0.2	2.5[a]	1.7 ± 0.3	2.5[a]
	nad5	0.001 ± 0.0002	0.01 ± 0.005	0.001 ± 0.0003	0.0001 ± 0.00003	0.0003 ± 0.0002		0.001 ± 0.0006	4[a]
	12s rRNA								
Lipogenesis	*acc*	0.0002 ± 0.00005	0.0002 ± 0.0001	0.0002 ± 0.00004	0.002 ± 0.0006	0.002 ± 0.0003		0.002 ± 0.001	
Oxidative stress	*sod1*	0.6 ± 0.07	0.8 ± 0.2	1.0 ± 0.2	0.60 ± 0.08	2.0 ± 0.6	3[a]	2.3 ± 0.7	3[a]
	sod2	0.1 ± 0.01	0.1 ± 0.04	0.1 ± 0.02	0.08 ± 0.02	0.15 ± 0.05		0.14 ± 0.06	
	cat	0.07 ± 0.01	0.08 ± 0.03	0.05 ± 0.01	0.02 ± 0.004	0.18 ± 0.13		0.15 ± 0.07	5[a]
Detoxification	*mt*	5.7 ± 1.1	8.4 ± 2.3	7.9 ± 1.8	3.5 ± 0.8	7.1 ± 2.2		6.4 ± 1.5	
DNA repair	*gadd*	0.008 ± 0.001	0.008 ± 0.003	0.004 ± 0.001	0.00007 ± 0.00002	0.00009 ± 0.00003		0.0002 ± 0.0001	2.5[a]

Function	Genes	40				
		Control	C1	Cd effect	C10	Cd effect
Mitochondrial metabolism	*cox1*	0.6 ± 0.1	1.5 ± 0.2		1.2 ± 0.2	2.5[a]
	nad5	0.0003 ± 0.001	0.007 ± 0.001	11[a]	0.004 ± 0.002	5[a]
	12s rRNA	0.0001 ± 0.00005	0.003 ± 0.001	11[a]	0.001 ± 0.0003	19.5[a]
Lipogenesis	*acc*	0.007 ± 0.003	0.02 ± 0.006	4[a]	0.015 ± 0.002	3[a]
Oxidative stress	*sod1*	0.7 ± 0.01	0.8 ± 0.3		0.5 ± 0.1	
	sod2	0.02 ± 0.003	0.05 ± 0.01	2.5[a]	0.03 ± 0.001	
	cat	0.005 ± 0.001	0.3 ± 0.1	12[a]	0.19 ± 0.03	49[a]
Detoxification	*mt*	2.5 ± 0.6	18.8 ± 3.9	7.5[a]	19.5 ± 4.4	7.5[a]
DNA repair	*gadd*	0.00001 ± 0.000004	0.00009 ± 0.00002	6.5[a]	0.0002 ± 0.0000 3	19.5[a]

Cd effect was indicated as induction factors

[a] Significant induction factor ($P < 0.05$)

differences were stronger between the three groups at the end of the 40 days of contamination. Indeed, ducks of C1 and C10 contaminated groups displayed 1.5 and 5-fold the MT level of control ducks, respectively. Discrepancies of MT levels followed experimental condition (control < C1 < C10; $P < 0.05$). From our results, kidneys, which are the final targets of Cd, displayed lower basal MT levels than liver. These lower basal levels could lead to the requirement of MT production when very high amount of Cd were bioaccumulated in kidneys. Cd appeared to bind to existing MT in liver and in contrast to induce new synthesis of MT in kidneys. These results differed from those of Vasconcelos et al. (2002) who found no increase in levels of protein in kidneys of rats contaminated by Cd.

Gene expression in response to Cd exposure

During this study, a partial catalase cDNA of 350 bp was sequenced. The corresponding protein [116 amino acids (aa)] presented high similarities with *Gallus gallus* catalase (99% identity) and with bird *Melopsittacus undulatus* catalase (98% identity). In the case of mitochondrial superoxide dismutase (Mn), the fragment that was sequenced (355 bp, 118 aa) corresponds to mitochondrial superoxide dismutase of birds *Taeniopygia guttata* (98% identity) and *Melopsittacus undulatus* (96% identity). For the sequenced growth arrest DNA damage gene (336 bp, 112 aa), high similarity was found with the corresponding gene of *Gallus gallus* (92% identity) and *Ornithorhynchus anatinus* (81% identity).

Cd is known to affect cellular DNA and to enhance oxidative stress (Bertin and Averbeck 2006). In birds, some authors have demonstrated the existence of oxidative stress by the enhancement of the plasma malondialdehyde (MDA) level as an indicator of lipid peroxidation and the up regulation of enzymes activity involved in hepatic antioxidant defence (Berglund et al. 2007; Erdogan et al. 2005). However, some of these studies do not address the particular impact of Cd in cellular metabolism because they were made on wild birds contaminated with a large variety of pollutants or only focused on one particular aspect of the bird metabolism. In this study, the impact of Cd appeared after 20 days of contamination for the two Cd contamination levels (Table 3). Our results showed the appearance of an oxidative stress after this period. It was demonstrated by the significant up regulation of genes involved in oxidative stress response such as *sod1* for the two contaminated groups and catalase in the case of the highest level of Cd (C10). Some studies have shown that high amounts of Cd are linked to a reduction of antioxidant activities (Yano and Marcondes 2005), whereas low dose exposures are associated with enhanced levels of *sod* (Bertin and Averbeck 2006). This reduction of antioxidant activities was not exactly observed in this study. However, after 40 days of Cd exposure, *sod1* returned to basal level and *sod2* was only up regulated for the lowest Cd contamination. In contrast, the enhancement of catalase in contaminated ducks at this exposure time appeared to be correlated to Cd level (induction factor: C1 = 12; C10 = 49). This result could be in relation with the significant production of reactive oxygen species (ROS) promoted by Cd and thus leading to the appearance of DNA strand damage after 20 days of contamination for the highest Cd level. Indeed, the DNA damage repairing *gadd* was highly expressed after this time of exposure and even more after 40 days (induction factor: C1 = 6.5; C10 = 19.5).

Our results showed the enhancement of mitochondrial metabolism after 20 days of contamination especially for ducks of the C10 condition and after 40 days of contamination for the two contamination conditions (Table 3). Complex IV (cytochrome *c* oxidase) of the mitochondrial respiratory chain is known to transfer electrons from the reduced cytochrome *c* to molecular oxygen. It contributes to the production of electrochemical proton gradient across the mitochondrial inner membrane leading to the production of ATP (Capaldi 1990). Previous studies have demonstrated that the inhibition of cytochrome *c* enhances ROS production (Campian et al. 2004). From our results, the up regulation of *cox1* as well as up regulation of *nad5* could have several purposes. First, it could represent a compensatory mechanism to restore the decrease in mitochondrial activity as previously described for bivalves (Achard-Joris et al. 2006) in order to maintain a sufficient number of functioning complexes and thus preserve respiratory activity. Secondly, the implementation of the defences against oxidative stress could entail an important energy expense for cells and consequently require the enhancement of the mitochondrial

metabolism *via* the Krebs cycle (up regulation of *acc*) and the respiratory chain (up regulation of *cox1* and *nad5*). In this context, we observed a significant enhancement of 12 s rRNA expression, which highlighted an increase of the number of mitochondria, for contaminated ducks after 40 days of experiment. Moreover, this 12 s rRNA up-regulation appeared to be in relation with Cd dose (induction factor: C1 = 4.5; C10 = 19.5).

Our results gave a first insight into the Cd cellular toxicity which can lead to many damages in birds. Indeed, we can see that Cd promoted the appearance of ROS via the observed enhancement of the expression of genes involved in various aspects of defence against oxidative stress. Cd also enhanced mitochondrial metabolism and led to the implementation of DNA repair system. In further studies, the comparison of the *mt* gene expression and the MT protein level in different organs would give more comprehensive clues on the Cd regulatory system. Further investigations could focus on other well known paths of Cd detoxification as for instance glutathione peroxidase. This information would be of great interest in defining molecular mechanism in response to metals in birds.

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References

Achard-Joris M, Gonzalez P, Marie V, Baudrimont M, Bourdineaud J-P (2006) Cytochrome *c* oxydase subunit I gene is up-regulated by cadmium in freshwater and marine bivalves. Biometals 19(3):237–244

Barjaktarovic L, Elliott JE, Scheuhammer AM (2002) Metal and metallothionein concentrations in scoter (*Melanitta* spp.) from the Pacific Northwest of Canada, 1989–1994. Arch Environ Contam Toxicol 43(4):486–491

Baudrimont M, Andres S, Durrieu G, Boudou A (2003) The key role of metallothioneins in the bivalve *Corbicula fluminea* during the depuration phase, after in situ exposure to Cd and Zn. Aquat Toxicol 63(2):89–102

Baudrimont M, Schäfer J, Marie V, Maury-Brachet R, Bossy C, Boudou A, Blanc G (2005) Geochemical survey and metal bioaccumulation of three bivalve species (*Crassostrea gigas*, *Cerastoderma edule* and *Ruditapes philippinarum*) in the Nord Médoc salt marshes (Gironde estuary, France). Sci Total Environ 337(1–3):265–280

Berglund ÅMM, Sturve J, Förlin L, Nyholm NEI (2007) Oxidative stress in pied flycatcher (*Ficedula hypoleuca*) nestlings from metal contaminated environments in northern Sweden. Environ Res 105(3):330–339

Bertin G, Averbeck D (2006) Cadmium: cellular effects, modifications of biomolecules, modulation of DNA repair and genotoxic consequences (a review). Biochimie 88(11):1549–1559

Berzina N, Markovs J, Isajevs S, Apsite M, Smirnova G (2007) Cadmium-induced enteropathy in domestic cocks: a biochemical and histological study after subchronic exposure. Basic Clin Pharmacol Toxicol 101(1):29–34

Boening DW (2000) Ecological effects, transport, and fate of mercury: a general review. Chemosphere 40:1335–1351

Campian JL, Qian M, Gao X, Eaton JW (2004) Oxygen tolerance and coupling of mitochondrial electron transport. J Biol Chem 279(45):46580–46587

Capaldi RA (1990) Structure and assembly of cytochrome *c* oxidase. Arch Biochem Biophys 280(2):252–262

Carpenè E, Andreani G, Monari M, Castellani G, Isani G (2006) Distribution of Cd, Zn, Cu and Fe among selected tissues of the earthworm (*Allolobophora caliginosa*) and Eurasian woodcock (*Scolopax rusticola*). Sci Total Environ 363(1–3):126–135

Dutton MD, Stephenson M, Klaverkamp JF (1993) A mercury saturation assay for measuring metallothionein in fish. Environ Toxicol Chem 12(7):1193–1202

Elliott JE, Scheuhammer AM (1997) Heavy metal and metallothionein concentrations in seabirds from the Pacific coast of Canada. Mar Pollut Bull 34(10):794–801

Erdogan Z, Erdogan S, Celik S, Unlu A (2005) Effects of ascorbic acid on cadmium-induced oxidative stress and performance of broilers. Biol Trace Elem Res 104(1):19–31

Eriyamremu G, Asagba S, Onyeneke E, Adaikpoh M (2005) Changes in carboxypeptidase A, dipeptidase and Na+/K+ ATPase activities in the intestine of rats orally exposed to different doses of cadmium. Biometals 18(1):1–6

Gómez G, Baos R, Gómara B, Jiménez B, Benito V, Montoro R, Hiraldo F, González MJ (2004) Influence of a mine tailing accident near Doñana National Park (Spain) on heavy metals, arsenic accumulation in 14 species of waterfowl (1998 to 2000). Arch Environ Contam Toxicol 47(4):521–529

Gonzalez P, Baudrimont M, Boudou A, Bourdineaud J-P (2006) Comparative effects of direct cadmium contamination on gene expression in gills, liver, skeletal muscles and brain of the zebrafish (*Danio rerio*). Biometals 19(3):225–235

Held DD, Hoekstra WG (1984) The effects of zinc deficiency on turnover of cadmium-metallothionein in rat liver. J Nutr 114(12):2274–2282

Hernández LM, Gómara B, Fernández M, Jiménez B, González MJ, Baos R, Hiraldo F, Ferrer M, Benito V, Suñer MA, Devesa V, Muñoz O, Montoro R (1999) Accumulation of heavy metals and As in wetland birds in the area around Doñana National Park affected by the Aznalcollar toxic spill. Sci Total Environ 242(1–3):293–308

Hughes MR, Smits JE, Elliott JE, Bennett DC (2000) Morphological and pathological effects of cadmium ingestion on Pekin ducks exposed to saline. J Toxicol Environ Health A 61(7):591–608

Kalisinska E, Salicki W, Myslek P, Kavetska KM, Jackowski A (2004) Using the Mallard to biomonitor heavy metal

contamination of wetlands in north-western Poland. Sci Total Environ 320(2–3):145–161

Laurin DE, Klasing KC (1990) Roles of synthesis and degradation in the regulation of metallothionein accretion in a chicken macrophage-cell line. Biochem J 268(2):459–463

Liu W, Zhu R-H, Li G-P, Wang D-C (2002) cDNA cloning, high-level expression, purification, and characterization of an avian Cu, Zn superoxide dismutase from Peking duck. Protein Expr Purif 25(3):379–388

Lock JW, Thompson DR, Furness RW, Bartle JA (1992) Metal concentrations in seabirds of the New Zealand region. Environ Pollut 75(3):289–300

Maury-Brachet R, Rochard E, Durrieu G, Boudou A (2008) The 'Storm of the Century' (December 1999) and the accidental escape of Siberian sturgeons (*Acipenser baerii*) into the Gironde estuary (Southwest France). Environ Sci Pollut Res Int 15:89–94

Moza U, De Silva SS, Mitchell BM (1995) Effect of sub-lethal concentrations of cadmium on food intake, growth and digestibility in the goldfish, *Carassius auratus* L. J Environ Biol 16:253–264

Nam D-H, Anan Y, Ikemoto T, Tanabe S (2005) Multielemental accumulation and its intracellular distribution in tissues of some aquatic birds. Mar Pollut Bull 50(11): 1347–1362

Pierron F, Baudrimont M, Lucia M, Durrieu G, Massabuau J-C, Elie P (2008) Cadmium uptake by the European eel: trophic transfer in field and experimental investigations. Ecotoxicol Environ Saf 70(1):10–19

Rothschild RFN, Duffy LK (2005) Mercury concentrations in muscle, brain and bone of Western Alaskan waterfowl. Sci Total Environ 349(1–3):277–283

Trust KA, Rummel KT, Scheuhammer AM, Brisbin IL Jr, Hooper MJ (2000) Contaminant exposure and biomarker responses in spectacled eiders (*Somateria fischeri*) from St. Lawrence Island, Alaska. Arch Environ Contam Toxicol 38(1):107–113

Vasconcelos MH, Tam S-C, Hesketh JE, Reid M, Beattie JH (2002) Metal- and tissue-dependent relationship between metallothionein mRNA and protein. Toxicol Appl Pharmacol 182(2):91–97

Wang Y, Fang J, Leonard SS, Krishna Rao KM (2004) Cadmium inhibits the electron transfer chain and induces reactive oxygen species. Free Radic Biol Med 36(11): 1434–1443

White DH, Finley MT (1978) Uptake and retention of dietary cadmium in mallard ducks. Environ Res 17(1):53–59

Yano CL, Marcondes MCCG (2005) Cadmium chloride-induced oxidative stress in skeletal muscle cells in vitro. Free Radic Biol Med 39(10):1378–1384