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Effects of dietary cadmium contamination on bird *Anas platyrhynchos*—Comparison with species *Cairina moschata*

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ABSTRACT

This study aimed to assess the effect of two dietary cadmium (Cd) levels (C1: 1 mg kg^{-1}; C10: 10 mg kg^{-1}) on bird *Anas platyrhynchos* exposed for 10, 20 and 40 days (5 animals per experimental condition). Ducks were able to accumulate high amounts of Cd, especially in kidneys (after 40 days: C1 8.1 ± 1 mg kg^{-1}, C10 37.7 ± 4.3 mg kg^{-1}). After 40 days, the lowest Cd level triggered oxidative stress and stimulated mitochondrial metabolism. At the same time, highest amounts of Cd (C10 group) only triggered repression of genes encoding for catalase and acetyl-CoA carboxylase, with repression factors of 1/50 and 1/5, respectively. High dose exposures were then associated with the repression of genes encoding for antioxidant, whereas low dose exposure triggered their induction. In contrast, the onset of MT gene expression appeared quickly for the C10 group even if a time delay was observed between gene expression and protein accumulation. Through the comparison of *A. platyrhynchos* and *Cairina moschata*, the response to Cd toxicity appeared species-dependent. Discrepancies between species could be explained by differential utilization of MT. This pathway of detoxification seemed sufficient to counter Cd toxicity.

1. Introduction

Cadmium (Cd) is a toxic metal present in many environments due to its widespread industrial use in mining, metallurgy, batteries and plastic stabilizers. Toxicological studies at cellular scale have shown that this element triggered severe consequences on many phyla (Risso-de Faverney et al., 2004; Bertin and Averbeck, 2006; Mouchet et al., 2006). Indeed, Cd inhibits the mitochondrial electron transfer chain and induces reactive oxygen species (ROS) production leading to DNA strand damage (Wang et al., 2004).

Few studies conducted on birds have focused on Cd impairment. Authors often investigate *in-situ* levels of metals on wild populations (Trust et al., 2000; Gómez et al., 2004). Nevertheless, the generation of oxidative stress by Cd has been previously demonstrated in birds. Indeed, Cd triggers the enhancement of plasma malondialdehyde level as an indicator of lipid peroxidation and induces up-regulation of enzymes activity involved in hepatic antioxidant defences (Erdogan et al., 2005; Berglund et al., 2007). However, some of these studies do not especially address Cd impact on cellular metabolism because they were performed on wild birds contaminated by many pollutants. Moreover, authors only focused on one specific aspect of bird metabolism.

France has a central position on the East-Atlantic axis of migration between Siberia, the North of Europe, the Eastern Europe and Western Africa. Wetlands situated on the Atlantic coast are important transition areas along the migratory route of numerous bird species such as mallards (*Anas platyrhynchos*), greylag goose (*Anser anser*), red knot (*Calidris canutus*) and gray plover (*Pluvialis squatarola*). The Gironde estuary hosts a wide population of mallards (*A. platyrhynchos*). Permanently present in this area, its population increases at the beginning of the autumn by the arrival of birds coming from Scandinavian and Western European countries. This ecosystem is characterized by a long-standing Cd contamination of the water and sediments. Geochemical surveys demonstrated that Cd originated from former ore treatment in the industrial basin of Decazeville, situated 400 km upstream from the Gironde estuary on the Lot River (Jouanneau et al., 1990).

The current study aimed to assess the effects of two dietary Cd levels on aquatic bird *A. platyrhynchos* (1 and 10 mg kg^{-1}).

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Cd concentrations, especially the lowest level, were used to investigate the impact of this toxic element at field contamination levels. The chosen concentrations were based on levels accumulated in molluscs of the Gironde estuary which could reach 24 mg kg^{-1} in oysters, 1.4 mg kg^{-1} in cockles and in clams (Baudrimont et al., 2005). To address this issue, Cd bioaccumulation was investigated at the organ level. Nine genes were selected encoding for proteins involved in detoxification mechanisms, mitochondrial metabolism, oxidative stress responses and DNA repair. Mitochondrial metabolism and lipogenesis were examined through the expression of the cytochrome C oxidase subunit 1 (*cox1*), NADH dehydrogenase subunit 5 (*nad5*), 12 s rRNA, and acetyl-CoA carboxylase (*acc*). The gene encoding for the growth-arrest-DNA-damage (*gadd*) protein involved in response to DNA damage repair, has been investigated. Moreover, in order to defend against Cd toxicity, organisms display different mechanisms. They could increase antioxidant defences such as cytoplasmic and mitochondrial superoxide dismutases (*sod1* and *sod2*) and catalase (*cat*). Organisms can also employ detoxification mechanisms such as metallothioneins (MT). These cysteine-rich and low molecular weight proteins are involved in homeostasis of essential metals such as zinc and protection against Cd toxicity. MT concentrations have been shown to be positively correlated with Cd levels in liver and kidneys of marine birds (Jordan et al., 1990; Elliott et al., 1992; Elliott and Scheuhammer, 1997). This biomarker is therefore expected to respond to Cd contamination and to be a precursor sign to Cd toxicity in the current study. This pathway of detoxification is investigated through the comparison between MT gene expressions and MT protein levels. Gene expressions were measured in liver, the main tissue for detoxification in ducks, by real-time PCR and normalized according to the expression of the reference gene: β-actin. Moreover, the current study aimed to compare the effects of Cd on *A. platyrhynchos* to those observed on other Anseriform *Cairina moschata* which was previously studied using the same experimental conditions and similar Cd levels (1 and 10 mg kg^{-1}) (Lucia et al., 2009). These two domesticated species could then be expected to have close response pattern to the contamination.

2. Materials and methods

2.1. Experimental design

Male ducks were raised in a breeding structure belonging to the French National Institute for Agricultural Research (INRA) at the "domaine d'Artiguères", Landes, France. The present work was carried out in agreement with the French legislation on animal experimentation and with the authorization from the French Ministry of Agriculture (Animal Health and Protection Directorate). 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 proteins. Between 4 and 10 weeks of age, ducks were fed *ad libitum* with growth food (granule with length of 4 mm) less enriched in proteins.

At 10 weeks of age, 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 under its $CdCl_2$ form (Merck). 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. Concentrations of Cd were checked in food: 0.05 ± 0.01, 1.02 ± 0.01 and 10.03 ± 0.04 mg kg^{-1} for control ducks, C1 and C10 conditions, respectively ($n=5$ per condition). The lowest level of contamination was chosen according to Cd concentrations encountered in the Gironde estuary (C1: 1 mg kg^{-1} of Cd). The upper level was chosen to compare effects of environmental level to acute metal concentrations (C10: 10 mg kg^{-1} of Cd). Feeding of ducks was carefully monitored to make sure that animals ate all the granules.

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 in liver, muscle and kidneys. Animals were sampled at three other times 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. Liver, kidneys and pectoral muscle were dissected and divided in two parts. The first part was stored at −80 °C under nitrogen atmosphere for metallothioneins (muscle, kidneys, liver) and for genetic analyses (liver only after immediate freezing with liquid nitrogen). The second part was stored at −20 °C before metal determination.

2.2. Metal determination

Metal determinations were realized on food and on five replicates per experimental condition. Food and 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 and incubated 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 atomization 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 method was simultaneously validated for each sample series by the analysis of standard biological reference materials (Tort-2: lobster hepatopancreas and Dolt-3: dogfish liver from NRC-CNRC; Ottawa, Canada). Values were in agreement with the certified ranges (Tort-2: 26.7 ± 0.6 mg kg^{-1}; Dolt-3: 19.4 ± 0.6 mg kg^{-1}).

2.3. Metallothionein quantification

Concentration of total MT protein was determined by the mercury-saturation assay in the liver, kidneys and muscle, using cold inorganic mercury (Dutton et al., 1993; Baudrimont et al., 2003). MT analysis was conducted on 5 replicates per exposure condition, the saturation assay being repeated twice per sample. This technique is based on the quantification of Hg bound to the saturated MT. The denaturation of non-MT proteins was performed using trichloroacetic acid and the excess Hg not bound to the MT was removed by scavenging with lyophilized beef hemoglobin (Sigma) prepared in 30 mM Tris–HCl buffer (pH 8.2 at 20 °C). The final supernatant was then quantitatively recovered and used for Hg determination by flameless atomic absorption spectrometry (AMA 254, Altec, Prague, Czech Republic). The detection limit was estimated at 1 ngHg. Owing to the fact that the exact quantity of Hg binding sites per MT molecule is unknown for this species, MT concentrations cannot be directly expressed in nmol MT g^{-1} (wet weight), but in nmol Hg g^{-1} (ww).

2.4. Sequencing of genes

Three partial genes sequences were determined 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 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. Consensus primer pairs for each gene investigated were determined in conserved region from multiple sequence alignment of avian or mammalian species using Clustal W software (Infobiogen) (Table 1), and used for PCR to obtain amplified cDNA fragment of the different genes. PCR products were cloned into pGEM-T vector and sequenced (Millegen, France).

Table 1
Consensus primers used to clone mitochondrial superoxide dismutase (*sod2*), catalase (*cat*) and growth arrest DNA damage protein (*gadd*) cDNA.

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

[a] Forward primers.
[b] Reverse primers.

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	ACAGGAAGTTACTCGCCTCT[a] TGGGGAACACAGCCCGC[b]
cox1	NC_009684	CCGACGATACTCGGACTACC[a] GGGCAGCCGTGGATTC[b]
nad5	NC_009684	ATTCGCCGCCACATGC[a] ATGGACCCGGAGCATAGGAA[b]
acc	EF990143	GTCCTCCAAGCCAAGCAATGTG[a] GGCCTTGATCATGACAGGGTAGCC[b]
12s rRNA	NC_009684	CCCTACCGAAGTATCCGCC[a] CCCACTGTTGCGCTCTCA[b]
sod1	Liu et al., 2002	GCGCACCATGGTGGTCCATG[a] GTCTTCACCAGTTTAACTGATACTCA[b]
sod2	EU598453	ACGCCGAGATCATGCAG[a] CGAAAGATTTGTCCAGAAGATGGT[b]
cat	EU598454	AGGCAAAAGTGTTTGAACACAT [a] ACAACATTGCATCCCGAATAAAG[b]
mt	AB258230	ctcccaggactgcccttgcg[a] CAGCTGCACTTGGCGGAGGG[b]
gadd	EU598455	GCGGACGAGGAGGAAGC[a] GGGGTTCGTGACCAGG[b]

Abbreviations: *act*—β-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] Forward primers.
[b] Reverse primers.

2.5. Real-time PCR

After extraction and reverse transcription (see above), real-time PCR reactions were performed in a Mx3000P (Stratagene) following the manufacturer 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 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 pairs 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.

2.6. 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 was assumed and homogeneity of variance, necessary for the use of this parametric test, was verified. If this assumption was not achieved despite $\log_{10}(x+1)$ transformation (Cochran C test), non-parametric analysis of variance was applied (Mann–Whitney *U*-test, Statistica 7.1). Spearman correlation test was applied to evaluate the relationship between the concentrations of Cd and metallothioneins.

3. Results

3.1. Cadmium bioaccumulation in bird tissues

No discrepancies were observed between control and contaminated ducks for total weight, liver and kidney weights throughout the experiment (Mann–Whitney *U*-test).

In liver and kidneys, Cd bioaccumulation was influenced by the contamination level (Fig. 1A, B). At each time of exposure, control ducks displayed significantly lower concentrations than ducks of C1 group (1 mg kg^{-1}) which themselves showed lower concentrations than C10 group (10 mg kg^{-1}; Control < C1 < C10, $p<0.05$, Mann–Whitney *U*-test). In the pectoral muscle, Cd accumulated after 10 days was the same for C1 and C10 groups (Fig. 1C; Mann–Whitney *U*-test). Concentrations remained stable throughout the experiment.

In liver and kidneys, Cd bioaccumulation was correlated to the time of exposure (Spearman correlation, $p<0.05$, $r=0.89$). Concentrations of C1 and C10 groups significantly increased between 10, 20 and 40 days of contamination. In kidneys, high concentrations were observed after 40 days of contamination, averaging 21.3 ± 1.6 mg kg^{-1} (SE, dw) for C1 ducks and 129.3 ± 16.2 mg kg^{-1} (SE, dw) for C10 ducks. Liver was the second most contaminated tissue (Mann–Whitney *U*-test). Cd levels reached on average 8.13 ± 0.98 mg kg^{-1} (SE, dw) for C1 group and 37.65 ± 4.29 mg kg^{-1} (SE, dw) for C10 group. In muscle, concentrations were very low compared to other tissues, averaging 0.85 ± 0.15 mg kg^{-1} (SE, dw) for C1 group and 0.77 ± 0.14 mg kg^{-1} (SE, dw) for C10 group.

3.2. Cadmium effect on gene regulation

A partial mitochondrial superoxide dismutase (*sod2*) cDNA of 355 bp was sequenced. The corresponding protein (118 amino acids (aa)) presented high similarities with mitochondrial superoxide dismutase protein from duck *C. moschata* (100% identity, 100% homology), from birds *Taeniopygia guttata* (98% identity, 98% homology) and *Melopsittacus undulatus* (96% identity, 99% homology). In the case of catalase (*cat*), a partial fragment (350 bp, 116 aa) was sequenced and corresponded to catalase of *Gallus gallus* (99% identity, 99% homology), *C. moschata* (98% identity, 99% homology) and *M. undulatus* (98% identity, 99% homology). For the sequenced growth arrest DNA damage protein (336 bp; 112 aa), high homology was found with the corresponding gene of *C. moschata* (100% identity, 100% homology) and *G. gallus* (92% identity, 92% homology).

In liver, results obtained by quantitative RT-PCR are presented in Table 3. For C1 group, up-regulation of genes involved in mitochondrial metabolism and oxidative stress occurred after 40 days of contamination. For C10 group, expression of genes was identical to control levels throughout the first 20 days of exposure. After 40 days, Cd triggered a repression of *acc* and *cat* genes.

3.3. Metallothionein quantification and gene expression level

Expression factors of *mt* gene are presented in Table 3. For C10 group, induction of *mt* appeared after 10 days of contamination (induction factors: 3) and continued to increase throughout the experiment (9-fold increase after 40 days). For C1 group, up-regulation of *mt* gene only appeared after 20 days of exposure (induction factor: 2) and increased significantly after 40 days of contamination (induction factor: 6.5, Mann–Whitney *U*-test). Moreover, positive correlations were observed between Cd bioaccumulation levels in liver and *mt* expression level (Spearman correlation, $T1-r=0.53$; $T2-r=0.61$; $T3-r=0.58$).

MT protein levels were quantified by mercury saturation assay in liver, kidneys and muscle (Fig. 2). Throughout the experiment, the highest protein levels were measured in liver (Mann–Whitney *U*-test). After 20 days, Cd triggered a significant 2.5-fold increase of MT in the liver of ducks belonging to the two contamination

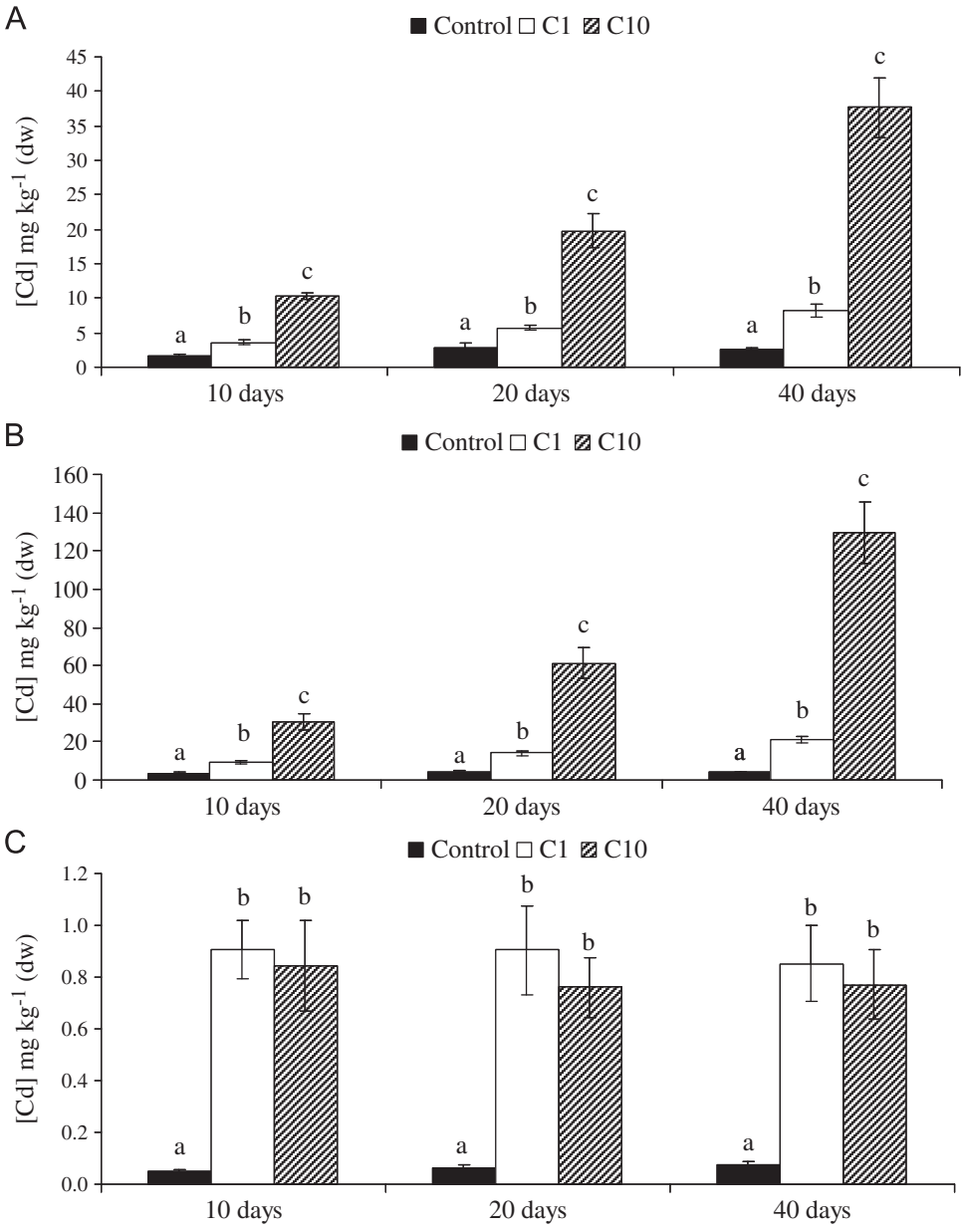

Fig. 1. Cd concentrations (mean ± SE, $n=5$) in liver (A), kidneys (B) and muscle (C) of control ■ and contaminated ducks (C1: □ 1 mg kg^{-1} and C10: ▨ 10 mg kg^{-1}) exposed for 10, 20 and 40 days; Letters on the graphs represent the significant differences between contamination levels independently for 10, 20 and 40 days of exposure time at the level $\alpha=0.05$.

Table 3
Gene expression compared to control (induction or repression factors) in liver from *A. platyrhynchos* after 10, 20 and 40 days of Cd contamination: C1 (1 mg kg^{-1} dw) and C10 (10 mg kg^{-1} dw).

Function	Genes	10 days		20 days		40 days	
		C1	C10	C1	C10	C1	C10
Mitochondrial metabolism	*cox1*	/	/	/	/	4[a]	/
	nad5	/	/	/	/	3.5[a]	/
	12s rRNA					/	/
Lipogenesis	*acc*	/	/	/	/	2.5[a]	1/5[a]
Oxidative stress	*sod1*	/	/	/	/	2.5[a]	/
	sod2	/	/	/	/	3[a]	/
	cat	/	/	/	/	/	1/50[a]
Detoxification	*mt*	/	3[a]	2	4[a]	6.5[a]	9[a]
DNA repair	*gadd*	/	/	/	/	/	/

/: identical to control level.

[a] Significant induction or repression factor (Mann–Whitney *U*-test; $p<0.05$).

groups (Fig. 2A). Nevertheless, MT concentrations did not increase between 20 and 40 days of the experiment (Mann–Whitney *U*-test). In kidneys, MT protein level significantly increased after 10 days for C1 and C10 groups. However, C10 group displayed higher level than C1 group (Mann–Whitney *U*-test: control < C1 < C10, Fig. 2B). In kidneys of C10 group, Cd also triggered a significant enhancement of between each time of exposure ($[MTs]_{10\ days} < [MTs]_{20\ days} < [MTs]_{40\ days}$). In contrast, this increase of MT protein level was not observed for C1 group, especially between 20 and 40 days. In muscle, MT levels of contaminated ducks were identical to controls (data not shown).

In liver and muscle, Cd bioaccumulation in tissues was not correlated with MT protein levels. In contrast, significant correlations were observed in kidneys between Cd concentrations after 10 and 40 days and corresponding MT concentrations (Spearman correlation, $p<0.05$; 10 days–$r=0.82$, 40 days–$r=0.91$).

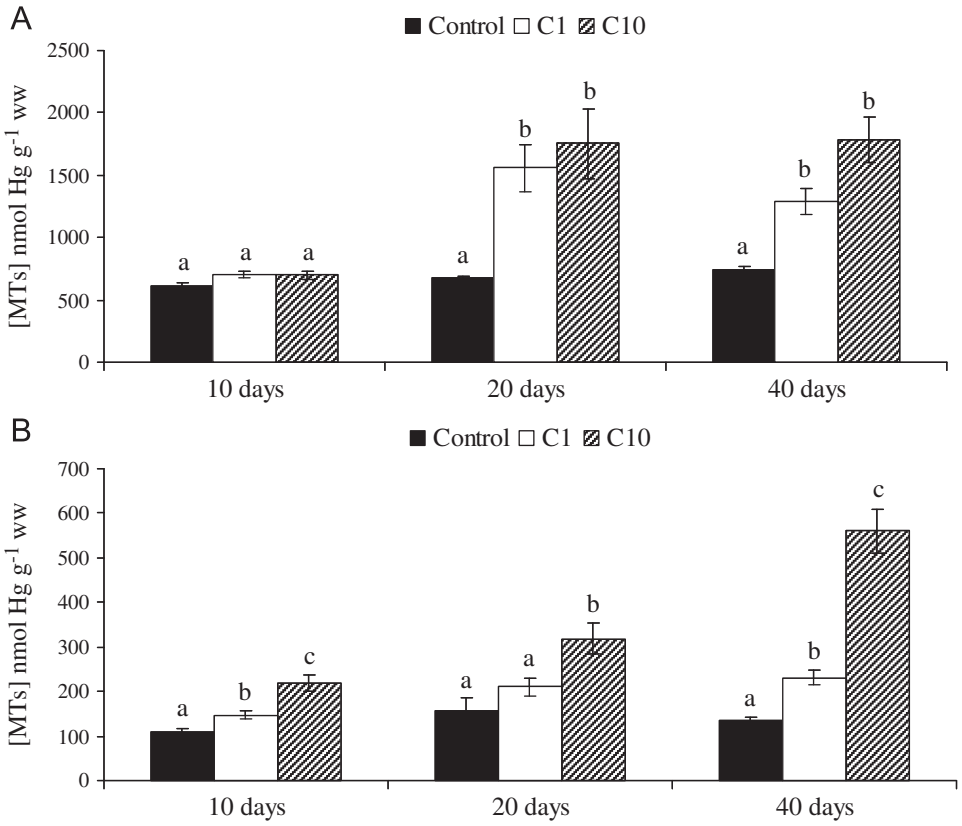

Fig. 2. Metallothionein concentrations (mean ± SE, $n=5$) in liver (A) and kidneys (B) of control ■ and contaminated ducks (C1: □ 1 mg kg^{-1} and C10: ▨ 10 mg kg^{-1}) exposed for 10, 20 and 40 days; Letters (a b c) on the graphs represent the significant differences between contamination levels independently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

4. Discussion

The current study aimed to assess the effect of two Cd concentrations close to environmental levels (C1: 1 and C10: 10 mg kg^{-1}), on bird *A. platyrhynchos*. All along this study, Cd measurements in tissues demonstrated that ducks were able to accumulate high amounts of this non-essential metal. In parallel, the investigation of MT gene expression evidenced that Cd contamination triggered the onset regulation of this gene in liver, demonstrating then the fundamental role of this detoxification process against Cd toxicity. However, MT only gave one indication on how aquatic birds counter Cd toxicity. It was therefore very interesting to study more detoxification processes that these species could set up. In this context, the study of cellular process under Cd contamination could bring important data on this matter. Indeed, Cd triggered oxidative stress and stimulated mitochondrial metabolism for low dose exposure. In contrast, high dose exposures were associated with the repression of genes encoding for antioxidant. Finally, the current study also attempted to compare the response of birds to Cd contamination. This comparison could enable us to see if two species of anseriformes could stimulate the same patterns to counter toxicity and to determine their respective sensibility to one contaminant. Eventually, these results could allow us to determine if the response of one species of bird might be extrapolated to species of the same order. In this case, it appeared that responses to Cd toxicity were species dependent.

4.1. Cadmium bioaccumulation in bird tissues

Birds of the current study accumulated very high amounts of Cd, especially in kidneys (Mayack et al., 1981; Elliott et al., 1992; Levengood and Skowron, 2007; Lucia et al., 2008). Nevertheless, the difference of Cd exposure between C1 and C10 groups (10-fold factor) was only partly reflected on organs accumulation. After 40 days, C10 group only displayed 5-fold the Cd concentration of C1 group in liver and 6-fold in kidneys. Despite the high levels of Cd bioaccumulated in tissues, body weight and organs weight were not impaired. This result was previously observed on mallards contaminated with similar or highest levels of Cd (2, 20 or 200 mg kg^{-1}) (White and Finley, 1978; Mayack et al., 1981). Previous authors used Cd concentrations above our levels (200 mg kg^{-1} ww; White and Finley, 1978). However, metal bioaccumulation in liver and kidneys were lowest compared to the current study (White and Finley, 1978). Recent reports have demonstrated that Cd could induce enteropathy in domestic cocks contaminated with levels of 50 mg kg^{-1} of Cd (Berzina et al., 2007). Histological data showed that $CdCl_2$ causes an increase in number of goblet cells and granular lymphocytes in the intestinal mucosa. Moreover, previous study on the European eels (*Anguilla Anguilla*) demonstrated that Cd bioaccumulated from Cd-enriched food in the digestive tract is not able to efficiently penetrate the intestinal mucosa and that Cd trigger intestinal toxicity (Pierron et al., 2008). These results could partially explain discrepancies of Cd bioaccumulation between the current study and other works using highest amounts of Cd to contaminate ducks. Acute contamination may thus trigger a lower penetration efficiency of the metal through the intestinal mucosa and a lower accumulation of Cd in the tissues. The experimental use of very high amounts of Cd irrelevant with field concentrations could lead to the misinterpretation of real metal bioaccumulation in wildlife. In future studies, working with actual environmental levels is necessary in order to better represent the Cd transfer and impairment in birds.

4.2. Response of metallothioneins to cadmium contamination

MT basal expression levels were correlated to Cd bioaccumulation, therefore contradicting a previous study performed on *A. platyrhynchos* where MT1 (isoform 1 of avian MTs) was not correlated to Cd concentrations (Nam et al., 2007). Although MT gene was quickly up-regulated in ducks of C10 group, induction appeared later with the lowest Cd concentration used in the current study. With low Cd concentrations, MT gene expression appeared to be a late hour biomarker, as previously described for zebrafish *Danio rerio* (Gonzalez et al., 2006) and duck *C. moschata* (Lucia et al., 2009). These results suggest that high metal amounts need to be reached in tissues in order to trigger the onset of MT. On the other hand, the high levels of MT proteins present in liver could very likely explain the delay observed between the two contamination groups. These levels could be sufficient to counter Cd toxicity with low Cd concentration. In contrast, the very high concentrations of Cd reached in tissues in C10 group triggered the onset of MT gene expression.

The current results demonstrated that up-regulation of MT gene expressions was not necessarily followed by an increase of MT protein levels in tissues, as previously observed in rat and zebrafish *D. rerio* (Vasconcelos et al., 2002; Gonzalez et al., 2006). This time delay could be explained by the transcriptional and the translational regulation of MT synthesis in *A. platyrhynchos*. Some authors have suggested the existence of factors affecting the translation of mRNA. For instance, sequences located on 3′-untranslated regions could influence both the translation efficiency and the degradation rate (Lemoine and Laulier, 2003). Moreover, The Cd level bioaccumulated in liver was not correlated to MT protein level in this organ. MT protein level was even between the two contamination levels. This result contrasted with previous investigations conducted on duck *C. moschata* where a narrow correlation was observed between these two parameters (Lucia et al., 2009). Nevertheless, the MT protein level similarity in *A. platyrhynchos* between the two Cd levels could be explained by two processes. First, the functional pool of MT in liver is sufficient to counter Cd toxicity. Secondly, it has been previously demonstrated that Cd is sequestered to binding MT of hepatocytosolic fraction in some avian species (Ikemoto et al., 2004; Nam et al., 2005). Once the MT present in the cytosol are saturated by metals and that the intracellular fluxes of toxic elements are beyond the biosynthesis capacities, protection by MT are overwhelmed and the metals may applied their toxicity (Winge et al., 1974). This mechanism could lead to the stabilization of MT levels over the range of the Cd concentrations applied, as stated for *Xenopus laevis* larvae (Mouchet et al., 2006). This stabilization phenomenon was observed in the current study and therefore led us to think that ducks have reached their biosynthesis capacities, especially for the highest concentration applied.

The kidney ability to synthesized MT is closely correlated to the Cd concentration applied. The fast induction of MT synthesis in this organ contrast with observations in liver. This discrepancy could be explained by the highest basal levels of MT proteins reported in liver, as already observed in white-trailed tropicbird from the Reunion Island (Kojadinovic et al., 2007) and surf scoter from Canada (Barjaktarovic et al., 2002). In kidneys, the lower functional pool of MT proteins combined to the higher Cd bioaccumulation in this organ led to the onset of MT synthesis. Therefore, this process is tissue dependent in *A. platyrhynchos*.

4.3. Cellular effects of cadmium

Few studies have focused on Cd cellular impairment in birds. However, investigations have demonstrated that Cd increased the plasma malondialdehyde level as an indicator of lipid peroxidation and could lower the activity of blood superoxide dismutase (Erdogan et al., 2005). In the current study, the lowest Cd level induced expression of genes encoding for antioxidant enzymes after 40 days of contamination. This result indicates that Cd generates oxidative stress in cells. Moreover, Cd triggered the induction of genes encoding for Nad5 and Cox1. Mitochondrial metabolism was then stimulated in order to provide sufficient energetic reserves to counter Cd toxicity. Surprisingly, the highest amount of Cd did not disrupt the expression of the majority of the genes encoding for proteins involved in mitochondrial metabolism or antioxidant enzymes. This stabilization to basal levels may suggest that this species set up an adaptive response with high amounts of Cd. Liver could bring other molecular mechanisms to counter Cd toxicity, as previously observed in *X. laevis* larvae (Mouchet et al., 2006). Possibly, cells of *A. platyrhynchos* may use other defensive strategies to fight against oxidative stress such as glutathione-S-transferase, glutathione peroxidase and glutathione reductase. Nevertheless, it has also been demonstrated that glutathione-S-transferase (GST) presented a decrease in activity for concentrations exceeding 5 μM in skeletal muscle cells (Yano and Marcondes, 2005). *A. Platyrhynchos* results evidenced a down-regulation for two genes. The first encoded for acetyl-CoA carboxylase and the second for catalase, an antioxidant enzyme. Similar results have been previously described in a normal rat liver cell line where superoxide dismutase (*sod*), catalase, glutathione reductase and glutathione peroxidase lost activities upon a 4-h exposure to levels of Cd^{2+} ranging from 100 to 300 μM (Ikediobi et al., 2004). In the current study, high dose exposure triggered the down-regulation of some antioxidant genes, whereas low dose enhanced the expression levels of *sod*. This reduction of antioxidant expression for high amounts of Cd may lead to the accumulation of ROS species and then to DNA damages even if *gadd* gene expression did not demonstrate the set up of DNA repair. This result may then represent the precursor sign of Cd toxic effects. Consequently, it may also be hypothesized that after 40 days of contamination with important Cd concentrations, detoxification mechanisms employed by *A. platyrhynchos* may not be sufficient enough to counter the metal toxicity. To answer this question, the experiment should be prolonged in further study and more detoxification pathways should be investigated.

4.4. Comparison with species Cairina moschata

A. Platyrhynchos results contradicted previous study led on duck *C. moschata* (Lucia et al., 2009). Although this latter species was exposed to the same conditions and similar Cd levels (1 and 10 mg kg^{-1}), metal concentrations in tissues were lower compared to *A. platyrhynchos* in the current study. For both Cd levels, the fast induction of genes involved in mitochondrial metabolism of *C. moschata* evidenced the energetic need required to fight against the metal effects. The enhancement of antioxidant defences (*sod1*, *sod2*, catalase) also demonstrated that Cd generated oxidative stress in this species (Lucia et al., 2009). Moreover, Cd induced DNA damages in *C. moschata*. This bird was thus widely affected by Cd toxicity in contrast to *A. platyrhynchos*. This comparison between *A. platyrhynchos* and *C. moschata* highlighted the divergent responses to Cd contamination according to species. The metal effects were therefore species dependent.

Discrepancies between species could be explained by two mechanisms. First, as discussed above, *A. platyrhynchos* may be able to set up an adaptive response to Cd exposure and bring other defense mechanisms. Second, MTs belong to the principal pathway of Cd detoxification (Ikemoto et al., 2004; Bertin and Averbeck, 2006; Nam et al., 2008) and play a predominant role in

Cd depuration (Baudrimont et al., 2003). In *A. platyrhynchos*, MT gene was quickly set up after Cd contamination. For the highest level of Cd, induction of this gene appeared after 10 days of contamination and increased throughout the experiment. For the lowest Cd concentration, up-regulation was observed after 40 days. In contrast, MT gene expression of *C. moschata* only increased after 40 days of Cd exposure for both levels. Moreover, *A. platyrhynchos* presented higher basal MT protein levels than *C. moschata* in liver and kidneys. The faster establishment of MT in *A. platyrhynchos* provides a protection system against Cd toxicity. This species may therefore be less submitted to cellular damages under environmental pressure than *C. moschata*.

5. Conclusion

The current comparison between *A. platyrhynchos* and *C. moschata* demonstrated that Cd induces species-dependent effects and triggers different defense strategies. Cd impairments in one particular species must not be extrapolated to the whole bird phylum. It is thus advisable to study the metal effects on species scale in further studies. Results of the current study focused on the combined analysis of Cd content in tissues, MT quantification and gene expression in liver. These analyses provided complementary information to understand Cd toxicity in birds. However, for a better understanding of all the processes involved in protection against this toxic element in birds, further investigations should focus on other pathways of Cd detoxification such as glutathione peroxidase and GST.

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