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Magali Lucia[a,*], Pierrick Bocher[a], Richard P. Cosson[b], Carine Churlaud[a], Paco Bustamante[a]

[a] Littoral, Environnement et Sociétés (LIENSs), UMR 7266 CNRS-Université de La Rochelle, 2 rue Olympe de Gouges, 17000 La Rochelle, France.

[b] Mer Molécules Santé (MMS), Université de Nantes, EA 2663, 2 rue de la Houssinière, 44322 Nantes, France.

***Corresponding Author:** Magali LUCIA

E-mail address: m.lucia33@laposte.net

Phone: + 33 5 46 45 72 17

FAX: + 33 5 46 50 76 63

Abstract: This study investigated sub-lethal effects and detoxification processes activated in free-ranging Red Knots (*Calidris canutus*) from the Pertuis Charentais on the Atlantic coast of France, and compared the results with previous data obtained on another shorebird species, the Black-tailed Godwit (*Limosa limosa*). The concentrations of 13 trace elements (Ag, As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, Zn) were assessed in the liver, kidneys, muscle and feathers. Stable isotope analyses of carbon and nitrogen were carried out to determine whether differences in diet explained variations in elemental uptake. The mRNA expression of relevant genes (cytochrome c oxidase 1, acetyl-CoA carboxylase, superoxide dismutase Cu/Zn and Mn, catalase, metallothionein, malic enzyme), antioxidant enzyme activities (catalase, glutathione peroxidase (GPx), superoxide dismutase), and metallothionein (MT) levels were investigated to shed light on trace element detoxification and toxic effects. Although Red Knots were characterized by elevated As and Se concentrations which were potentially toxic, most elements were usually below toxicity threshold levels. The results strongly suggested a dietary specialization of Red Knots, with individuals feeding on higher trophic status prey experiencing higher As, Hg and Se burdens. Red knots and Godwits also showed discrepancies in elemental accumulation and detoxification processes. Higher As and Se concentrations in Red Knots enhanced catalase gene expression and enzyme activity, while Godwits had higher Ag, Cu, Fe and Zn levels and showed higher MT production and GPx activity. The results strongly suggest that detoxification pathways are essentially trace element- and species-specific.

Keywords: Metal; Bioaccumulation; Biomarker; Waterbird; Gene expression

Introduction

Over the last three decades, the massive dispersal of toxic compounds into coastal areas as a result of human activities has greatly impacted and degraded those ecosystems, which rank today among the most endangered ecosystems in the world (Vitousek et al. 1997; Halpern et al. 2008). The loss and degradation of habitats at different points along shorebird flyways have partly caused their decline (Stroud et al. 2006). On a global scale, with respect to known population trends, 46% of wader populations are in decline (Delany and Scott 2006; Stroud et al. 2006). The Red Knot (*Calidris canutus*) is a shorebird species highly specialized for long distance migrations. Two subspecies, *C. c. islandica* (the so-called Nearctic Knots) and *C. c. canutus* (the so-called Afro-Siberian Knots) reach the north of Europe in late July and August after breeding, where they possibly mingle on some sites like the Wadden Sea or the Pertuis Charentais (Piersma et al. 1993; Nebel et al. 2000; Koopman 2002; Bocher et al. 2012). Demographic studies in the Wadden Sea demonstrated the loss of 25% of Red Knots *C. c. islandica* population between 1997 and 2003 (van Gils et al. 2006). Similarly, *C. c. canutus* is considered to possibly decreasing in numbers (Spaans et al. 2011).

The Pertuis Charentais on the French central Atlantic coast is the main wintering and/or stopover site during Spring and Fall migration for shorebirds in France (Mahéo 2011). This area is subject to trace element inputs through river discharges (Pigeot et al. 2006) which lead to subsequent bioaccumulation in the macrofaunal community (Bustamante and Miramand 2004, 2005) and, therefore, in the prey of shorebirds. Consequently, shorebirds could be exposed during the wintering period to non-essential (and thus potentially toxic beyond a threshold) elements such as silver (Ag), cadmium (Cd), lead (Pb) and, mercury (Hg) and also to essential elements that could be toxic at high levels such as arsenic (As), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), selenium (Se) and zinc (Zn).

Reporting population declines at the community scale provides a late-stage signal of potential contaminant effects. In this context, investigating sub-lethal effects of contaminants in wild individuals to provide early diagnosis of endangered species poses a major challenge (Adams et al. 2001). Nonetheless, in contrast to terrestrial birds and some waterbirds (e.g. Anatidae) for which oxidative stress related to trace elements has been demonstrated in the wild or experimentally (Hoffman et al. 2000; Hoffman 2002; Mateo et al. 2003; Berglund et al. 2007; Lucia et al. 2009), cellular damages due to trace elements are relatively unexplored in shorebirds. Filling in these gaps in the data is crucial for these endangered species. Yet, a previous study on the Black-tailed Godwit (*Limosa limosa*) from the Pertuis Charentais demonstrated possible production of reactive oxygen species, and suggested that in order to counter trace element toxicity, additional energy needs are required, especially to sustain detoxification mechanisms (Lucia et al. 2012). However, even though Red Knots and Godwits are both specialized to feed on mudflats, their morphology and feeding behaviours are different. A comparison of these shorebird species could, therefore, provide new clues on sub-lethal effects and detoxification mechanisms implemented by free-ranging birds and reveal possible species-specific responses to trace element accumulation.

The objective of the current study was to compare the detoxification processes and sub-lethal effects of trace elements in free-ranging Red Knots to previous results obtained on Black-tailed Godwits (Lucia et al. 2012) wintering or staging in the Pertuis Charentais. The concentrations of the 13 trace elements cited above were determined in the liver, kidneys, muscle and feathers of Red Knots. The first two tissues are specifically involved in detoxification of trace elements, while muscle could function as an important storage tissue for some elements and feathers as a pathway for their excretion (Lewis and Furness 1991). Since ingestion of food is considered to be the main exposure pathway for shorebirds, stable isotopes of carbon and nitrogen were also analysed in Red Knots to determine whether differences in trophic position or diet explained variations in elemental uptake (Jardine et al. 2006). Second, seven genes were selected in order to study trace element

effects on key metabolic functions such as lipogenesis and mitochondrial metabolism as well as detoxification processes and oxidative stress responses. Mitochondrial metabolism was investigated using cytochrome c oxidase subunit 1 (*cox1*) and lipogenesis was investigated through expression of acetyl-CoA carboxylase (*acc*) and malic enzyme (*me*). Moreover, organisms display different mechanisms to counter trace element toxicity. Among them, metallothioneins (MT), low molecular weight cysteine-rich proteins, are known to be involved in homeostasis of essential metals such as Cu and Zn as well as in protection against toxic metals such as Ag, Cd and Hg. Another means employed to counter trace element toxicity is to increase antioxidant defenses such as Cu/Zn and Mn superoxide dismutase (*sod1* and *sod2*, respectively) and catalase (*cat*). Expression of these genes and the activities of the corresponding enzymes were studied, as well as the activity of glutathione peroxidase (GPx). Finally, some elements such as Cd induce lipid peroxidation of hepatocyte membranes (Stohs and Bagchi 1995). Lipid peroxidation was therefore investigated via malondialdehyde (MDA) contents.

Material and Methods

Study site and sampling

Fifteen Red Knots that accidentally died during mist net capture for ringing purposes, were collected between October 2007 and February 2011 in the Pertuis Charentais, on the Atlantic coast of France (Figure 1). Birds were sampled at three sites: Ré Island (n = 3), Yves Bay (n = 3) and Marennes-Oléron Bay (n = 9).

When it was possible during the dissection, sex and age class (juvenile/adult) were determined for each individual. The liver, kidneys, pectoral muscle and ventral feathers were sampled, weighed (wet weight, ww), placed in individual plastic bags and stored at -20°C. Liver, kidneys and muscle samples were later freeze-dried and weighed again (dry weight, dw). Freeze-dried tissues were then ground and stored in individual plastic vials until further trace element, isotopic and MT analyses.

Ventral feathers were washed to remove oil and dirt in a chloroform-methanol solution (2:1) in an ultrasonic bath for two minutes. Afterwards, they were rinsed in two consecutive pure methanol baths for a few seconds and dried at 40 °C for 48 hours prior to being used for trace element and stable isotope analyses.

In order to specifically investigate mRNA expressions in the liver, kidneys and muscle, and enzymatic activities in the liver, four Red Knots accidentally dead in nets were collected at Marennes-Oléron in February 2011. For these analyses, the liver, kidneys and muscle were immediately dissected in the field after their deaths and divided into two parts. The first part was immediately frozen in liquid nitrogen and stored at -80°C for enzymatic and genetic analyses. The second part was stored at -20°C and subjected to the same treatment as previously described for trace element, stable isotope and MT determinations.

Trace element determinations

Total Hg analyses were carried out in the Red Knot by atomic absorption spectrophotometry with an Advanced Mercury Analyser (ALTEC AMA 254) on dried tissue aliquots (liver, kidneys, pectoral muscle, ventral feathers) ranging from 4 to 50 mg (± 0.01 mg) depending on the Hg concentration in the tissue following the protocol described in Bustamante et al. (2006). Mercury analyses were run under a thorough quality controlled program that includes analysis of Certified Reference Material (CRM; lobster hepatopancreas TORT-2; National Research Council, Canada). CRM aliquots were treated and analysed according to the same conditions as the samples. The results were in good agreement with the certified values, with a mean recovery rate of 92%. The detection limit was 5 ng Hg g^{-1} dw.

Ag, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Se and Zn were analysed using a Varian Vista-Pro ICP-OES and a Thermo Fisher Scientific XSeries II ICP-MS in the liver, kidneys, pectoral muscle and ventral feathers. Aliquots of the biological samples (30–300 mg) were digested with 6 ml 67–70%

HNO_3 and 2 ml 34–37% HCl (Fisher Scientific, trace element grade quality), with the exception of feathers (3 ml HNO_3 and 1 ml HCl). Acidic digestion of the samples was carried out overnight at room temperature, then using a Milestone microwave (30 min with constantly increasing temperature up to 120°C, then 15 min at this maximal temperature). Each sample was brought up to a volume of 50 ml (or 25 ml for feathers) with milli-Q water. Three control samples (two CRMs and one blank) treated and analysed the same way as the samples were included in each analytical batch. The CRMs were dogfish liver DOLT-4 (NRCC) and lobster hepatopancreas TORT-2 (NRCC). Quantification limits and mean recovery rates were, respectively, equal to 0.1 µg L^{-1} and 82% for Ag, 1 µg L^{-1} and 98% for As, 0.1 µg L^{-1} and 94% for Cd, 0.1 µg L^{-1} and 99% for Co, 0.1 µg L^{-1} and 102% for Cr, 5 µg L^{-1} and 93% for Cu, 20 µg L^{-1} and 86% for Fe, 5 µg L^{-1} and 90% for Mn, 0.2 µg L^{-1} and 101% for Ni, 0.1 µg L^{-1} and 86% for Pb, 0.5 µg L^{-1} and 109% for Se, and 20 µg L^{-1} and 98% for Zn. Trace element concentrations are expressed in µg g^{-1} dw. Values below the quantification limit were taken into account in calculating of the means by assigning them values one-half of the detection limit for the given element (e.g. a value < 0.02 was reported as 0.01 µg g^{-1} dw).

Nitrogen and carbon stable isotope analysis

Cleaned feathers of red knots were chopped using surgical scissors and accurately weighed (± 0.001 mg) to a range between 0.200 and 0.500 mg. Liver and muscle samples were also weighed with the same accuracy and in the same range of masses. All samples were placed in tin capsules for carbon and nitrogen stable isotope analysis and analysed using an elemental analyser (Flash EA 1112 fitted with a “No Blank” option, Thermo Scientific, Milan, Italy) coupled to an isotope ratio mass spectrometer (Delta V Advantage, Conflo IV interface, Smart EA option, Thermo Scientific, Bremen, Germany). The results are reported in δ unit notation (expressed in per mil relative to

standards: Vienna Pee Dee Belemnite for $\delta^{13}C$ and N_2 in air for $\delta^{15}N$). The analytical precision of the measurements was < 0.06 ‰ and < 0.1 ‰ for carbon and nitrogen, respectively.

Metallothionein determination

Approximately 100 mg of each freeze-dried liver aliquot was homogenized on ice in 6 ml 100 mM Tris buffer with β-mercaptoethanol at pH = 8.1 and then centrifuged (30,000 g; 30 min; 4°C). Before determining the MT level, the supernatant aliquot was submitted to heat denaturation (95°C; 15 min), placed on ice for 10 min and centrifuged (10,000 g; 10 min, 4°C) to separate heat-stable proteins from denatured proteins. The supernatant from the second centrifugation was then frozen (-20°C) until MT quantification.

Red Knots were analysed for their hepatic MT levels. Differential pulse polarographic analysis (DPP) was used to determine the level of MT in the heat-denatured soluble fraction. DPP is a technique based on -SH compound determination according to the Brdička reaction (Brdička 1933) as described by Thompson and Cosson (1984). A Model 303A static Hg drop electrode was used. Certified rabbit liver MT (Sigma Chemical Co., St. Louis, MO) were used to carry out the calibration according to the method of standard additions. The system consisted of a bevelled capillary, an Hg working electrode, a platinum counter electrode and an Ag/AgCl reference electrode. Results are expressed in µg of MT per g of dry homogenized tissue.

Sequencing of genes

Five DNA fragments of the Red Knot were sequenced for β-actin (*act*), acetyl-CoA carboxylase (*acc*), Cu/Zn superoxide dismutase (*sod1*), metallothionein (*mt*) and NADP-dependent malic enzyme (*me*).

A quantity of 40 mg of liver was homogenized for extraction of total RNA using the Absolutely Total RNA Miniprep kit (Agilent Technologies, USA) according to the manufacturer's instructions.

All RNA extracted was of good quality after evaluation by electrophoresis on a 1% agarose-formaldehyde gel, and the concentration was determined by spectrophotometry. First-strand cDNA was synthesized from 5 µg of previously extracted total RNA with the AffinityScript Multiple Temperature cDNA Synthesis kit (Agilent Technologies, USA) according to manufacturer's instructions.

The non-specific primers used to obtain amplified cDNA fragments of the different genes by PCR were determined after multiple sequence alignments of birds or mammalian species using Clustal W software (Infobiogen), or were qPCR primers based on duck *Cairina moschata* and *Anas platyrhynchos* (Supplementary Material Table S1). Amplified products were cloned into pGEM-T vector (Promega) and sequenced (Millegen, France).

For Mn superoxide dismutase (*sod2*), catalase (*cat*) and cytochrome c oxidase subunit 1 (*cox1*) primer pairs from *Cairina moschata* and *Anas platyrhynchos* were used to amplify products less than 200 bp. Cloning and sequencing demonstrated that the primer pairs chosen from conserved regions of the *C. moschata* and *A. platyrhynchos* genes could be used for *C. canutus* for qPCR analysis (Table Supplementary Material Table S2).

Quantitative real-time PCR

The mRNA expression levels of eight genes were investigated in the liver, kidneys and muscle of red knots by quantitative real-time PCR: β-actin, cytochrome C oxidase subunit 1, acetyl-CoA carboxylase, Cu/Zn superoxide dismutase, Mn superoxide dismutase, catalase, metallothionein, and NADP-dependent malic enzyme. Quantitative real-time PCR reactions were performed in an Mx3005P QPCR Systems (Agilent Technologies, USA) following the manufacturer's instructions (one cycle at 95°C for 10 min followed by 40 amplification cycles at 95°C for 30s, 60°C for 30s and 72°C for 30s). 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 (Agilent Technologies,

USA), enabling 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) (Supplementary Material Table S2).

Reaction specificity was determined for each reaction from the dissociation curve of the PCR product. This dissociation curve was obtained by monitoring the SyberGreen fluorescence level during gradual heating of the PCR products from 60 to 95°C. Relative gene expression levels were normalized according to β-actin gene expression which was stable across tissues and samples. Induction and repression factors were calculated between the Red Knot and the Black-tailed Godwit (Lucia et al. 2012) following the formula: $2^{(-\Delta CT\ BQN)}/2^{(-\Delta CT\ RK)}$, where ΔCT is the Threshold Cycle of the Black-tailed Godwit (BTG) and the Red Knot (RK) normalized according to their respective β-actin CT.

Antioxidant enzymes and lipid peroxidation

Glutathione peroxidase (GPx) activity was determined in the liver of red knots according to the method of Paglia and Valentine (1967) using a glutathione peroxidase assay kit (RS504/RS505, RANDOX, France) as previously described (Lucia et al. 2012).

Hepatic superoxide dismutase (SOD) and catalase (CAT) activities were determined according to the methods of Wooliams et al. (1983) and Deisseroth and Dounce (1970), respectively, using assay kits (SD125, RANDOX, France and CAT100, Sigma Aldricht, USA) as previously described (Lucia et al. 2012). The results are presented in units of SOD or CAT per mg of protein.

All activities were expressed in relation to protein concentration, measured according to the Bradford method with slight modifications, using bicinchoninic acid and 4% copper sulfate (Smith et al. 1985). Serum albumin was used as a standard (Sigma-Aldrich, France).

Lipid peroxidation levels were assessed in liver according to malondialdehyde (MDA) content, determined using a commercial MDA assay kit (Oxis International, USA), following the

manufacturer's instructions. The results are presented in nmol MDA per g of tissue. Antioxidant enzyme activity and lipid peroxidation were measured in UV microplates (Greiner Bio One, Germany) using a spectrophotometer (SAFAS Flx-Xenius, Monaco).

Statistical analysis

As normality and homogeneity of variance were not achieved despite $\log_{10}(x+1)$ transformation (Cochran C test), non-parametric analysis of variance was applied to assess differences in trace element concentrations between tissues (liver, kidneys, muscle and feathers), sexes or age classes in the Red Knot (Kruskal-Wallis and Mann-Whitney U-test, Statistica 7.1). Non-parametric analyses of variance (Mann-Withney U-test) were also employed to study differences in MT concentration, mRNA expression, enzymatic activities and trace element concentrations between the Red Knot (current study) and the Black-tailed Godwit (Lucia et al. 2012). Moreover, the Spearman test was applied to all correlations achieved in this study.

Distance-based Redundancy Analysis (dbRDA) was used to analyse the relations between trace element concentrations measured in Red Knots and Godwits and mRNA expressions, enzyme activities and MT protein concentrations. Trace element concentrations as well as mRNA expressions, enzyme activities and MT protein concentrations were first standardised according to the maximum value measured for each of these variables. Standardised trace elements were then used to produce a distance matrix among individual birds using Euclidean distance. This distance matrix was used to produce an ordination of individual birds constrained by the standardised values for mRNA expressions, enzyme activities and MT protein concentrations. Two separate analyses were performed on (1) liver data and (2) kidneys data. These analyses were performed using the PERMANOVA + package (Anderson et al. 2008) from PRIMER v6 (Clarke and Gorley 2006).

Results

Trace element bioaccumulation

The mean concentrations and ranges of the 13 trace elements in four tissues of 15 Red Knots sampled in the Pertuis Charentais are presented in Table 1. Although concentrations were usually low, especially for Ag, Co, Cu, Cr, Hg, Mn, Ni, Pb, and Zn, elevated concentrations were observed for As, Cd and Se. Moreover, important variations in individual concentrations were observed for the majority of the elements. For example, two birds exceeded Cd concentrations of 10.0 µg g^{-1} in their kidneys while the others were below 2.0 µg g^{-1} dw. Hg concentrations appeared to be highest in feathers, with one individual reaching 4.5 µg g^{-1} dw (Kruskal-Wallis test, $p < 0.05$). For the essential elements, As and Se, 73% of the individuals exceeded Se concentrations of 10.0 µg g^{-1} (Outridge et al. 1999) and 47% exceeded 30.0 µg g^{-1} (Skorupa et al. 1996) in the liver. Similarly, 80% of the Red Knots exceeded As concentrations of 3.0 µg g^{-1} in the liver.

Many correlations were observed between elements in the four tissues studied. As and Se were positively correlated (liver: R = 0.71; kidneys: R = 0.89; muscle: R = 0.85; feathers: R = 0.66), as well as Cu and Zn (liver: R = 0.66; kidneys: R = 0.74; muscle: R = 0.90). As for the non-essential elements, Cd was significantly correlated with Zn in internal tissues (liver: R = 0.71; kidneys: R = 0.69; muscle: R = 0.62) and with Pb (muscle: R = 0.66; feathers: R = 0.66). Moreover, when Se:Hg molar ratios were calculated in the livers of Red Knots, the ratios always exceeded one for all the birds and ranged between 5 and 594.

Influence of individual characteristics (sex, age class, body weight)

No differences in trace element concentrations were observed according to the sex of the birds. In contrast, the results highlighted discrepancies in element concentrations between age classes, especially for Cd. Indeed, Cd concentrations in adult birds were significantly above concentrations in juveniles in muscle (0.057 ± 0.093 *vs* 0.010 ± 0.001 µg g^{-1} dw, respectively; Mann-Whitney U-

test, p = 0.025) and below those of juveniles in the feathers (0.010 ± 0.001 *vs* 0.025 ± 0.011 µg g^{-1} dw, respectively; p = 0.012).

Trace element concentrations demonstrated positive relationships with the body weight of the Red Knots. Indeed, in kidneys, Ag, As, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Se and Zn concentrations were all positively correlated with weight (correlation coefficients were between 0.48 for As and 0.83 for Pb). The same trend was observed in the feathers only for As (R = 0.53), Hg (R = 0.59), Pb (R = 0.50) and Se (R = 0.65).

Influence of trophic position and feeding habitat

The relationship between trophic position assessed through determination of $\delta^{15}N$ and trace element concentrations in the Red Knots was investigated in three tissues: liver, muscle and feathers. Depending on the tissue, As, Hg and Se concentrations were positively correlated with $\delta^{15}N$ (Spearman correlation test, all $p < 0.05$; Figure 2).

The relationship between the foraging habitats of birds, assessed through determination of $\delta^{13}C$, and their trace element concentrations was also studied in the three tissues. The only significant correlation was between Hg concentrations and $\delta^{13}C$ in the liver and was negative (R = -0.82, Spearman correlation test, $p < 0.05$).

Trace element detoxification and effects: comparison with the Black-tailed Godwit

The trace element concentrations in three tissues (liver, kidneys, muscle) of the Red Knot and the Black-tailed Godwit are presented in Table 2. The Red Knot had significantly lower Ag, Cu, Fe and Zn concentrations in the liver, Co, Hg and Zn concentrations in the kidneys, and Zn concentrations in muscle compared with the Black-tailed Godwit. Conversely, Se concentrations were higher for the Red Knot in the three tissues, as well as As and Mn concentrations in kidneys and muscle, and Pb concentrations in the kidneys.

During the current study, a Red Knot partial β-actin cDNA of 887 bp was sequenced. The corresponding protein of 295 amino acids (aa) presented strong similarities with the Black-tailed Godwit β-actin (99% identity). In the case of acetyl-CoA carboxylase (*acc*), the fragment sequenced (245 bp, 81 aa) corresponded to *acc* of the Greylag Goose (*Anser anser*; 100% identity) and the Black-tailed Godwit (99% identity). The partial Cu/Zn superoxide dismutase (*sod1*) cDNA sequenced (273 bp, 40 aa) presented strong similarities with Dunlin *sod1* (*Calidris alpina*; 98% identity) and the metallothionein (*mt*) cDNA sequenced (224 bp, 62 aa) closely matched those of the Black-tailed Godwit (98% identity) and the Great Cormorant (*Phalacrocorax carbo*; 95% identity) *mt*. The last gene sequenced, NADP-dependent malic enzyme (215 bp, 71 aa), presented high similarities with the malic enzyme of the Muscovy Duck (*Cairina moschata*; 100% identity) and the Black-tailed Godwit (100% identity).

The relative mRNA expression of seven genes either induced or repressed in the Red Knot compared to the Black-tailed Godwit and involved in detoxification processes, defence against oxidative stress, lipogenesis, or mitochondrial metabolism were investigated in the liver, kidneys and muscle (Table 3). The results demonstrated that *cat* gene expression in the Red Knot was 50-fold the level of Black-tailed Godwit *cat* expression in the liver and kidneys (induction factor: 50). In contrast, this gene appeared to be down-regulated in muscle of the Red Knot compared to the other species. The Red Knot also seemed to highly express the *acc* gene in the muscle. The only other gene that differed between the two species was the *mt* gene. Indeed, this gene appeared to be three to seven times lower in the three tissues of the Red Knot compared to the Black-tailed Godwit (repression factor between 1/3 and 1/7; Table 3).

MT concentrations in the livers of Red Knots were on average 3582 ± 1656 µg g^{-1} dw (n = 15), showing a wide variability among individuals. Nonetheless, MT concentrations were positively correlated with concentrations of Cd (R = 0.69; Spearman test, $p < 0.05$), Co (R = 0.83), Cu (R = 0.86), Hg (R = 0.70), Mn (R = 0.79), Pb (R = 0.77) and Zn (R = 0.88). MT concentrations in the

Red Knots were significantly below those in Godwits previously reported by Lucia et al. (2012) (7615 ± 4340 µg g^{-1} dw (n = 31); Mann-Withney U-test, p = 0.002).

Results of enzymatic activity assays demonstrated that the Red Knot displayed higher hepatic catalase and SOD activities than the Black-tailed Godwit (Figure 3). Conversely, this species showed higher GPx activity than the Red Knot. For lipid peroxidation, no discrepancies appeared between the two species. Enzyme activities were positively correlated with elemental concentrations. Indeed, catalase activity significantly increased with As and Se concentrations (R = 0.76 and R = 0.74, respectively; Spearman test, $p < 0.05$), whereas SOD activity was positively linked only with Se concentration only (R = 0.76). GPx activity appeared to be influenced by several elements. Indeed, this enzyme was correlated with Ag (R = 0.81), Cu (R = 0.83), Fe (R = 0.74) and Zn (R = 0.76) concentrations.

A distance-based Redundancy Analysis (dbRDA) was performed on the liver and kidneys data (Figure 4). The first-two axes extracted 80.1% and 80.7% of the variance of the data clouds in the liver and kidneys, respectively. The first axis discriminated the two species. In the liver, the dbRDA demonstrated that the higher As and Se concentrations in Reds Knots were linked with *cat* gene expression, as well as SOD and catalase enzyme activities (Figure 4). In contrast, the higher Ag, Cu, Fe and Zn concentrations in Godwits were positively linked with *mt* gene expression, the concentration of MT proteins and GPx activity (Figure 4). In the kidneys, higher As, Mn, Pb and Se concentrations were correlated with elevated mRNA expression of *acc*, *cat*, *cox1*, *me*, *sod1* and *sod2* mRNAs. In muscle, Spearman correlations demonstrated that *acc* gene expression was positively correlated with As (R = 0.82), Hg (R = 0.90), Mn (R = 0.92), and Se (R = 0.82) concentrations.

Discussion

Trace element distribution and toxicity significance

The current study, one of the first investigating trace elements in the Red Knot *C. canutus*, demonstrated generally low elemental concentrations in the investigated tissues. Nevertheless, several elements reached levels above potentially toxic thresholds, especially As and Se in the liver and kidneys as well as Cd in the kidneys. These results are in accordance with a previous study in the Southwest Atlantic coast of France where three individuals of this species were reported to have high As and Se concentrations (Lucia et al. 2010).

In birds, inorganic As is considered highly toxic in comparison with organic compounds of this element and may disrupt reproduction, act as an endocrine disruptor, trigger sub-lethal effects or even induce individuals' death (Eisler 1994; Kunito et al. 2008). As concentrations in living organisms are generally low (< 1 µg g^{-1} ww, approximately 5 µg g^{-1} dw; Braune and Noble 2009). Sixty percent of the birds were above this threshold. However, all the Red Knots were below 50 µg g^{-1} dw, a threshold associated with direct toxic effects in seabirds (Neff 1997). Even if As is mostly accumulated in its organic form (Neff 1997), many organic arsenicals undergo biotransformation and organisms could be exposed to toxic intermediates (Albert et al. 2008). Moreover, Red Knots prey on benthic macroinvertebrate (Quaintenne et al. 2010) which can accumulate inorganic forms of As including As(V) or As(III) (Baumann and Fisher 2011). Consequently, birds may be able to accumulate both inorganic and organic As that could trigger sub-lethal effects in the Red Knot. In the future, further investigations should be performed to assess the different forms of As accumulated by Red Knots in order to empirically determine whether birds can accumulate the more toxic forms.

The results highlighted discrepancies in Cd concentrations between age classes. Indeed, Cd concentrations of adult birds were significantly above concentrations of juveniles in muscle but below juveniles in feathers. Age is one of the main factors explaining Cd accumulation, as observed in waterfowl from southern Spain (Gómez et al. 2004). With continued exposure, even at low levels, Cd is accumulated throughout the life span of birds (Dailey et al. 2008). The lower Cd

concentration in the feathers of adults compared to young birds could be a consequence of the balance between elemental uptake during the feather's formation and elemental removal during the moult (Lewis and Furness 1991). Adults may therefore be able to deposit Cd in their plumage and subsequently eliminated it through moulting. Nonetheless in this study Cd mainly accumulated in the kidneys, which represent the major internal organ for accumulation of this non-essential element (Barjaktarovic et al. 2002; Gómez et al. 2004; Kojadinovic et al. 2007). The toxicity of this metal was previously demonstrated in vertebrates and birds in particular (Hughes et al. 2000). Although the current results were below concentrations observed in previous studies where adult Barau's Petrel from the Reunion island were reported to have accumulated 66.8 ± 31.8 µg g^{-1} (Kojadinovic et al. 2007), low environmental levels such as 4.7 ± 0.7 µg g^{-1} in the liver of birds experimentally contaminated could trigger the appearance of sub-lethal effects (Lucia et al. 2009). Lucia et al. (2009) observed the appearance of oxidative stress and up-regulation of genes involved in mitochondrial metabolism. Cd is indeed able to inhibit the electron transfer chain and induce reactive oxygen species (Wang et al. 2004; Bertin and Averbeck 2006). Similar endpoints could be expected in Red Knots given their exposure to Cd in the environment.

Although Se is essential to living organisms' metabolism, this element is considered highly toxic depending upon the chemical form ingested (Stewart et al. 1999). Effects of Se include direct mortality, reproductive impairment with teratogenesis, growth reduction, histopathological lesions and alterations in hepatic glutathione metabolism (Hoffman 2002). Some authors have suggested an effect threshold of 30 µg g^{-1} dw in the liver of birds (Skorupa et al. 1996). Moreover, hepatic Se concentrations exceeding 10 µg g^{-1} dw are associated with lower reproductive success in breeding females as well as reduced adult weight gain (Outridge et al. 1999). In the current study, 73% of Red Knots exceeded 10 µg g^{-1} dw and 47% reached or were above 30 µg g^{-1} dw. Nevertheless, the co-accumulation of Se and Hg could reduce the toxic effects of both trace elements (Peterson et al. 2009). A Se:Hg molar ratio approaching one suggests the existence of mercuric selenide (HgSe)

which is a well-known non-toxic form in marine mammals and birds (Koeman et al. 1973; Martoja and Berry 1980; Nigro and Leonzio 1996; Ikemoto et al. 2004). In the current study, Hg and Se concentrations were not correlated in internal tissues of Red Knots and molar ratios were always above one. Consequently, the excess hepatic Se and the high concentrations reached in each tissue strongly support a possible toxicity of this element for the Red Knot.

Relation between trace element concentrations, trophic level and feeding habitat

Stable isotopes of carbon and nitrogen are useful tools to study trophic relationships and prey origins to elucidate inter- and intraspecific dietary patterns, and determine if variations in elemental uptake could be explained by differences in foraging strategy (Jardine et al. 2006; Michener and Kaufman 2007). Depending on the tissue, As, Hg and Se were positively linked to the trophic level of birds as reflected by $\delta^{15}N$ values. This result highlights the dietary specialization occurring in one particular species with individuals feeding on high trophic status prey and thus experiencing high As, Hg and Se exposure. Indeed, despite their trophic specialization (Piersma et al. 1998), Red Knots are able to feed on a variety of benthic macroinvertebrates that differ in profitability as well as digestive quality (van Gils et al. 2005). In their European wintering grounds, most of the diet consist of by the balthic clam *Macoma balthica*, the edible cockle *Cerastoderma edule* and the mudsnail *Hydrobia ulvae* (Quaintenne et al. 2010). These authors have demonstrated variations in the diet composition of Red Knots between sites but also within sites in Western Europe, corroborating the observed dietary specialization of individuals. The variation in $\delta^{15}N$ signatures between individuals from the current study could partly come from the different life status and origin of the birds. However, when taken into account, these parameters triggered no statistical discrepancies in $\delta^{15}N$ signatures. Nonetheless, this dietary specialization could explain the different trace element accumulations observed in Red Knots of the Pertuis Charentais.

Hepatic Hg concentrations were also negatively linked to $\delta^{13}C$, which is mainly used to indicate the foraging habitats of predators (Rubenstein and Hobson 2004). This result highlighted the fact that the more birds fed on continental prey impoverished in ^{13}C, the more Hg concentrations increased. Inter-individual feeding behaviour discrepancies could partly be the origin of the important variations in trace element accumulation observed in free-ranging Red Knots.

Trace element detoxification and effects: comparison with the Black-tailed Godwit

This study was designed to investigate the detoxification of trace elements in shorebird species, which are vulnerable to high exposure when they feed on coastal prey during their migration. A previous study led of the Black-tailed Godwit gave some insight about the possible effects triggered by a combination of contaminants in free-ranging species difficult to obtain from a fairly large number of individuals (Lucia et al. 2012). The objective was thus to compare the genetic and enzymatic responses of both Red Knots and Godwits.

The Red Knot and the Black-tailed Godwit were characterized by differential elemental bioaccumulation. The Red Knot had notably higher As and Se concentrations while the Black-tailed Godwit had higher hepatic Ag, Cu, Fe and Zn levels. This discrepancy resulted in differential gene expression, hepatic enzyme activities and MT concentrations between the two species. First, the *cat* gene was up-regulated in the liver and kidneys of Red Knots compared to Godwits. This pattern was also reflected in the catalase enzyme, whose activity was higher in the Red Knot. Correlation tests and redundancy analyses in both tissues demonstrated that catalase gene expression and enzyme activity were enhanced by higher As and Se concentrations. Bioaccumulation of both trace elements appeared to trigger oxidative stress in the Red Knot. Se especially has the property to react with thiols which generate reactive oxygen species (Spallholz and Hoffman 2002). Experimental studies with selenomethionine, an organic form of Se, have shown oxidative stress in wild aquatic birds (Hoffman 2002). Symptoms included alterations in hepatic glutathione metabolism towards a

more oxidized state, accompanied by lipid peroxidation. Glutathione is an important antioxidant and a cofactor for antioxidant enzymes such as GPx. GSH depletion possibly triggered by Se could partly explain the reduced GPx activity observed in Red Knots in comparison to Black-tailed Godwits. Consequently, Red Knots may rely on other antioxidant defenses such as catalase or SOD whose activities were higher in this species.

Secondly, synthesis of MT is well known to be induced by several metals such as Ag, Cd, Cu, Hg and Zn (Scheuhammer 1987; Kägi 1991; Elliott et al. 1992). The synthesis of MT diverged between the two shorebird species. The gene encoding for MT was indeed down-regulated in the Red Knot in comparison to the Black-tailed Godwit. This down-regulation was also reflected in the MT protein concentration in Red Knots, which was two-fold lower. The higher hepatic Ag, Cu, Fe and Zn concentrations in Black-tailed Godwits might explain this difference. Indeed, mRNA expression of *mt* was positively influenced by these elements in the liver. Godwits therefore appeared to mainly use this detoxification system to counteract the toxicity of trace elements.

The use of sensitive biomarkers, such as mRNA expression, demonstrated that trace elements may trigger species-dependent effects and induce different detoxification pathways in two free-ranging shorebird species feeding in the same habitats during winter migration. In the case of the Red Knot, antioxidant enzymes such as catalase and SOD seemed to be the main protective mechanisms against oxidative stress possibly triggered by As and Se levels. Conversely, the Black-tailed Godwit strongly stimulated the production of MT and had higher GPx activity. Although both species used different detoxification systems, their MDA concentrations were similar. These different response pathways could therefore give the birds similar levels of protection against lipid peroxidation and likely trace element toxicity.

This study highlighted for the first time that two relatively closely related shorebird species (Scolopacidae) exploiting the same feeding habitats in the Pertuis Charentais during migration stopover and/or wintering, and subject to several contaminants in the natural environment, may

develop divergent adaptive responses. Red Knots seemed to suffer from stronger oxidative stress than Godwits, probably triggered by higher As and Se concentrations. Nevertheless, our results tend to demonstrate that energy expenditure and the general health of shorebirds seem not to suffer strongly from trace element bioaccumulation. These results suggest that trace element effects and detoxification pathways are both species-dependent and trace element specific.

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Table 1 Trace element concentrations (Mean ± SD and range, µg g^{-1} dw) in the tissues of 15 Red Knots from the Pertuis Charentais (Atlantic coast of France)

Element		Liver (*n=15*)	Kidneys (*n=14*)	Muscle (*n=12*)	Feathers (*n=15*)
Ag	Mean ± SD	0.26 ± 0.80	0.05 ± 0.06[a]	0.12 ± 0.16[a]	0.11 ± 0.08
	Min-Max	< 0.02-3.13	< 0.02-0.23	< 0.02-0.55	0.03-0.28
As	Mean ± SD	7.26 ± 5.08[a]	4.43 ± 3.55[a]	3.28 ± 1.61[a]	1.19 ± 1.64
	Min-Max	< 0.22-16.36	0.16-13.87	< 0.19-6.04	< 0.2-5.92
Cd	Mean ± SD	0.87 ± 2.40[a]	3.70 ± 9.00[a]	0.03 ± 0.06[a]	0.03 ± 0.05
	Min-Max	0.03-9.49	0.11-33.29	< 0.02-0.22	< 0.02-0.21
Co	Mean ± SD	0.10 ± 0.06	0.18 ± 0.08	0.04 ± 0.01	0.07 ± 0.04
	Min-Max	0.03-0.26	0.08-0.40	< 0.02-0.06	0.03-0.16
Cr	Mean ± SD	0.15 ± 0.33	0.63 ± 0.86	0.18 ± 0.19	0.41 ± 0.66
	Min-Max	< 0.02-1.32	< 0.04-2.33	0.05-0.72	0.07-2.71
Cu	Mean ± SD	24.4 ± 29.0	20.7 ± 5.6	29.0 ± 6.2	19.3 ± 4.0
	Min-Max	8.0-125	12.1-32.2	16.9-41.5	12.0-26.4
Fe	Mean ± SD	1208 ± 934	537 ± 137	291 ± 20	160 ± 99
	Min-Max	389-4062	338-810	250-335	71-373
Hg	Mean ± SD	0.73 ± 0.45[a]	0.71 ± 0.40[ab]	0.28 ± 0.12[ab]	1.26 ± 1.05[b]
	Min-Max	0.10-1.53	0.22-1.76	0.07-0.52	0.42-4.45
Mn	Mean ± SD	8.0 ± 2.6[a]	8.1 ± 3.2[a]	2.2 ± 0.5[a]	8.0 ± 4.0
	Min-Max	4.4-12.5	2.3-12.5	1.2-2.9	2.6-15.2
Ni	Mean ± SD	0.63 ± 2.36	0.45 ± 0.34[a]	0.07 ± 0.09[a]	2.6 ± 5.5
	Min-Max	< 0.04-9.2	< 0.05-1.0	< 0.03-0.34	0.31-22
Pb	Mean ± SD	0.07 ± 0.03[a]	0.40 ± 0.28[a]	0.01 ± 0.01	0.77 ± 0.38
	Min-Max	0.02-0.12	0.07-0.93	< 0.02-0.04	0.30-1.70
Se	Mean ± SD	31.0 ± 22.8[ab]	20.1 ± 14.5[a]	5.7 ± 2.9[ab]	3.7 ± 2.1[b]
	Min-Max	2.9-64.8	2.4-36.9	1.2-11.3	1.0-7.1
Zn	Mean ± SD	105 ± 65	84 ± 15	36 ± 4.1	174 ± 20
	Min-Max	45-192	67-119	28-41	140-206

Significant correlations between tissues for each trace element are indicated by letters at the level $\alpha = 0.05$ (Spearman correlation test). Two identical letters mean that tissues are positively correlated (R ranged between 0.58 and 0.96). No letters means that no correlations have been found for the considered tissues

Table 2 Trace element concentrations (Mean ± SD, µg g^{-1} dw) in the tissues of Red Knots (RK, n = 4) and Black-tailed Godwits (BTG, n = 3; Lucia et al., 2012) from the Pertuis Charentais

	Liver		Kidneys		Muscle	
Element	RK	BTG	RK	BTG	RK	BTG
Ag	0.05 ± 0.04*	2.77 ± 1.99	0.02 ± 0.01	0.03 ± 0.02	0.02 ± 0.01	0.02 ± 0.01
As	11.09 ± 4.05	5.60 ± 2.27	5.62 ± 1.17*	1.97 ± 0.41	3.52 ± 0.44*	1.94 ± 0.60
Cd	0.38 ± 0.18	0.93 ± 1.2	0.95 ± 0.55	3.45 ± 4.36	0.01 ± 0.01	0.09 ± 0.15
Co	0.11 ± 0.02	0.17 ± 0.09	0.20 ± 0.02*	0.38 ± 0.09	0.04 ± 0.01	0.05 ± 0.02
Cr	0.09 ± 0.04	0.11 ± 0.04	1.90 ± 0.42	1.08 ± 1.00	0.25 ± 0.32	0.29 ± 0.20
Cu	20.9 ± 6.4*	312 ± 217	25.9 ± 5.2	20.4 ± 8.6	26.2 ± 3.7	54.7 ± 28.0
Fe	516 ± 117*	2169 ± 1007	488 ± 71	631 ± 173	291 ± 13	372 ± 89
Hg	0.97 ± 0.24	1.19 ± 0.45	0.88 ± 0.17*	1.33 ± 0.25	0.29 ± 0.06	0.23 ± 0.02
Mn	11.2 ± 1.4	9.5 ± 2.3	12.3 ± 0.2*	9.3 ± 1.2	2.7 ± 0.3*	1.8 ± 0.2
Ni	0.02 ± 0.001	0.02 ± 0.001	0.77 ± 0.23	0.60 ± 0.48	0.15 ± 0.13	0.15 ± 0.08
Pb	0.09 ± 0.02	0.09 ± 0.07	0.69 ± 0.17*	0.21 ± 0.09	0.01 ± 0.001	0.01 ± 0.001
Se	53.8 ± 11.7*	14.7 ± 1.1	31.9 ± 4.2*	12.7 ± 4.5	6.61 ± 0.91*	2.76 ± 0.06
Zn	99 ± 6*	192 ± 74	87 ± 5*	145 ± 36	31 ± 2*	57 ± 18

Comparison between red knots and black-tailed godwits was notified for each tissues with p level indication at the level $\alpha = 0.05$ (* $p < 0.05$; Mann-Whitney U-test)

Table 3 Gene expression (induction or repression factors) in the liver, kidneys and muscle of the Red Knot (n = 4) compared to the Black-tailed Godwit (n = 3) sampled at Marennes-Oléron Bay (Pertuis Charentais, Atlantic coast of France) in September 2010

Functions	Genes	Liver	Kidneys	Muscle
Mitochondrial metabolism	*cox1*			
Lipogenesis	*me*			
	acc			5*
Oxidative stress	*sod1*			
	sod2			
	cat	50*	50*	1/7*
Detoxification	*mt*	1/7*	1/3*	1/5*

Significant induction and repression factors (* $p < 0.05$; Mann-Whitney U-test) are indicated by positive and fractionated values, respectively. Blank cells: not statistically significant

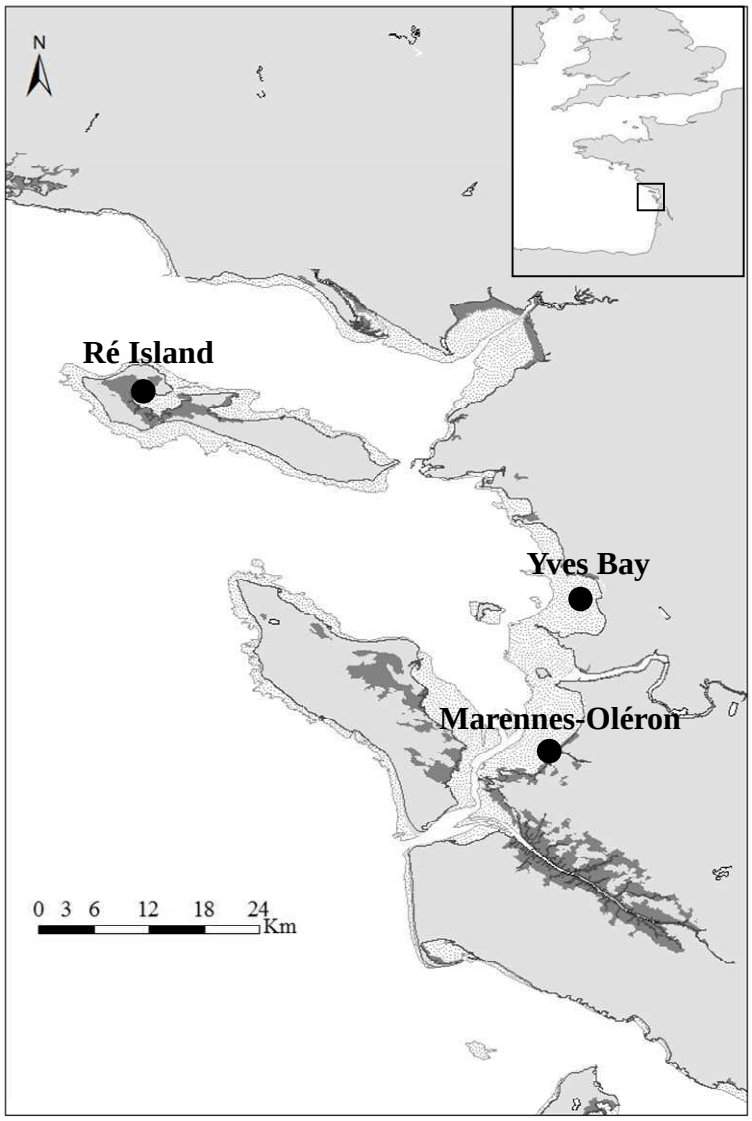

Fig. 1 Pertuis Charentais study site on the French Atlantic coast with the sampling stations: Ré Island, Yves Bay, and Marennes-Oléron Bay

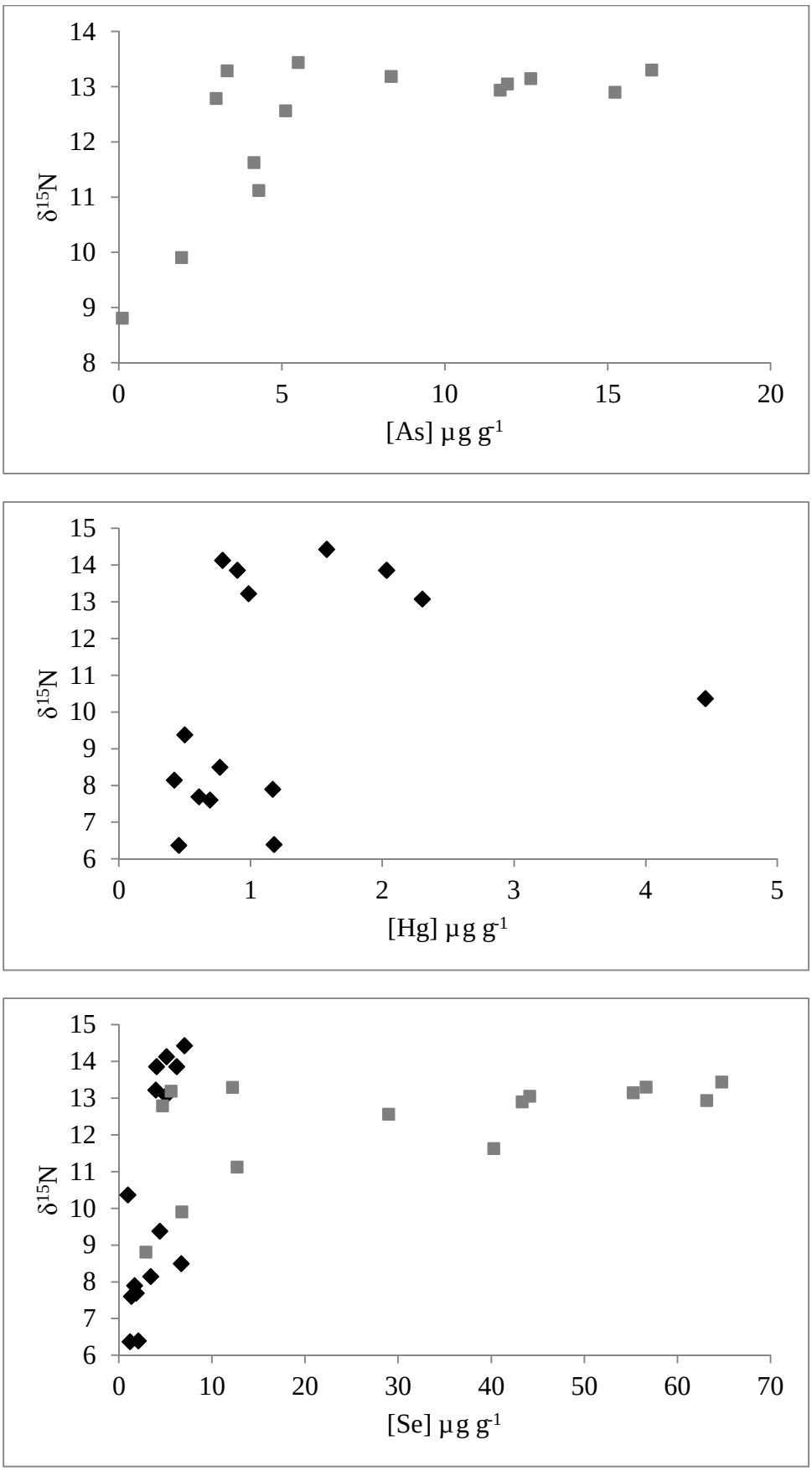

Fig. 2 $\delta^{15}N$ versus As, Hg and Se concentrations in the liver ■ (As, Se) and feathers ◆ (Hg, Se).

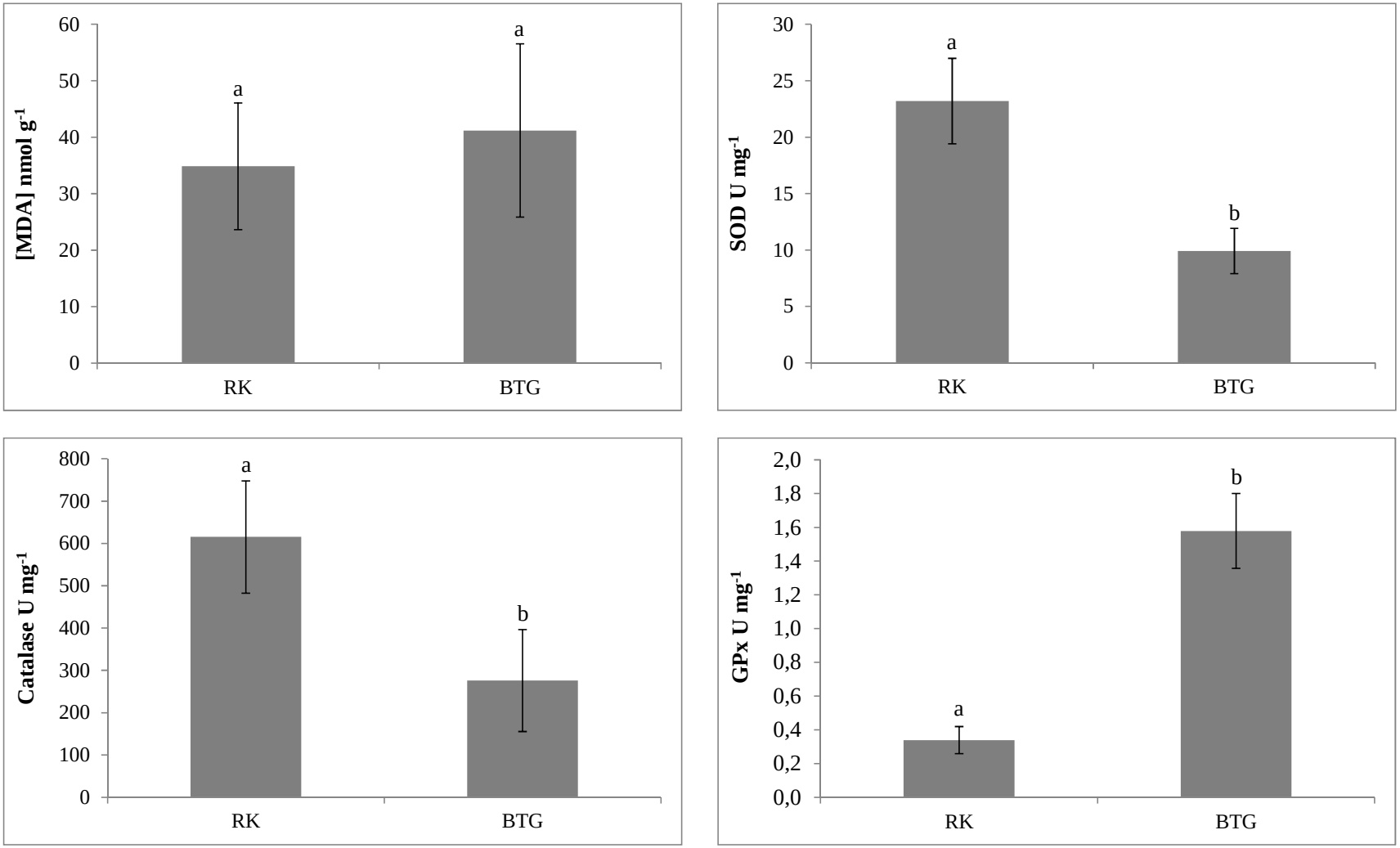

Fig. 3 Malondialdehyde concentrations (nmol MDA per g of tissue), superoxide dismutase (units of SOD per mg of proteins), catalase (units of catalase per mg of proteins) and glutathione peroxidase activities (units of GPx per mg of proteins) in liver of Red Knots (RK: Mean ± SD; n = 4) and Black-tailed Godwits (BTG: Mean ± SD; n = 3). Significant differences between species are indicated by letters at the level $\alpha = 0.05$ (Mann-Whitney U-test)

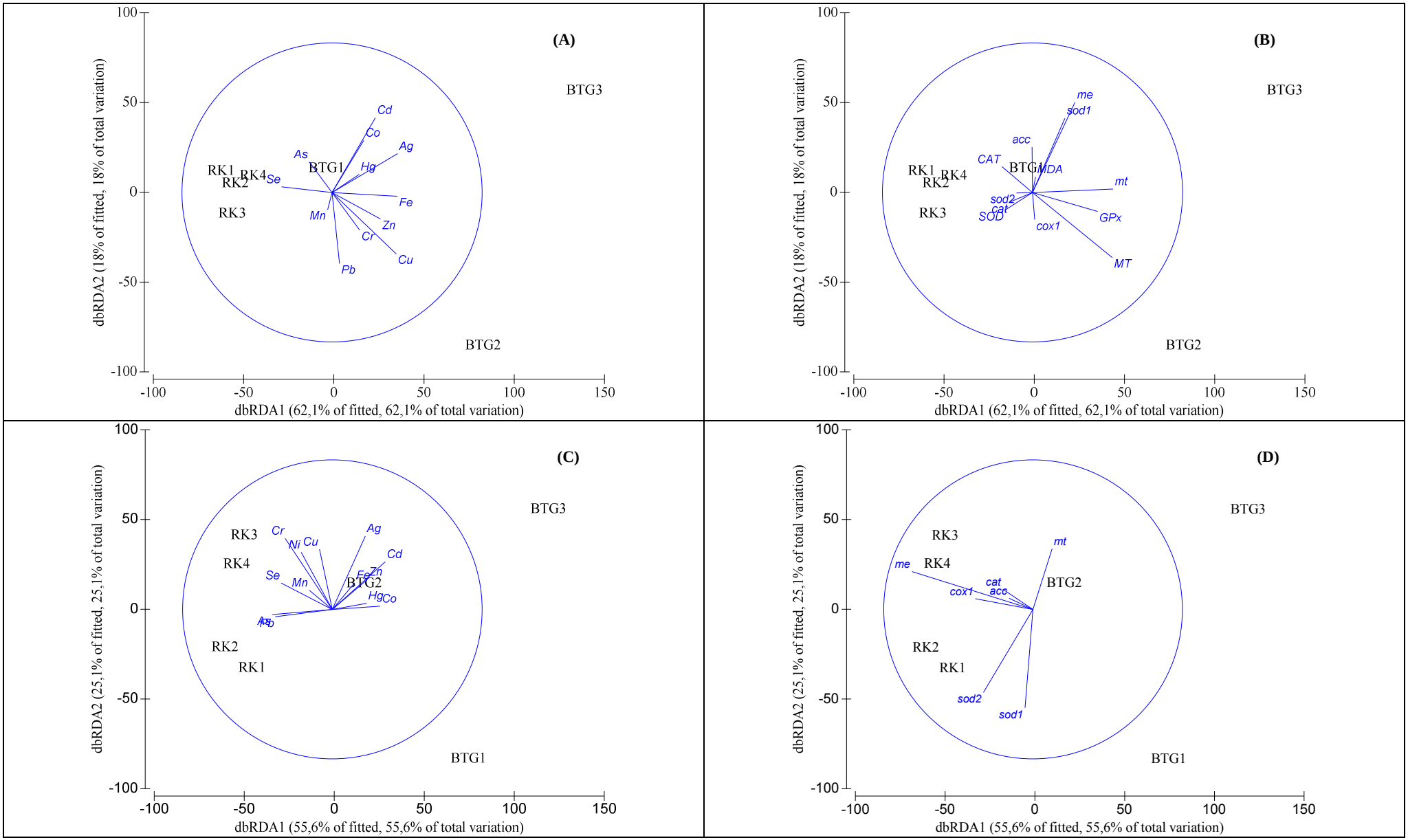

Fig. 4 Distance-Based Redundancy Analysis based on the trace elements concentrations in the liver (**A**-**B**) and kidneys (**C**-**D**) of the Red Knots (RK; n = 4) and the Black-tailed Godwits (BTG; n = 3). **(A-C)** Relation of the trace elements with the first two dimensions; **(B-D)** Relation of the mRNA expressions and the enzyme activities with the first two dimensions. Abbreviations: *cox1* - cytochrome C oxidase subunit 1; *acc* - acetyl-CoA carboxylase; *sod1* - superoxide dismutase (Cu/Zn); *sod2* - mitochondrial superoxide dismutase (Mn); *cat* - catalase; *mt* - metallothionein; *me* - NADP-dependent malic enzyme; CAT - catalase enzyme activity; SOD - superoxide dismutase enzyme activity; GPx - glutathione peroxidase; MDA - malondialdehyde

Supplementary material for the paper: “Evidence of species-specific detoxification processes for trace elements in shorebirds”

Magali LUCIA[a]*, Pierrick BOCHER[a], Richard P. COSSON[b], Carine CHURLAUD[a], Paco BUSTAMANTE[a]

[a] Littoral, Environnement et Sociétés (LIENSs), UMR 7266 CNRS-Université de La Rochelle, 2 rue Olympe de Gouges, 17000 La Rochelle, France.

[b] Mer Molécules Santé (MMS), Université de Nantes, EA 2663, 2 rue de la Houssinière, 44322 Nantes, France.

*Corresponding author E-mail: m.lucia33@laposte.net

CONTENT

Table S1 Primer pairs used to clone partial mRNA sequences of β-actin (*act*), acetyl-CoA carboxylase (*acc*), Cu/Zn superoxide dismutase (*sod1*), metallothionein (*mt*) and NADP-dependent malic enzyme (*me*) genes of the Red Knot

Gene name	Primers (5'-3')
act	TGACCCTGAAGTACCCCATTG[a]
	CTGCTTGCTGATCCACATCTG[b]
acc	GTCCTCCAAGCCAAGCAATGTG[a]
	GGCCTTGATCATGACAGGGTAGCC[b]
sod1	GCGCACCATGGTGGTCCATG[a]
	GTCTTCACCAGTTTAACTGATACTCA[b]
mt	TGGACCCCCAGGACTGC[a]
	CCGGCTATTTACAGGCGGA[b]
me	ATCAAGGCTATTGTGGTGACAG[a]
	ATTCTCTTGTGTCTCAGCCC[b]

[a] Forward primers
[b] Reverse primers

Table S2 Specific primers used for qPCR

Gene name	Accession number	Specific primers (5'-3')
act	JN122335	GTGCTGCTCACAGAGGC[a]
		GGCATGGGGAAGGGCATAA[b]
cox1	NC_009684	CCGACGATACTCGGACTACC[a]
		GGGCAGCCGTGGATTC[b]
acc	JN122336	GTCCTCCAAGCCAAGCAATGTG[a]
		GGCCTTGATCATGACAGGGTAGCC[b]
sod1	JN205797	GCGCACCATGGTGGTCCATG[a]
		GTCTTCACCAGTTTAACTGATACTCA[b]
sod2	EU598450	ACGCCGAGATCATGCAG[a]
		CGAAAGATTTGTCCAGAAGATGGT[b]
cat	EU598454	AGGCAAAAGTGTTTGAACACAT [a]
		ACAACATTGCATCCCGAATAAAG [b]
mt	JN205798	TGGACCCCCAGGACTGC[a]
		CCGGCTATTTACAGGCGGA[b]
me	JN122337	ATCAAGGCTATTGTGGTGACAG[a]
		ATTCTCTTGTGTCTCAGCCC[b]

Abbreviations: *act* - β-actin; *cox1* - cytochrome C oxidase subunit 1; *acc* - acetyl-CoA carboxylase; *sod1* - superoxide dismutase (Cu/Zn); *sod2* - mitochondrial superoxide dismutase (Mn); *cat* - catalase; *mt* - metallothionein; *me* - NADP-dependent malic enzyme. [a] Forward primers. [b] Reverse primers