Science of the Total Environment 423 (2012) 73–83

Contents lists available at SciVerse ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Insight on trace element detoxification in the Black-tailed Godwit (*Limosa limosa*) through genetic, enzymatic and metallothionein analyses

Magali Lucia [a,*], Pierrick Bocher [a], Richard P. Cosson [b], Carine Churlaud [a], Frédéric Robin [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, BP 92208, 44322 Nantes Cedex 3, France

ARTICLE INFO

Article history:
Received 18 November 2011
Received in revised form 3 February 2012
Accepted 3 February 2012
Available online 14 March 2012

Keywords:
Metals
Mercury
Bioaccumulation
Biomarker
Shorebird
Genomic expression

ABSTRACT

Trace element concentrations (Ag, As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, Zn) were investigated in the liver, kidneys, muscle and feathers of 31 black-tailed godwits (*Limosa limosa*) accidentally killed during catches by mist net in the Pertuis Charentais, Atlantic coast of France. Analyses of carbon and nitrogen stable isotope ratios were carried out in liver, muscle and feathers in order to elucidate dietary patterns and to determine whether differences in diet explained the variation in elemental uptake. This study also aimed to have a preliminary assessment of sub-lethal effects triggered by trace elements through the investigation of gene expressions by quantitative real-time PCR, antioxidant enzyme activities (catalase, superoxide dismutase, glutathione peroxidase), and metallothionein (MT) levels. The results showed that Cr and Ni concentrations in tissues of adults were lower than in juveniles in part because adults may have eliminated these trace elements through moulting. Except for Cd and Ni, trace element concentrations were negatively correlated to the body mass of godwits. Ag, As, Hg and Se concentrations were positively linked with the trophic position of birds. The diet could be considered as a fundamental route of exposure for these elements demonstrating therefore the qualitative linkage between dietary habits of godwits and their contaminant concentrations. Our results strongly suggest that even though trace element concentrations were mostly below toxicity threshold level, the elevated concentrations of As, Ag, Cd, Cu, Fe and Se may however trigger sub-lethal effects. Trace elements appear to enhance expression of genes involved in oxidative stress defence, which indicates the production of reactive oxygen species. Moreover, birds with the highest concentrations appeared to have an increased mitochondrial metabolism suggesting that the fight against trace element toxicity requires additional energetic needs notably to produce detoxification mechanisms such as metallothioneins.

1. Introduction

Coastal ecosystems rank among the most endangered ecosystems in the world due to human activities (Halpern et al., 2008; Vitousek et al., 1997). These ecosystems are mainly threatened by the reduction/degradation of habitats, excess exploitation of biological resources, invasive species and climate change (Thomas et al., 2004). The degradation and/or the alteration of the habitats are partly the result of the massive dispersion of toxic compounds generated by human activities in environments (Nriagu, 1996). Coastal ecosystems indeed constitute the ultimate destination of most of these compounds through erosion and drainage of rivers catchment areas.

The adverse impact of these compounds on wild populations and communities has been clearly documented (De Luca-Abbott et al., 2001; Scheuhammer, 1987; Spallholz and Hoffman, 2002). Among wild populations, birds exploiting intertidal system are particularly vulnerable to contaminants because they are relatively long-lived species that bioaccumulate contaminants throughout their whole life and feed at the top of their food chain (Burger, 1993). Furthermore, the loss or degradation of habitats anywhere along shorebird's flyway has triggered the decline of their population. Actually, on a worldwide scale, 48% of shorebird species are declining (Delany and Scott, 2006; Stroud et al., 2006).

Impact assessment of toxic compounds on wild populations at community scales by examination of population dynamics provides late-stage signals of the environment's degradation where contaminants have already overwhelmed the various defence mechanisms at lower levels of biological organization (i.e. sub-cellular response). In this context, the study of sub-lethal contaminant effects on wild populations is a major challenge for ecotoxicological approaches since its aim is to develop early-stage metrics of contamination on populations considered for ecological risk assessment (ERA) programs (Adams et al., 2001). These methodologies focus on the sub-organismal level of biological organization by searching for biomarkers that can be used as functional measures of exposure to stressors expressed as biochemical or physiological variations induced by anthropogenic perturbations (Adams et

* Corresponding author. Tel.: +33 5 46 45 72 17; fax: +33 5 46 50 76 63.
E-mail address: m.lucia33@laposte.net (M. Lucia).

0048-9697/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.scitotenv.2012.02.005

al., 2001; McCarty and Munkittrick, 1996). However, few studies have focused on cellular damages related to contaminant loads in birds. As an example, a study recently analysed the cellular effects of Cd on domestic ducks through experimentally enhanced contaminations (Lucia et al., 2009), shedding new light on the sub-lethal effects of Cd in birds. Nevertheless, the use of captive birds to assess what can happen in free-ranging birds is equivocal and even controversial (Piersma and van Gils, 2011). Thus, there is a great need to verify the consistency between experimental and field approaches.

The Atlantic coast of France appears as a central position on the East-Atlantic flyway of waterbirds between breeding sites in high arctic area and/or in Northern Europe; and Southern Europe and Western Africa used as wintering area (Delany et al., 2009). The Pertuis Charentais on the French central Atlantic coast is the major wintering site for shorebirds in France (Mahéo, 2010). The area is also used as a stopover during spring and fall migration (Delany et al., 2009). Most of the shorebirds feed directly on local large intertidal mudflats for refuelling during migratory events or to ensure a minimal body mass for winter survival (Alerstam et al., 2003; Alerstam and Lindström, 1990). However, this area is subject to trace element contaminations through river discharges (Pigeot et al., 2006) which lead to the subsequent bioaccumulation in macrofaunal community in general (Bustamante and Miramand, 2004, 2005), and in the prey of shorebirds in the study area. Consequently, birds could be exposed during this period of their life cycle to non-essential (and thus potentially toxic beyond a threshold) elements such as silver (Ag), cadmium (Cd), lead (Pb), mercury (Hg) and 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). The Black-tailed Godwit (*Limosa limosa*) is one of the most abundant shorebird species using the Pertuis Charentais to forage in winter or during migration (Triplet and Mahéo, 2007). Two subspecies were recorded in the study area with *L. l. islandica* wintering from September to March and *L. l. limosa* only present during fall and spring migrations (Robin, 2011). Because of its concentration on a restricted number of sites during migration, wintering and breeding period, this species is particularly sensitive to habitat loss, degradation and pollution of these areas (Gill et al., 2001; Groen and Hemerik, 2002; Lourenço and Piersma, 2008; Roodbergen et al., 2008; Schekkerman et al., 2009). The Black-tailed Godwit is now classified in decline throughout much of its range (Gill et al., 2007) and thus listed on Annex II/2 of the EU Birds Directive.

Henceforth, the current study had two objectives. First, this work aimed to assess the concentrations of the 13 trace elements cited above in the tissues of free-ranging godwits. Since ingestion of food is the main route of exposure for shorebirds, their trace element concentrations are linked to their feeding behaviour. Stable isotopes have been analysed in liver, muscle and ventral feathers to elucidate dietary patterns, and so determine whether differences in foraging strategy explained the variation in elemental uptake (Jardine et al., 2006). Secondly, this study aimed to realize a preliminary assessment of potential sub-lethal effects of trace elements on godwits at different cellular levels. Thus, we investigated 1) the expressions of nine genes involved in detoxification process, oxidative stress, lipogenesis, peripheral lipid transfer and in the control of energetic metabolism, 2) the activities of antioxidant enzymes (catalase, superoxide dismutase, glutathione peroxidase) and lipid peroxidation (malondialdehyde), and 3) the levels of metallothioneins which are involved in the homeostasis of essential metals such as Zn and in protection against toxic metals such as Cd.

2. Materials and methods

2.1. Study site and sampling

Thirty-one black-tailed godwits (*Limosa limosa*), accidentally dead during catches by mist net for ringing process, were collected between September 2007 and September 2010 in the Pertuis Charentais, Atlantic coast of France (Fig. 1). Birds were sampled at three Natural Reserves: Ré Island (n = 2), Yves marshes (n = 5) and Marennes-Oléron Bay (n = 24) which are submitted to different pollutant inputs (Luna-Acosta et al., 2010).

When it was possible during the dissection, sex and age classes (juvenile/adult) were determined for each individual. The liver, kidneys, pectoral muscle and ventral feathers were sampled, weighted (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 metallothionein (MT) analyses. Ventral feathers were washed to remove oil and dirt in a chloroform–methanol solution (2:1) in an ultrasonic bath for 2 min. Afterwards, they were rinsed in two consecutive pure methanol baths for a few seconds and dried at 40 °C for 48 h.

In order to specifically investigate genetic expressions in the liver, kidneys and muscle, and enzymatic activities in the liver, three godwits were sampled at Marennes-Oléron in September 2010. For these analyses, the liver, kidneys and muscle were immediately dissected on the fieldwork after their death and divided in two parts. The first part was frozen in liquid nitrogen immediately after the bird death and then stored at −80 °C for enzymatic and genetic analyses. The second part, including the ventral feathers, was stored at −20 °C and was submitted to the same treatment as previously described for trace element, stable isotope and MT determinations.

2.2. Trace element determination

Trace elements were determined in the liver, kidneys, muscle and the whole ventral feathers. Total Hg analyses were carried out with an Advanced Mercury Analyser (ALTEC AMA 254), on dried tissue aliquots ranging from 4 to 50 mg, weighed to the nearest 0.01 mg

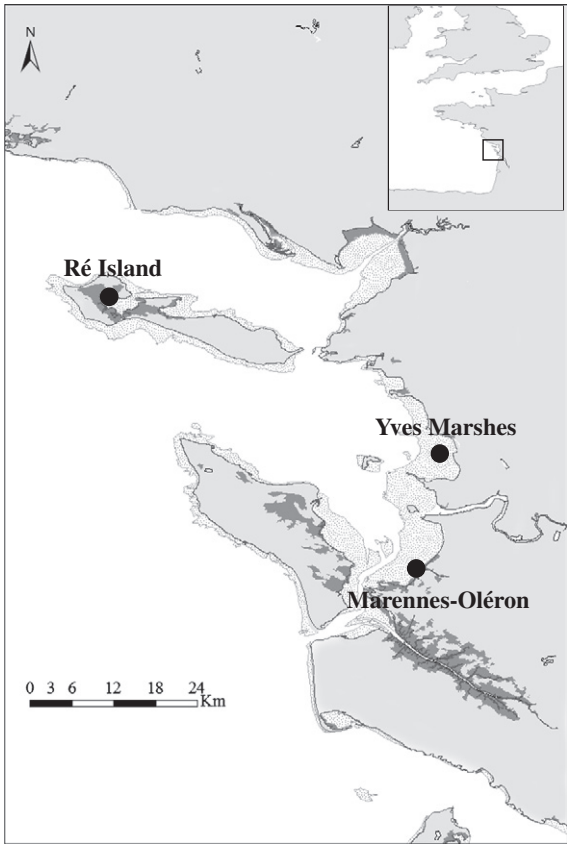

Fig. 1. Pertuis Charentais study site with the sampling stations: Ré Island, Yves marshes and Marennes-Oléron.

(Bustamante et al., 2006). For Hg determination, the metal was evaporated by progressive heating up to 800 °C, then held under oxygen atmosphere for 3 min, and finally amalgamated on gold net. Afterwards, the net was heated to liberate the collected Hg, which was measured by atomic absorption spectrophotometry. Mercury analyses were run according to a thorough quality control program including the analysis of a NRC reference material (lobster hepatopancreas TORT-2; National Research Council, Canada). Standard 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 ThermoFisherScientific XSeries 2 ICP-MS (Métian et al., 2008). 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) (except for the 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 completed to 50 ml (or 25 ml for the feathers) with milli-Q water. Three control samples (two Certified Reference Materials, CRMs, and one blank) treated and analysed in the same way as the samples were included in each analytical batch. 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, 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 the calculation of the means as half of the detection limit of the given element (e.g. value<0.02 was considerate as a concentration of 0.01 μg g^{-1} dw).

2.3. Nitrogen and carbon stable isotope analysis

The basic isotopic concept is that an animal's chemical composition is directly influenced by what it consumes (Michener and Kaufman, 2007). Consumers are enriched in ^{15}N relative to their food and consequently stable nitrogen isotope measurements ($\delta^{15}N$) serve as indicators of a consumer trophic position. By contrast, stable carbon signatures ($\delta^{13}C$) vary little along the food chain and, in the marine environment, $\delta^{13}C$ values are mainly used to indicate the foraging habitats of predators (Rubenstein and Hobson, 2004). The stable isotope method is based on time-integrated assimilated food. The isotopic signature of liver and muscle was respectively considered as representative of the isotopic niche of birds during weeks and months preceding sampling while the signature of feathers was representative of the moulting (inter-breeding) period (Cherel et al., 2008).

Cleaned feathers were chopped using surgical scissors and accurately weighed out to a range comprised between 0.200 and 0.500 ± 0.001 mg. Liver and muscle samples were also precisely weighed (0.200 to 0.500 ± 0.001 mg). All samples were placed in tin capsules for carbon and nitrogen stable isotope analysis and were 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 as per mil (‰) and are expressed in the δ unit notation as deviations from standards (Vienna Pee Dee Belemnite for $\delta^{13}C$ and N_2 in air for $\delta^{15}N$) following the formula:

$$\delta \text{ isotope} = \left(\frac{R_{sample}}{R_{standard}} - 1\right) * 1000$$

where δ isotope is the sample ratio (^{13}C or ^{15}N) relative to a standard (traceable to a primary international standard), and *R* is the ratio of heavy to light isotope ($^{13}C/^{12}C$ or $^{15}N/^{14}N$) in the *sample* or *standard*. $\delta^{13}C$ and $\delta^{15}N$ are reported relative to their primary international standard. The analytical precision of the measurements was <0.06‰ and <0.1‰ for carbon and nitrogen, respectively.

2.4. Metallothionein determination

Birds were analysed for their hepatic MT levels. Aliquots of approximately 100 mg were homogenized on ice in 6 ml 100 mM Tris buffer with β-mercaptoethanol at pH = 8.1 and then centrifuged (30000 g; 30 min; 4 °C). Before MT dosage, the supernatant aliquot was submitted to heat denaturation (95 °C; 15 min), placed on ice 10 min and centrifuged (10000 g; 10 min, 4 °C) to separate the heat-stable proteins from the denatured proteins. The supernatant stemming from the second centrifugation was frozen (−20 °C) until MT quantification.

Differential pulse polarographic analysis (DPP) was used to determine the amount of MTs in the heat-denatured soluble fraction. DPP is a technique based on –SH compound determination according to Brdička reaction (Brdička, 1933) as described by Thompson and Cosson (1984). Model 303A static mercury drop electrode was used. Certified rabbit liver MTs (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, a Hg working electrode, a Pt counter electrode, and an Ag/AgCl reference electrode. Results are expressed in μg of MTs per g of dry homogenized tissue (μg g^{-1} dw).

2.5. Sequencing of genes

Ten DNA fragments were searched for: β-actin (*act*), cytochrome c oxidase subunit 1 (*cox1*), acetyl-CoA carboxylase (*acc*), catalase (*cat*), Cu/Zn superoxide dismutase (*sod1*), superoxide dismutase 2 (*sod2*), metallothionein (*mt*), lipoprotein lipase (*lpl*), fatty acid synthase (*fas*) and NADP-dependent malic enzyme (*me*).

A quantity of 40 mg of fresh liver was homogenized to extract total RNAs using Absolutely Total RNA Miniprep kit (Agilent Technologies, USA), 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 (Agilent Technologies, USA), 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) or were qPCR primers obtained on duck *Cairina moschata* (Table 1). Amplified products were cloned into pGEM-T vector (Promega) and sequenced (Millegen, France). For superoxide dismutase 2 and cytochrome c oxidase subunit 1 primer pairs from *C. moschata* and *Anas platyrhynchos* were also used to amplify less than 200 bp of these genes. Cloning and sequencing demonstrated that primer pairs chosen in conserved regions of *C. moschata* and *A. platyrhynchos* could be used for *L. limosa* during qPCR analysis (Table 2).

2.6. Quantitative real-time PCR

Quantitative real-time PCR reactions were performed on the liver, kidneys and muscle in a Mx3005P (Agilent Technologies, USA) following the manufacturer's instructions (1 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

Table 1
Primer pairs used to clone partial mRNA sequences of β-actin (*act*), cytochrome C oxidase subunit 1 (*cox1*), acetyl-CoA carboxylase (*acc*), catalase (*cat*), Cu/Zn superoxide dismutase (*sod1*), Mn superoxide dismutase (*sod2*), metallothionein (*mt*), lipoprotein lipase (*lpl*), fatty acid synthase (*fas*) and NADP-dependent malic enzyme (*me*) genes.

Gene name	Primers (5′-3′)
act	TGACCCTGAAGTACCCCATTG[a]
	CTGCTTGCTGATCCACATCTG[b]
cox1	CCGACGATACTCGGACTACC[a]
	GGGCAGCCGTGGATTC[b]
acc	GTCCTCCAAGCCAAGCAATGTG[a]
	GGCCTTGATCATGACAGGGTAGCC[b]
cat	AAGATGTTGTTTTCACTGATGAG[a]
	ATCCATATCCATTCATATGGCC[b]
sod1	GCGCACCATGGTGGTCCATG[a]
	GTCTTCACCAGTTTAACTGATACTCA[b]
sod2	ACGCCGAGATCATGCAG[a]
	CGAAAGATTTGTCCAGAAGATGGT[b]
mt	TGGACCCCCAGGACTGC[a]
	CCGGCTATTTACAGGCGGA[b]
lpl	ATCCATATCCATTCATATGGCC[a]
	GTCCACCAGTCTGACCAGCTGAAG[b]
fas	ATAACTTGGAGTTCTTCCTAAC[a]
	GGAAGGATAGTTGCCTGATG[b]
me	ATCAAGGCTATTGTGGTGACAG[a]
	ATTCTCTTGTGTCTCAGCCC[b]

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

product template, 12.5 μL of Brilliant master mix including the SyberGreen I fluorescent dye (Agilent Technologies, USA), 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.

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

Gene name	Accession number/reference	Specific primers (5′-3′)
act	JF913946	CCAACTGGGATGACATGGAGAAG[a]
		CCAGAGGCATACAGGGACAA[b]
cox1	NC_009684[c]	CCGACGATACTCGGACTACC[a]
		GGGCAGCCGTGGATTC[b]
acc	JN122328	GTCCTCCAAGCCAAGCAATGTG[a]
		GGCCTTGATCATGACAGGGTAGCC[b]
sod1	JN205793	GCGCACCATGGTGGTCCATG[a]
		GTCTTCACCAGTTTAACTGATACTCA[b]
sod2	EU598450[c]	ACGCCGAGATCATGCAG[a]
		CGAAAGATTTGTCCAGAAGATGGT[b]
cat	JN122327	TGAAGTCACGCATGACATTACCC[a]
		TGGATGAAGGACGGAAACAACATT[b]
mt	JN205794	TGGACCCCCAGGACTGC[a]
		CCGGCTATTTACAGGCGGA[b]
me	JN122330	ATCAAGGCTATTGTGGTGACAG[a]
		ATTCTCTTGTGTCTCAGCCC[b]
lpl	JN122329	GCCGTAAGAACCGCTGC[a]
		AGTGCCATAGAGAGAGATCAGG[b]
fas	JF913947	GCTCCAAAGGCTCTGCG[a]
		AGCACAACAGGCATTTGCTC[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; *lpl* — lipoprotein lipase; *fas* — fatty acid synthase.
[a] Forward primers.
[b] Reverse primers.
[c] Accession number/reference of *C. moschata* and *A. platyrhynchos*.

2.7. Antioxidant enzymes and lipid peroxidation

Glutathione peroxidase (GPx), Superoxide dismutase (SOD), catalase (CAT) activities and malondialdehyde (MDA) concentrations were assayed in the livers of three godwits. GPx activity was determined according to the method of Paglia and Valentine (1967), using a glutathione peroxidase assay kit (RS504/RS505, RANDOX, France). GPx catalyses the oxidation of reduced glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH the oxidized glutathione (GSSG) is immediately converted to the reduced form with concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance was measured at 340 nm. The results are presented in units of GPx per mg of protein.

SOD activity was determined according to the method of Wooliams et al. (1983) and using a superoxide dismutase assay kit (SD125, RANDOX, France). This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye, assessed at 505 nm. The superoxide dismutase activity was then measured by the degree of inhibition of this reaction. One unit of SOD was that which causes a 50% inhibition of the rate of reduction of INT. The results are presented in units of SOD per mg of protein.

CAT activity was determined according to the method of Deisseroth and Dounce (1970) and using a catalase assay kit (CAT100, Sigma Aldrich, USA). Samples were mixed (v:v) with hydrogen peroxide. Hydrogen peroxide degradation kinetics were assessed at 280 nm. The results are expressed in units of CAT per mg of protein.

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

Lipid peroxidation levels were assessed via MDA contents determined using a commercially available MDA assay kit (MDA assay kit, Oxis International, USA). The method was based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA. The blue product was quantified by measuring absorbance at 586 nm (Gérard-Monnier et al., 1998). The results are presented in nmol of MDA per g of tissue.

Materials used to measure spectrophotometrically antioxidant enzymes activity and lipid peroxidation were UV microplates (Greiner Bio One, Germany) and a spectrophotometer (SAFAS Flx-Xenius, Monaco).

2.8. Statistical analysis

Normality and homogeneity of variance, necessary for the use of analysis of variance parametric test, were checked by Cochran C test. These assumptions were not achieved despite $\log_{10}(x+1)$ transformation. Non-parametric analysis of variance was thus applied to assess differences in trace element concentrations between tissues (liver, kidneys, muscle and ventral feathers), gender or age classes (Kruskal–Wallis and Mann–Whitney *U*-test, Statistica 7.1).

Spearman correlation test was applied to analyse if trace element were linked together in a same tissue and if MT levels, antioxidant activities and MDA were correlated to trace element concentrations. For each trace element, Spearman correlations have also been applied to investigate if concentrations were linked between tissues. Moreover, this test was applied to see if trace element concentrations were influenced by individual characteristic such as body weight.

3. Results

3.1. Concentrations of trace element in the tissues

Table 3 shows the mean concentrations and ranges of the 13 trace elements in four tissues of 31 black-tailed godwits sampled in the

Table 3
Trace element concentrations (Mean ± SD and range, μg g^{-1} dw) in the tissues of 31 black-tailed godwits from the Pertuis Charentais (Atlantic coast of France).

Element		Liver ($n=30$)	Kidneys ($n=30$)	Muscle ($n=31$)	Feathers ($n=30$)
Ag	Mean ± SD	4.01 ± 3.90^{a}	0.09 ± 0.15^{b}	0.05 ± 0.05^{b}	0.04 ± 0.02^{b}
	Min–max	0.11–12.6	<0.03–0.66	<0.02–0.18	<0.02–0.12
Cd	Mean ± SD	0.59 ± 0.77^{a}	3.70 ± 5.37^{a}	0.05 ± 0.07^{b}	0.02 ± 0.02^{b}
	Min–max	0.03–3.04	0.10–19.66	<0.02–0.22	<0.02–0.09
Hg	Mean ± SD	1.97 ± 1.69^{a}	1.43 ± 1.21^{a}	0.40 ± 0.33^{b}	1.11 ± 0.63^{a}
	Min–max	0.08–4.98	0.07–4.60	0.03–1.43	0.24–2.79
Pb	Mean ± SD	0.18 ± 0.26^{a}	0.29 ± 0.26^{a}	0.02 ± 0.01^{b}	1.13 ± 1.12^{c}
	Min–max	0.03–1.42	0.06–1.08	<0.02–0.05	0.18–5.76
As	Mean ± SD	3.25 ± 2.01^{a}	1.81 ± 0.85^{a}	1.97 ± 1.02^{a}	0.73 ± 0.47^{b}
	Min–max	<0.18–8.09	<0.35–3.51	<0.17–4.26	<0.2–2.04
Co	Mean ± SD	0.17 ± 0.09^{a}	0.34 ± 0.15^{b}	0.05 ± 0.03^{c}	0.09 ± 0.08^{c}
	Min–max	0.05–0.45	0.15–0.74	<0.02–0.13	0.02–0.35
Cr	Mean ± SD	0.68 ± 1.82^{a}	0.25 ± 0.46^{a}	0.39 ± 0.35^{b}	0.37 ± 0.34^{b}
	Min–max	<0.02–7.93	<0.03–2.06	0.05–1.41	0.07–1.39
Cu	Mean ± SD	231 ± 243^{a}	18 ± 10^{b}	41 ± 23^{a}	19 ± 4.5^{b}
	Min–max	10–804	10–56	20–119	11–34
Fe	Mean ± SD	2108 ± 1345^{a}	656 ± 183^{b}	386 ± 147^{bc}	235 ± 253^{c}
	Min–max	684–6449	359–1133	216–868	35–1082
Mn	Mean ± SD	8.2 ± 2.7^{a}	8.3 ± 5.7^{a}	2.0 ± 0.7^{b}	13 ± 10^{a}
	Min–max	4.4–15	4.3–34	1.3–4.9	1.4–43
Ni	Mean ± SD	1.43 ± 4.63^{a}	0.21 ± 0.21^{b}	0.21 ± 0.15^{b}	1.60 ± 2.10^{c}
	Min–max	<0.04–18.3	0.08–1.13	<0.04–0.65	0.30–8.80
Se	Mean ± SD	16 ± 11^{a}	11 ± 6.5^{a}	3.2 ± 1.3^{b}	1.9 ± 0.8^{c}
	Min–max	4.2–50	2.8–28	1.5–6.2	1.0–4.7
Zn	Mean ± SD	159 ± 75^{ac}	107 ± 39^{a}	57 ± 22^{b}	160 ± 18^{c}
	Min–max	56–230	67–244	35–117	129–227

Significant differences between tissues for each metal are indicated by letters at the level $\alpha = 0.05$ (Kruskal–Wallis test).

Pertuis Charentais. Trace element concentrations strongly varied among tissues. The liver and kidneys displayed elevated concentrations of non-essential elements, especially of Ag and Cd. For example, kidneys showed Cd concentrations reaching up 20 μg g^{-1} dw in one individual. Moreover, 11 birds had values above 3 μg g^{-1} in this tissue. In the case of Ag, 13 individuals had values above 5.0 μg g^{-1} in the liver. In comparison, Hg concentrations were relatively low. Muscle showed the lowest Hg concentrations compared to the other tissues whose concentrations ranged between 1.1 ± 0.6 and 2.0 ± 1.7 μg g^{-1}. However, Hg concentrations around 5.0 μg g^{-1} were recorded in the liver of six individuals displayed and in the kidneys of one bird. Overall, concentrations of Pb were low in godwit tissues. Nevertheless, feathers appeared to have the highest Pb concentrations among all the considered tissues.

Concerning essential elements, elevated concentrations have been found for As, Cu, Fe and Se (Table 3). Nonetheless, essential elements concentrations greatly varied among tissues. Thus, As concentrations were in the same range for liver, kidneys and muscle but lower in feathers. The highest concentrations of Cu and Fe were found in the liver. Kidneys, as liver, had also elevated concentrations for Se.

For each trace element, correlations between tissue concentrations were investigated. Ag, As, Cd, Co, Cu, Fe, Mn and Zn concentrations were positively correlated between internal tissues but not between internal tissues and ventral feathers (Spearman correlation test, $p<0.05$). In contrast, Hg concentrations were positively correlated between all the tissues considered, as well as Se concentrations. Pb concentrations in the muscle were also linked to concentrations in the feathers ($R=0.39$). No significant correlations among tissues were found for Cr, as well as for Ni.

Many correlations appeared between trace elements in each of the four tissues studied (Table 4). The correlations between trace element concentrations appeared however to vary in regard of tissues, especially for feathers. This result can be particularly observed for correlations between Cd and the other trace elements.

3.2. Influence of biometrics characteristics on trace element concentrations

The sex of 23 birds was successfully determined during dissections. No discrepancies were observed between males ($n=11$) and females ($n=12$) for most of the considered trace elements, with the exception of Ni and Se in feathers. Indeed, females displayed significantly higher concentrations than males for Se (2.1 ± 0.9 *vs* 1.5 ± 0.3 μg g^{-1} dw, respectively; Mann–Whitney *U*-test, $p=0.030$). On the contrary, males had significantly higher Ni concentrations than females (0.7 ± 0.5 *vs* 2.4 ± 2.9 μg g^{-1} dw, respectively; Mann–Whitney *U*-test, $p=0.049$).

When birds were categorized in two age classes (juveniles and adults), no differences were observed in muscle and kidneys. However, juveniles displayed higher Cr concentrations in liver than for adults (1.4 ± 2.6 *vs* 0.1 ± 0.1 μg g^{-1} dw, respectively; Mann–Whitney *U*-test, $p=0.025$) and Ni in feathers (2.6 ± 2.7 *vs* 0.7 ± 0.5 μg g^{-1} dw, respectively; Mann–Whitney *U*-test, $p=0.018$).

Trace element concentrations were negatively correlated to the body weight of the birds which was on average 245 ± 64 g. The increase of Pb concentrations in the liver, muscle and ventral feathers were correlated to a decrease in body weight ($R=-0.61$, $R=-0.85$, $R=-0.40$, respectively, $p<0.05$, Spearman correlation test). The same results have been observed for Hg (liver: $R=-0.62$, kidneys: $R=-0.63$, feathers: $R=-0.56$), Ag (liver: $R=-0.63$, kidneys: $R=-0.50$), As (liver: $R=-0.49$), Cr (liver: $R=-0.47$), Zn (liver: $R=-0.70$, kidneys: $R=-0.65$, muscle: $R=-0.85$), Co (liver: $R=-0.69$, kidneys: $R=-0.40$, muscle: $R=-0.49$), Cu (liver: $R=-0.63$, kidneys: $R=-0.66$, muscle: $R=-0.75$), Fe (liver: $R=-0.62$, muscle: $R=-0.87$), Mn (liver: $R=-0.40$, kidneys: $R=-0.43$, feathers: $R=-0.52$), and Se (liver: $R=-0.56$, kidneys: $R=-0.48$). In contrast, no significant correlations were found between Cd and Ni concentrations and body weight.

3.3. Influence of trophic position and feeding habitat

The relation between the trophic position assessed through the determination of $\delta^{15}N$ and trace element concentrations of godwits was studied in three tissues: liver, muscle and feathers (Fig. 2). Ag, As, Hg and Se concentrations increased in the three tissues with the trophic level (Ag: liver $R=0.46$, muscle $R=0.54$, feathers $R=0.42$; As: liver $R=0.45$, muscle $R=0.70$, feathers $R=0.48$; Hg: liver $R=0.70$, muscle $R=0.62$, feathers $R=0.40$; Se: liver $R=0.62$, muscle $R=0.64$, feathers $R=0.44$, Spearman correlation test, $p<0.05$).

Table 4
Correlation between trace elements in the liver, kidneys, muscle and feathers of Black-tailed Godwit ($p<0.05$; Spearman correlation test).

Elements	Liver	Kidneys	Muscle	Feathers
Ag	+As, +Co, +Cu, +Fe, +Hg, +Ni, +Pb, +Se, +Zn	+Co, +Cu, +Hg, +Mn, +Se, +Zn	+As, +Co, +Cu, +Fe, +Se, +Zn	+Cd, +Se
Cd		+Fe	+Co	+Ag, +Co, +Ni, +Pb
Hg	+Ag, +As, +Co, +Cu, +Fe, +Pb, +Se, +Zn	+Ag, +Co, +Cu, +Fe, +Mn, +Se, +Zn	+As, +Co, +Cu, +Fe, +Pb, +Se, +Zn	+Pb
Pb	+Ag, +As, +Co, +Cu, +Fe, +Hg, +Mn, +Ni, +Se, +Zn		+Co, +Cu, +Fe, +Hg, +Se, +Zn	+Cd, +Co, +Cr, +Fe, +Hg, +Mn, +Ni
As	+Ag, +Co, +Cu, +Hg, +Pb, +Se, +Zn	+Co, +Se	+Ag, +Cu, +Fe, +Hg, +Se, +Zn	+Se
Co	+Ag, +As, +Cr, +Cu, +Fe, +Hg, +Mn, +Ni, +Pb, +Se, +Zn	+Ag, +As, +Cu, +Hg, +Mn, +Se, +Zn	+Ag, +Cd, +Cu, +Fe, +Hg, +Pb, +Se, +Zn	+Cd, +Cr, +Fe, +Mn, +Ni, +Pb
Cr	+Co, +Ni		+Ni	+Co, +Fe, +Mn, +Ni, +Pb
Cu	+Ag, +As, +Co, +Fe, +Hg, +Pb, +Se, +Zn	+Ag, +Co, +Hg, +Mn, +Se, +Zn	+Ag, +As, +Co, +Fe, +Hg, +Pb, +Se, +Zn	
Fe	+Ag, +Co, +Cu, +Hg, +Mn, +Ni, +Pb, +Se, +Zn	+Cd, +Hg	+Ag, +As, +Co, +Cu, +Hg, +Pb, +Se, +Zn	+Co, +Cr, +Mn, +Pb
Mn	+Co, +Fe, +Pb, +Se, +Zn	+Ag, +Co, +Cu, +Hg, +Se, +Zn		+Co, +Cr, +Fe, +Ni, +Pb
Ni	+Ag, +Co, +Cr, +Fe, +Pb		+Cr	+Cd, +Co, +Cr, +Mn, +Pb
Se	+Ag, +As, +Co, +Cu, +Fe, +Hg, +Mn, +Pb, +Zn	+Ag, +As, +Co, +Cu, +Hg, +Mn, +Zn	+Ag, +As, +Co, +Cu, +Fe, +Hg, +Pb, +Zn	+Ag, +As
Zn	+Ag, +As, +Co, +Cu, +Fe, +Hg, +Mn, +Pb, +Se	+Ag, +Co, +Cu, +Hg, +Mn, +Se	+Ag, +As, +Co, +Cu, +Fe, +Hg, +Pb, +Se	

For other trace elements, this relation was only observed for Co in liver ($R=0.38$) and muscle ($R=0.48$), and for Cd in feathers ($R=0.44$).

The relation between the foraging habitats of birds ($\delta^{13}C$) and their trace element concentrations was also investigated in the three tissues. In the liver, a significant negative correlation appeared between Cd concentrations and $\delta^{13}C$ ($R=-0.40$, Spearman correlation test, $p<0.05$). In the case of muscle, Ag, As and Se concentrations significantly increased with $\delta^{13}C$ ($R=0.68$, $R=0.53$ and $R=0.41$, respectively). For the ventral feathers, the same pattern was observed between As and Se concentrations and $\delta^{13}C$ ($R=0.41$, $R=0.39$, respectively).

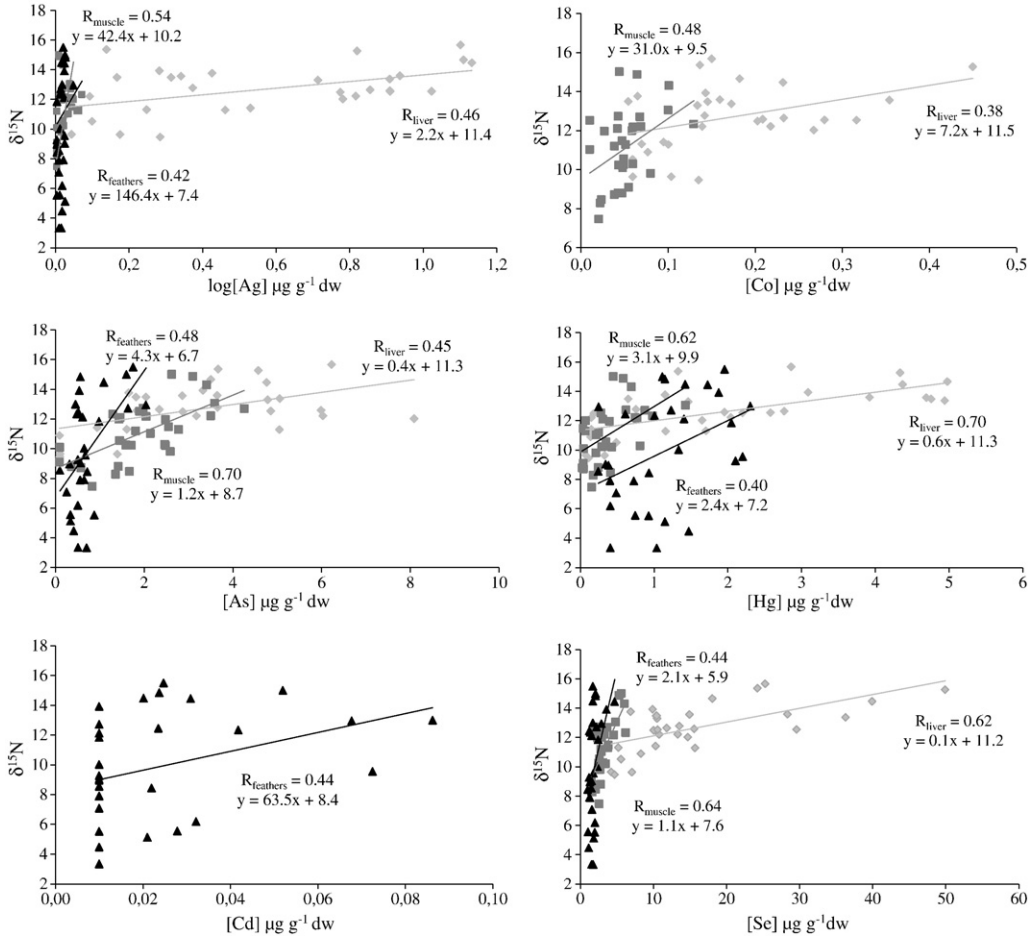

Fig. 2. Significant positive relationship between trophic level ($\delta^{15}N$) and Ag, As, Cd, Co, Hg and Se concentrations in liver ♦ (Ag, As, Co, Hg, Se), muscle ■ (Ag, As, Co, Hg, Se) and feathers ▲ (Ag, As, Cd, Co, Hg, Se). Ag concentrations were expressed in log. Correlation coefficients and equations of the trend curves were indicated for each tissue and trace element.

3.4. Trace element detoxification

Among the relationships between trace elements, Hg and Se were found to be positively linked in liver, kidneys and muscle (Table 4). The Se:Hg molar ratios were calculated in liver of godwits. The ratios always exceeded 1 for all the birds and ranged between 5.6 and 183.2.

Metallothionein (MT) concentrations in the liver of godwits were on average 7615 ± 4340 μg g^{-1} dw, showing an important variability among individuals. With the exception of Fe and Ni, MT concentrations were positively correlated with almost all trace elements studied (Ag: $R = 0.63$; As: $R = 0.46$; Hg: $R = 0.52$; Pb: $R = 0.56$; Cr: $R = 0.52$; Cd: $R = 0.56$; Mn: $R = 0.49$; Co: $R = 0.76$; Cu: $R = 0.65$; Zn: $R = 0.71$; Se: $R = 0.53$, Spearman test, $p < 0.05$).

Results for genetic and enzymatic analyses were obtained for three individuals from one site (Marennes-Oléron Bay). Two out of the three birds, BTG2 and BTG3, appeared to display lower SOD, CAT and GPx activities in the liver compared to BTG1 (Fig. 3). MDA levels were lower than in BTG1. It is also important to notice that BTG2 and BTG3 displayed higher MT levels than BTG1 (BTG2: 12 249 μg g^{-1}; BTG3: 12 377 μg g^{-1}; and BTG1: 2 928 μg g^{-1}). This assessment was put in regard of the trace element concentrations in the liver. It appeared that BTG2 and BTG3 were often above or even twice hepatic concentrations of BTG1 for most of the elements, except for Ni, Pb in BTG3, and Cd and Co in BTG2. For example, Hg concentrations were 1.2 and 1.7 μg g^{-1} dw, for BTG2 and BTG3 respectively, and 0.8 μg g^{-1} dw for BTG1 (Table 5). Concentrations of As were 5.1 and 8.1 μg g^{-1} dw, for BTG2 and BTG3 respectively, and 3.7 μg g^{-1} dw for BTG1. Zn concentrations reached 241 and 227 μg g^{-1} dw in BTG2 and BTG3 respectively, but 107 μg g^{-1} dw in BTG1 liver. Cd concentrations of BTG3 were also higher than BTG2 and BTG1 (2.4, 0.2 and 0.3 μg g^{-1} dw, respectively) as well as Ag concentrations (5.1, 1.9 and 1.4 μg g^{-1} dw, respectively).

During this study, a partial β-actin cDNA of 887 bp was sequenced. The corresponding protein (295 amino acids (aa)) presented high similarities with the Wild Turkey (*Meleagris gallopavo*) β-actin (100% identity). In the case of acetyl-CoA carboxylase, the fragment that was sequenced (245 bp, 81 aa) corresponds to acetyl-CoA carboxylase of the Greylag Goose (*Anser anser*; 98% identity) and Wild Turkey (98% identity). For the sequenced catalase gene (492 bp; 163 aa), high similarity was found with the corresponding gene of the Red Junglefowl (*Gallus gallus*; 96% identity) and Budgerigar (*Melopsittacus undulates*; 98% identity). The partial Cu/Zn superoxide dismutase cDNA sequenced (273 bp, 39 aa) presented high similarities with the Budgerigar (97% identity) and the metallothionein cDNA sequenced (220 bp, 61 aa) with the Zebra Finch (*Taeniopygia guttata*; 80% identity). The sequenced lipoprotein lipase (713 bp, 237 aa) and fatty acid synthase (410 bp, 136 aa) correspond respectively to those of the Muscovy Duck (*C. moschata*; 98% identity) and Red Junglefowl (93% identity). The last gene sequenced, NADP-dependent malic enzyme (215 bp, 71 aa), presented high similarities with malic enzyme of the Muscovy Duck (100% identity).

Expressions of nine genes involved in mitochondrial metabolism, lipogenesis, peripheral lipid transfer, defence against oxidative stress and detoxification were studied in the three individuals (Table 6). Differences occurred in the gene expressions in regard to tissues (liver, muscle and kidneys). Indeed, muscle displayed higher differential expressions compared to kidneys for *cox1*, *sod1*, *cat* and *lpl* (Kruskal–Wallis test, $p < 0.03$). Moreover, kidneys demonstrated lower *mt* gene expression compared to liver ($p = 0.02$). Although no correlations could be obtained between trace element concentrations and differential gene expressions, BTG1 often demonstrated lower gene expressions in the liver and muscle than BTG2 and especially BTG3 for genes involved in mitochondrial metabolism (*cox1*), defence against oxidative stress (*sod1*, *sod2*, *cat*) and detoxification (*mt*) which corroborate the trends observed for MT protein levels. Moreover, BTG2 and BTG3 displayed higher expression of *fas* in the liver compared to BTG1.

4. Discussion

4.1. Trace element distribution

Many studies have used feathers to study trace element concentrations of birds in part because this non-invasive method allows a large sampling and therefore studies of bird populations (e.g., Burger and Gochfeld, 2004). Some authors have suggested that feathers are useful indicators of trace element contamination because the proportion of the body burden stored in the feathers is relatively constant for most elements (Burger, 1993). In the current study, Hg, Pb and Se feather concentrations have appeared good indicators of internal tissue concentrations. Nonetheless, kidneys and liver showed high Ag, As and Cd concentrations not reflected in the feathers which often displayed concentrations below quantification limits. Kidneys and liver are considered as important organs involved in detoxification processes. Consequently, these organs store high levels of non-essential elements such as Cd in vertebrate top predators (Arai et

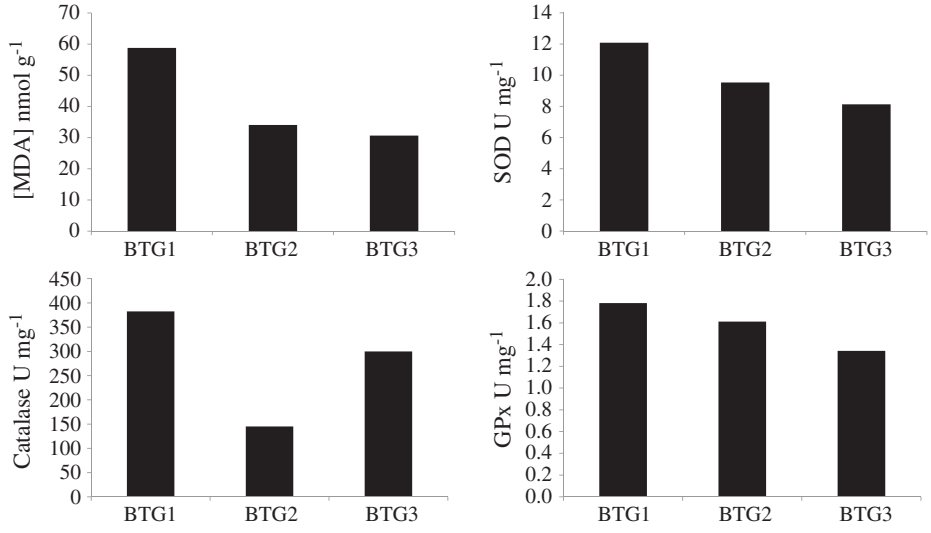

Fig. 3. Malondialdehyde concentrations (nmol per g of tissue), superoxide dismutase, catalase and glutathione peroxidase activities (units per mg of proteins) in liver of three godwits: BGT1, BTG2 and BTG3.

Table 5
Trace element concentrations (μg g^{-1} dw) in the liver of 3 black-tailed godwits from the Pertuis Charentais: BTG1, BTG2 and BTG3.

Element	BTG1	BTG2	BTG3
Ag	1.4	1.9	5.1
Cd	0.3	0.2	2.4
Hg	1.2	1.2	1.6
Pb	0.27	0.26	0.10
As	3.7	5.1	8.1
Co	0.14	0.10	0.27
Cr	0.07	0.15	0.12
Cu	84	516	334
Fe	1070	2390	3047
Mn	7.0	11.4	10.1
Ni	0.02	0.02	0.02
Se	13.6	15.7	14.7
Zn	107	241	227

al., 2004; Gallien et al., 2001; Stewart et al., 1996). The lower trace element concentrations in feathers (Ag, As, Cd) may also be the result of moulting. Indeed, the rate and extent of this mechanism provides an excretory outlet and therefore a detoxification process for trace elements (Elliott and Scheuhammer, 1997).

4.2. Trace element concentrations with respect to individual characteristics

Sex and age of vertebrate top predators are known to be involved in the variation of tissue trace element concentrations (Gochfeld et al., 1996; Kojadinovic et al., 2007a,b). Nevertheless, this effect is often dependent on the trace element studied. Our results have demonstrated the complex relationship between elemental uptake and individual characteristics. For example, Cr and Ni concentrations were higher in the liver and feathers of juveniles, respectively, compared to adults. This pattern could be the consequence of the balance between elemental uptake during feather's formation and elemental removal during the moult. Since trace element concentrations in feathers correspond in part to the dietary input since the last moult, their analysis in ventral feathers of juvenile birds reflects therefore maternal deposition into the eggs prior to laying and the dietary elemental intake since hatching (Donaldson and Braune, 1999). The concentrations of Cr and Ni in adult feathers were lower than in young birds because these elements may have been transferred into their plumage and subsequently eliminated through moulting.

4.3. Relation between trace element concentrations, trophic level and feeding habitat

The knowledge of the feeding ecology of birds is essential to understand contaminant pathways in ecosystems. Conventional approaches for quantifying dietary composition and determining shorebird foraging zones used traditionally field observation and gut or faeces content analyses (Quaintenne et al., 2010; Weber and Haig, 1997). Nevertheless, these approaches are difficult to obtain over long periods and on a fairly large number of individuals. To overcome this problem, stable isotopes of carbon and nitrogen are currently used to examine the trophic relationships and/or origins of prey to elucidate broad-scale, inter- and intraspecific dietary patterns, and so to determine whether differences in foraging strategy explain the variation in elemental uptake (Jardine et al., 2006; Michener and Kaufman, 2007). The current study investigated inter-individual variations in diets of godwits that could explain exposure to contaminants. Concentrations of four trace elements in the liver, muscle and feathers including Ag, As, Hg and Se increased with $\delta^{15}N$. Because of the importance of diet as a route of exposure for trace elements, particularly for Hg and Se (Hall et al., 1997), this result demonstrates the qualitative linkage between dietary habits of godwits and their contaminant concentrations. Variability in Ag, As, Hg and Se concentrations were particularly high in this species. Godwits periodically switch diets in response to changes in habitat or prey availability (e.g. migration, breeding period; Gill et al., 2001; Perez-Hurtado et al., 1997; Roodbergen et al., 2008) clearly leading to drastic change in trace element exposure. Nonetheless, the significant relationships between $\delta^{15}N$ and these elements suggest that some of these variations can be linked to dietary specialization with individuals that fed higher trophic status prey experiencing higher Ag, As, Hg and Se burdens. Bearhop et al. (2000) found a similar pattern when they investigated the influence of trophic status in the bioaccumulation of Hg in blood and feathers of Great Skua (*Catharacta skua*) chicks. In parallel, the increase of feather Cd concentrations with $\delta^{15}N$ could be the result of higher trophic level prey items accumulating higher Cd directly from the environment rather than through the food chain.

Stable-carbon signatures ($\delta^{13}C$) vary little along the food chain and, in the marine environment, $\delta^{13}C$ values are mainly used to indicate the foraging habitats of predators (Rubenstein and Hobson, 2004). A clear positive relationship was observed between Ag, As and Se concentrations and $\delta^{13}C$ demonstrating that individuals feeding on coastal prey are more likely to be contaminated with these elements than birds feeding on more terrestrial ones. Our results highlight the inter-individual divergence of feeding behaviours within this shorebird species that could partly explain discrepancies of trace element bioaccumulation between individuals.

4.4. Toxicity significance

This study is one of the first investigating trace element levels of one shorebird species. Concentrations of Cd, Cu, Hg, Ni, Pb and Zn observed in feathers were in accordance with a previous study that measured elemental levels in wing feathers and eggs of godwits present in their breeding ground in The Netherlands (Roodbergen et al., 2008). Generally, most of trace elements were below concentrations of toxicological concern. However, some individuals reached high values of Ag, As, Cd,

Table 6
Differential gene expressions compared to β-actin in liver, muscle and kidney from three godwits: BTG1, BTG2 and BTG3 sampled at Marennes-Oléron Bay (Pertuis Charentais, Atlantic coast of France) in September 2010.

		BTG1			BTG2			BTG3		
Functions	Genes	Liver	Muscle	Kidney	Liver	Muscle	Kidney	Liver	Muscle	kidney
Mitochondrial metabolism	*cox1*	0.001	0.07	0.005	0.005	0.11	0.001	0.005	0.07	0.001
Lipogenesis	*me*	0.06	0.02	0.006	0.06	1.8	0.04	1.40	0.11	0.02
	acc	0.15	0.09	0.01	0.11	0.16	0.01	0.330	0.08	0.01
	fas	0.08	0.01	0.001	0.23	0.06	0.003	0.55	0.01	0.001
Peripheral lipid transfer	*lpl*	0.04	0.99	0.004	0.03	1.9	0.02	0.03	0.93	0.01
Oxidative stress	*sod1*	0.002	0.006	0.0002	0.002	0.03	0.0001	0.011	0.01	0.0001
	sod2	0.00002	0.003	0.0004	0.00005	0.02	0.0004	0.0001	0.006	0.0001
	cat	0.005	0.96	0.001	0.002	4.3	0.0001	0.023	2.4	0.002
Detoxification	*mt*	6.6	0.58	0.6	164	77	9	347.3	18	7

Cu, Fe and Se in the liver and kidneys. In a review of the literature, Eisler (1996) reported lower to similar hepatic Ag levels in diving ducks from the San Francisco Bay. This review also reported that Common Eider (*Somateria mollissima*) from contaminated area in Norway reaching hepatic concentration of 44 μg g^{-1} dw appeared outwardly unaffected. Although Ag and its compounds are not known to be mutagenic, teratogenic, or carcinogenic, studies on humans and domestic animals suggested growth retardation, liver and kidney damage at elevated levels (Eisler, 1996). Godwits from the Pertuis Charentais also displayed high Ag concentrations in their liver but the assessment on their health is difficult because of the lack of studies of Ag toxicity on free-ranging birds. Highly toxic inorganic As found in some seabirds may act as an endocrine disruptor, induce the death of an individual, trigger sub-lethal effects, or disrupt reproduction (Eisler 1994; Kunito et al. 2008). As concentrations in living organisms are known to be usually low (<1 μg g^{-1} ww, approximately 5 μg g^{-1} dw; Braune and Noble, 2009). Thirty percent of godwits reached or were above this level but below values known to produce direct toxic effects (i.e., 50 μg g^{-1} dw) in seabirds (Neff, 1997). Generally, most of this element is accumulated under its organic forms (Neff, 1997). The current study only gave the total As concentrations of godwits. Consequently, this element could not be considered as a threat for the survival of birds but could be possibly involved in sub-lethal effects. Cd concentrations in kidneys were elevated and this metal is known to trigger sub-lethal effects in birds (Lucia et al., 2009). Indeed, previous experimental studies have demonstrated that these concentrations were associated with increased functioning of mitochondrial metabolism, and thus stronger energetic needs, as well as oxidative stress (Lucia et al., 2009, 2010). Even if Cu and Fe are essential elements and therefore regulated by homeostatic mechanism, birds could be submitted to deleterious effects at high concentrations. Our results were in the range of hepatic Cu concentrations (i.e., 187 to 323 μg g^{-1}) reported to trigger acute Cu poisoning in Canada Goose (*Branta canadensis*) (Henderson and Winterfield, 1975). However, this threshold should be treated with caution. Indeed, herbivorous birds are naturally exposed to much lower Cu concentrations than insectivores and are likely to be more sensitive to Cu. This is particularly true for godwits, which feed on benthic invertebrates in estuaries that could have high Cu concentrations even when collected from pristine sites. Mean hepatic Fe concentrations were particularly high in some individuals reaching approximately twice the levels measured in Double-Crested Cormorant (*Phalacrocorax auritus*) and Herring Gull (*Larus argentatus*) from the Canadian Atlantic coast (Elliott et al., 1992).

Among all the elements, Se is considered as highly toxic to living organisms depending upon its chemical forms ingested (Stewart et al., 1999). Se has the property to react with thiols which generate reactive oxygen species (Spallholz and Hoffman, 2002). Based on a review of the literature, Outridge et al. (1999) suggested that hepatic Se concentrations > 10 μg g^{-1} dw are associated with reduced adult weight gain, lower reproductive success in breeding female and increased teratogenesis in their offspring. Moreover, Se concentrations exceeded 33 μg g^{-1} dw could be considered harmful to the health of birds (Heinz, 1996). Hepatic Se concentrations exceeded 10 μg g^{-1} dw in 75% of the females and 73% of all the birds (males + females) sampled in the current study and 10% of all of the birds analysed had Se concentrations superiors to 33 μg g^{-1} dw. Skorupa et al. (1996) suggested higher thresholds. These authors noted that the median background liver concentration for insectivores was 8.2 μg g^{-1} and suggested an effect threshold of 30 μg g^{-1} in the liver. They also cautioned that liver Se is a poor indicator of Se poisoning in birds. However, all the tissues have reached important Se concentrations in the current study. In contrast, Hg concentrations were relatively low except in the kidneys of some individuals. At high exposures, Se and Hg can each be individually toxic, but evidence supports that their co-accumulation reduces each other's toxic effects (Peterson et al., 2009). Hg is demethylated in the presence of Se and stored as non-toxic insoluble tiemannite granules (Nigro and Leonzio, 1996). A Se:Hg molar ratio approaching 1 strongly suggest the existence of mercuric selenide (HgSe). Hg and Se concentrations were highly correlated in internal tissues of godwits and molar ratios always exceeded 1. Presumably, godwits are protected against Hg toxicity but this result raises concern about the surplus of Se and its possible toxicity for this species.

In this study, birds were submitted to a decrease of their body weight with the increase of elemental concentrations. Trace elements may trigger long-term toxic effects such as stronger energetic needs to fight against the production of reactive oxygen species.

4.5. Metallothioneins

The important significant positive relationships between MT, low molecular weight and cysteine-rich proteins, concentrations and trace element concentrations detected in the current study could imply common uptake or similar regulation and detoxification mechanisms. One of the possible detoxification mechanisms which would explain concomitant trace element accumulation is the simultaneous binding of Ag, Cd, Cu, Hg and Zn to MT. The synthesis of MTs can indeed be induced by several elements such as Cd, Cu, Hg and Zn (Kägi, 1991; Scheuhammer, 1987). Two of the most important influences on hepatic MT levels were Cu and Zn. Toxic effects possibly triggered by the high Cu concentrations found in godwits may be counter by the important implementation of this detoxification system.

Mercury is stated to have the highest affinity to MT, greater than Cd, Zn, and Cu (Cosson et al., 1991). Inorganic Hg can induce the synthesis of MT and will bind with the protein (Scheuhammer, 1987). The positive relationship between Hg concentrations and MT levels in the liver of godwits suggested that Hg bound to these proteins as a detoxification mechanism of this non-essential element.

MT concentrations have been shown to be positively correlated with Cd levels in the liver and kidneys of marine birds (Elliott et al., 1992; Elliott and Scheuhammer, 1997). The role of this protein in Cd detoxification was previously demonstrated (Lucia et al., 2009). Such relationship was indeed observed in the current study. Ag concentrations recorded in liver were also linked to individual MT levels. A previous study has demonstrated that around 30–40% of Ag tissue content was bound to MT in hepatocytosol of Mallard (*A. platyrhynchos*), Great Cormorant (*Phalacrocorax carbo*) and Spot-billed Duck (*A. poecilorhyncha*) (Nam et al., 2005). High Ag concentrations reached in the liver of godwits could have thus triggered the production of this detoxification protein.

4.6. Genetic and enzymatic insights on detoxification process

Three godwits have been sampled in order to have a first look into the sub-lethal effects and detoxification mechanisms triggered by a combination of contaminants measured in birds from Marennes-Oléron Bay. Although the number of individuals is currently too small to make a full examination and to conclude about trace elements effects on genetic expressions and enzymatic activities in godwits, it allowed a first insight into the possible ongoing defence mechanisms developed by this species. To the best of our knowledge, this study is actually the first report of genetic impairments in free-ranging birds. Two of the birds displayed high elemental concentrations relative to the third one. Although no correlation could be obtained between trace element concentrations and differential gene expressions, these two individuals appeared to have higher basal gene expression of *cox1* than the bird showing lower elemental accumulation. This up-regulation could have several purposes. First, it could represent a compensatory mechanism to restore the decrease in mitochondrial activity in order to maintain a sufficient number of functioning complexes in the respiratory chain. Secondly, the implementation of defences against oxidative stress in cytosol and mitochondria (*sod1*, *sod2* and *cat*) of the birds with higher elemental levels may trigger important energy needs that could be

provided through the enhancement of the mitochondrial metabolism. This effect was previously described for Cd exposure in domestic duck (Lucia et al., 2009), methylmercury and polymetallic gradient exposures (Cd, Zn) in the zebrafish *Danio rerio* (Gonzalez et al., 2005; Orieux et al., 2011). Both individual with higher elemental concentrations appeared to have higher *fas* (fatty acid synthase) gene basal expression. Consequently, trace elements may stimulate the production of fatty acids in the liver of godwits as previously observed for Cd contaminated European eels (*Anguilla anguilla*; Pierron et al., 2007). These authors suggested that Cd exposure leaded to lipid mobilization in this species. This phenomenon could contribute to the renewal of damaged membrane lipid and/or because triglyceride is an energy-dense substance, could indicate increased energetic needs. The last hypothesis seems to be the one to apply in this study as MDA levels did not demonstrate lipid peroxidation in godwits as a consequence of high Cd and other trace element concentrations. Low MDA levels might be the result of efficient detoxification systems such as MT implemented by individuals with higher trace element concentrations. Indeed, both birds with high trace element concentrations displayed 4-fold MT levels than godwit with lower elemental concentrations. This pattern is moreover strengthened by the higher *mt* gene basal expression of these two individuals.

Conversely, discrepancies were observed between the basal gene expression of SOD, and its enzyme activity. Although godwits with higher elemental concentrations appeared to have higher basal gene expression, their SOD activities were similar or even lower than in the individual with the lowest elemental levels. This might result from a time delay between gene expression and enzyme production, suggesting that SOD synthesis is regulated at the transcriptional level as well as the translational level. This may also demonstrate that above a certain concentration threshold, individuals have developed other detoxification process such as MT to prevent from trace element toxic effects. Therefore, this mechanism may be the major detoxification process implemented by this species. Our results suggest that even though trace element concentrations were mostly below toxicity threshold level, individuals could be submitted to sub-lethal effects such as increased energetic needs. The use of sensitive biomarkers such as gene expressions in future studies could be therefore valuable to evaluate early-stage effects of environmental exposure to stressors and its repercussion on population health.

Acknowledgements

The authors wish to thank the University of La Rochelle for its financial support through a post-doctoral grant to ML, as well as Natural Reserves of the Pertuis Charentais (Moëze-Oléron, Marais d'Yves, Lilleau des Niges) for their technical assistance during bird catches. We especially thank P. Delaporte, P. Rousseau, J. Gautier and J. Gonin from the Moëze-Oléron Natural Reserve. We also thank P. Richard and G. Guillou (UMR LIENSs) for technical support during stable isotope analysis. Authors are grateful to the anonymous referee for their important comments on this manuscript. This work was financially supported by the CPER (Contrat de Projet Etat-Région) and the CNRS.

References

Adams SM, Giesy JP, Tremblay LA, Eason CT. The use of biomarkers in ecological risk assessment: recommendations from the Christchurch Conference on Biomarkers in Ecotoxicology. Biomarkers 2001;6:1–6.
Alerstam T, Lindström A. Optimal bird migration: the relative importance of time, energy and safety. In: Gwinner E, editor. Bird migration: the physiology and ecophysiology. New York: Springer; 1990. p. 331–51.
Alerstam T, Hedenström A, Akesson S. Long-distance migration: evolution and determinants. Oikos 2003;103:247–60.
Arai T, Ikemoto T, Hokura A, Terada Y, Kunito T, Tanabe S, et al. Chemical forms of mercury and cadmium accumulated in marine mammals and seabirds as determined by XAFS analysis. Environ Sci Technol 2004;15:6468–74.
Bearhop S, Waldron S, Thompson D, Furness R. Bioamplification of mercury in Great Skua *Catharacta skua* Chicks: the influence of trophic status as determined by stable isotope signatures of blood and feathers. Mar Pollut Bull 2000;40:181–5.
Braune BM, Noble DG. Environmental contaminants in Canadian shorebirds. Environ Monit Assess 2009;148:185–204.
Brdička AR. Polarographic studies with the dropping mercury method. A new test for proteins in the presence of cobalt salts in ammoniacal solution of ammonium chloride. Collect Czech Chem Commun 1933;7:112–28.
Burger J. Metals in avian feathers: bioindicators of environmental pollution. Rev Environ Toxicol 1993;5:203–311.
Burger J, Gochfeld M. Marine birds as sentinels of environmental pollution. Ecohealth 2004;1:263–74.
Bustamante P, Miramand P. Interspecific and geographical variations of trace element concentrations in Pectinidae from European waters. Chemosphere 2004;57:1355–62.
Bustamante P, Miramand P. Subcellular and body distributions of 17 trace elements in the variegated scallop *Chlamys varia* from the Charente-Maritime coast (Bay of Biscay, France). Sci Total Environ 2005;337:59–79.
Bustamante P, Lahaye V, Durnez C, Churlaud C, Caurant F. Total and organic Hg concentrations in cephalopods from the North East Atlantic waters: influence of geographical origin and feeding ecology. Sci Total Environ 2006;368:585–96.
Cherel Y, Le Corre M, Jaquemet S, Ménard F, Richard P, Weimerskirch H. Resource partitioning within a tropical seabird community: new information from stable isotopes. Mar Ecol Prog Ser 2008;366:281–91.
Cosson RP, Amiard-Triquet C, Amiard JC. Metallothionein and detoxification. Is the use of detoxification proteins for MTs a language abuse? Water Air Soil Pollut 1991;57–58:555–67.
De Luca-Abbott SB, Wong BS, Peakall DB, Lam PK, Young L, Lam MH, et al. Review of effects of water pollution on the breeding success of waterbirds, with particular reference to ardeids in Hong Kong. Ecotoxicology 2001;10:327–49.
Deisseroth A, Dounce AL. Catalase: physical and chemical properties, mechanisms of catalysis, and physiological role. Physiol Rev 1970;50:319–75.
Delany S, Scott D. Waterbird population estimates. 4th ed. Wageningen: Wetlands International; 2006.
Delany S, Scott D, Dobman T, Stroud D. An atlas of wader population in Africa and Western Eurasia. Wageningen: Wetlands International; 2009.
Donaldson GM, Braune BM. Sex-related levels of selenium, heavy metals, and organochlorine compounds in American White Pelicans (*Pelecanus erythrorhyncos*). Arch Environ Contam Toxicol 1999;37:110–4.
Eisler R. A review of arsenic hazards to plants and animals with emphasis on fishery and wild life resources. In: Nriagu JO, editor. Arsenic in the environment. Part II: human health and ecosystem effectsNew York: Wiley; 1994. p. 185–259.
Eisler R. Silver hazards to fish, wildlife and invertebrates: a synoptic review. US Department of the Interior, National Biological Service, Report 32; 1996.
Elliott JE, Scheuhammer AM. Heavy metal and metallothionein concentrations in seabirds from the Pacific coast of Canada. Mar Pollut Bull 1997;34:794–801.
Elliott JE, Scheuhammer AM, Leighton FA, Pearce PA. Heavy metal and metallothionein concentrations in Atlantic Canadian seabirds. Arch Environ Contam Toxicol 1992;22:63–73.
Gallien I, Caurant F, Bordes M, Bustamante P, Miramand P, Fernandez B, et al. Cadmium-containing granules in kidney tissue of the Atlantic white-sided dolphin (*Lagenorhyncus acutus*) off the Faroe Islands. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 2001;130:389–95.
Gérard-Monnier D, Erdelmeier I, Régnard K, Moze-Henry N, Yadan J, Chaudière J. Reactions of 1-methyl-2-phenylindole with malondialdehyde and 4-hydroxyalkenals. Analytical applications to a colorimetric assay of lipid peroxidation. Chem Res Toxicol 1998;11:1176–83.
Gill JA, Norris K, Potts PM, Gunnarsson TG, Atkinson PW, Sutherland WJ. The buffer effect and large-scale population regulation in migratory birds. Nature 2001;412:436–8.
Gill JA, Langston RHW, Alves JA, Atkinson PW, Bocher P, Vieira NC, et al. Contrasting trends in two Black-tailed Godwit populations: a review of causes and recommendations. Wader Study Group Bull 2007;114:43–50.
Gochfeld M, Belant JL, Shukla T, Benson T, Burger J. Heavy metals in laughing gulls: gender, age and tissue differences. Environ Toxicol Chem 1996;15:2275–83.
Gonzalez P, Dominique Y, Massabuau JC, Boudou A, Bourdineaud JP. Comparative effects of dietary methylmercury on gene expression in liver, skeletal muscle, and brain of the Zebrafish (*Danio rerio*). Environ Sci Technol 2005;39:3972–80.
Groen NM, Hemerik L. Reproductive success and survival of black-tailed godwits *Limosa limosa* in a declining local population in The Netherlands. Ardea 2002;90:239–48.
Hall BD, Bodaly RA, Fudge RJP, Rudd JWM, Rosenberg DM. Food as the dominant pathway of methylmercury uptake by fish. Water Air Soil Pollut 1997;100:13–24.
Halpern BS, Walbridge S, Selkoe KA, Kappel CV, Micheli F, D'Agrosa C, et al. A global map of human impact on marine ecosystems. Science 2008;319:948–52.
Heinz GH. Selenium in birds. In: Beyer WN, Heinz GH, Redmon-Norwood AW, editors. Environmental contaminants in wildlife: interpreting tissue concentrations. Boca Raton: SETAC CRC, Lewis Publishers; 1996. p. 447–58.
Henderson BM, Winterfield RW. Acute copper toxicosis in the Canada goose. Avian Dis 1975;19:385–7.
Jardine TD, Kidd KA, Fisk AT. Applications, considerations, and sources of uncertainty when using stable isotope analysis in ecotoxicology. Environ Sci Technol 2006;40:7501–11.
Kägi JH. Overview of metallothionein. Methods Enzymol 1991;205:613–26.
Kojadinovic J, Bustamante P, Churlaud C, Cosson RP, Le Corre M. Mercury in seabird feathers: Insight on dietary habits and evidence for exposure levels in the western Indian Ocean. Sci Total Environ 2007a;384:194–204.
Kojadinovic J, Cosson RP, Le Corre M, Bustamante P. Trace elements in three marine birds breeding on Reunion Island (Western Indian Ocean). Part 2: factors influencing their detoxification. Arch Environ Contam Toxicol 2007b;52:431–40.
Kunito T, Kubota R, Fujihara J, Agusa T, Tanabe S. Arsenic in marine mammals, seabirds, and sea turtles. Rev Environ Contam Toxicol 2008;195:31–69.

Lourenço PM, Piersma T. Stopover ecology of black-tailed godwits *Limosa limosa limosa* in Portuguese rice fields: a guide on where to feed in winter. Bird study 2008;55:194–202.
Lucia M, André JM, Gonzalez P, Baudrimont M, Gontier K, Maury-Brachet R, et al. Impact of cadmium on aquatic bird *Cairina moschata*. Biometals 2009;22:843–53.
Lucia M, André JM, Gonzalez P, Baudrimont M, Bernadet MD, Gontier K, et al. Effect of dietary cadmium on lipid metabolism and storage of aquatic bird *Cairina moschata*. Ecotoxicology 2010;19:163–70.
Luna-Acosta A, Bustamante P, Godefroy J, Fruitier-Arnaudin I, Thomas-Guyon H. Seasonal variation of pollution biomarkers to assess the impact on health status of juvenile Pacific oysters *Crassostrea gigas* exposed *in situ*. Environ Sci Pollut Res 2010;17:999-1008.
Mahéo R. Limicoles séjournant en France (Littoral). 31ème contribution française aux dénombrements internationaux des oiseaux d'eau organisés par Wetlands International. Office national de la chasse et de la faune Sauvage; 2010.
McCarty LS, Munkittrick KR. Environmental biomarkers in aquatic toxicology: fiction, fantasy, or functional? Hum Environ Risk Assess 1996;2:268–74.
Métian M, Bustamante P, Hédouin L, Warnau M. Accumulation of nine metals and one metalloid in the tropical scallop *Comptopallium radula* from coral reefs in New Caledonia. Environ Pollut 2008;152:543–52.
Michener RH, Kaufman L. Stable isotope ratios as tracers in marine food webs: an update. In: Michener RH, Lajtha K, editors. Stable isotopes in ecology and environmental science. Blackwell Publishing; 2007. p. 238–82.
Nam DH, Anan Y, Ikemoto T, Kim EY, Tanabe S. Distribution of trace elements in subcellular fractions of three aquatic birds. Mar Pollut Bull 2005;51:750–6.
Neff JM. Ecotoxicology of arsenic in the marine environment. Environ Toxicol Chem 1997;16:917–27.
Nigro M, Leonzio C. Intracellular storage of mercury and selenium in different marine vertebrates. Mar Ecol Prog Ser 1996;135:137–43.
Nriagu JO. A history of global metal pollution. Science 1996;272:223–4.
Orieux N, Cambier S, Gonzalez P, Morin B, Adam C, Garnier-Laplace J, et al. Genotoxic damages in zebrafish submitted to a polymetallic gradient displayed by the Lot River (France). Ecotoxicol Environ Saf 2011;74:974–83.
Outridge PM, Scheuhammer AM, Fox GA, Braune BM, White LM, Gregorich LJ. An assessment of the potential hazards of environmental selenium for Canadian wildlife. Environ Rev 1999;7:81–96.
Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 1967;70:158–69.
Perez-Hurtado A, Goss-Custard JD, Garcia F. The diet of wintering waders in Cádiz Bay, southwest Spain. Bird Study 1997;44:45–52.
Peterson SA, Ralston NVC, Peck DV, Van Sickle J, Robertson JD, Spate VL, et al. How might selenium moderate the toxic effects of mercury in stream fish of the Western U.S.? Environ Sci Technol 2009;43:3919–25.
Pierron F, Baudrimont M, Bossy A, Bourdineaud JP, Brèthes D, Elie P, et al. Impairment of lipid storage by cadmium in the European eel (*Anguilla anguilla*). Aquat Toxicol 2007;81:304–11.
Piersma T, van Gils JA. The flexible phenotype: a body-centred integration of ecology, physiology and behaviour. 1st ed. New York: Oxford University Press Inc.; 2011.
Pigeot J, Miramand P, Guyot T, Sauriau PG, Fichet D, Le Moine O, et al. Cadmium pathways in an exploited intertidal ecosystem with chronic cadmium inputs (Marennes-Oléron, Atlantic coast, France). Mar Ecol Prog Ser 2006;307:101–14.
Quaintenne G, van Gils JA, Bocher P, Dekinga A, Piersma T. Diet selection in a molluscivore shorebird across Western Europe: does it show short- or long-term intake rate-maximization? J Anim Ecol 2010;79:53–62.
Robin F. Dynamique de la distribution, sélection de l'habitat et stratégie d'alimentation chez la barge à queue noire *limosa limosa* à l'échelle des sites d'hivernage français. France: University of La Rochelle; 2011.
Roodbergen M, Klok C, van der Hout A. Transfer of heavy metals in the food chain earthworm Black-tailed Godwit (*Limosa limosa*): comparison of a polluted and a reference site in The Netherlands. Sci Total Environ 2008;406:407–12.
Rubenstein DR, Hobson KA. From birds to butterflies: animal movement patterns and stable isotopes. Trends Ecol Evol 2004;19:256–63.
Schekkerman H, Teunissen W, Oosterveld E. Mortality of Black-tailed Godwit *Limosa limosa* and Northern Lapwing *Vanellus vanellus* chicks in wet grasslands: influence of predation and agriculture. J Ornithol 2009;150:133–45.
Scheuhammer AM. The chronic toxicity of aluminium, cadmium, mercury, and lead in birds: a review. Environ Pollut 1987;46:263–95.
Skorupa JP, Morman SP, Sefchick-Edwards JS. Guidelines for interpreting selenium exposure of biota associated with nonmarine aquatic habitats. U.S. Fish and Wildlife Service, National Irrigation Water Quality Program; 1996.
Smith P, Khron R, Hermanson G, Mallia A, Gartner F, Provanzano M, et al. Measurement of protein using bicinchoninic acid. Anal Biochem 1985;150:76–85.
Spallholz JE, Hoffman DJ. Selenium toxicity: cause and effects in aquatic birds. Aquat Toxicol 2002;57:27–37.
Stewart FM, Furness RW, Monteiro LR. Relationships between heavy metal and metallothionein concentrations in lesser black-backed gulls, *Larus fuscus*, and Cory's shearwater, *Calonectris diomedea*. Arch Environ Contam Toxicol 1996;30:299–305.
Stewart MS, Neldner KH, Spallholz JE, Pence BC. Selenite, selenocystamine, and selenomethionine have disparate abilities to impose oxidative stress and induce apoptosis in mouse keratinocytes. Free Radic Biol Med 1999;26:42–8.
Stroud DA, Baker A, Blanco DE, Davidson NC, Delany S, Ganter B, et al. The conservation and population status of the world's waders at the turn of the millennium. In: Boere GC, Galbraith CA, Stroud DA, editors. Waterbirds around the world. Edinburgh: The Stationery Office; 2006. p. 643–8.
Thomas CD, Cameron A, Green RE, Bakkenes M, Beaumont LJ, Collingham YC, et al. Extinction risk from climate change. Nature 2004;427:145–8.
Thompson JAJ, Cosson RP. An improved electrochemical method for the quantification of metallothionein in marine organism. Mar Environ Res 1984;11:137–52.
Triplet P, Mahéo R. Le Drean Quenec'hdu S. La Barge à queue noire *Limosa limosa islandica* hivernant en France-Littoral Manche Atalntique, 1977–2006. Alauda 2007;75: 389–98.
Vitousek PM, Mooney HA, Lubchenco J, Melillo JM. Human domination of Earth's ecosystems. Science 1997;277:494–9.
Weber LM, Haig SM. Shorebird diet and size selection of Nereid Polychaetes in South Carolina coastal diked wetlands. J Field Ornithol 1997;68:358–66.
Wooliams JA, Wiener G, Anderson PH, McMurray CH. Variation in the activities of glutathione peroxidase and superoxide dismutase and in the concentration of copper in the blood in various breed crosses of sheep. Res Vet Sci 1983;34:253–6.