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Environmental Toxicology and Chemistry, Vol. 25, No. 2, pp. 412–418, 2006

Printed in the USA
0730-7268/06 $12.00 + .00

METALLOTHIONEIN GENE EXPRESSION AND PROTEIN LEVELS IN TRIPLOID AND DIPLOID OYSTERS *CRASSOSTREA GIGAS* AFTER EXPOSURE TO CADMIUM AND ZINC

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(*Received* 17 *February* 2005; *Accepted* 12 *July* 2005)

Abstract—Quantitative real-time polymerase chain reaction (PCR) was used to compare for the first time the differential expression of metallothionein (MT) isoform genes, together with biosynthesis of the total MT proteins, in the gills of triploid and diploid juvenile Pacific oyster *Crassostrea gigas* in response to cadmium (Cd) and zinc (Zn) exposure. Oysters were exposed to Cd (0.133 μM), Zn (15.3 μM), and Cd+Zn for 14 d. Results showed similar response capacities to metal exposures in the two populations. No significant difference was revealed in terms of MT gene expression, MT protein synthesis, and Cd accumulation. However, triploid oysters bioaccumulated Zn 30% less efficiently than diploid oysters. Among the three MT isoform genes, *CgMT2* appeared to be more expressed than *CgMT1*, whereas *CgMT3* appeared to be anecdotal (10^6 times lower than *CgMT2*). *CgMT2* and *CgMT1* gene expression levels were increased sevenfold in the presence of Cd, whereas Zn appeared to have no effect. A twofold increase in MT protein levels occurred in response to Cd exposure. Discrepancies between mRNA and protein levels suggest that in *C. gigas* MT are regulated at the transcriptional level, as well as at the translational level.

Keywords—*Crassostrea gigas* Diploid Triploid Metallothionein Expression levels

INTRODUCTION

Triploidy in oysters has been developed to enhance the production and selling period of commercial bivalve species [1,2]. Triploid individuals are characterized by three sets of chromosomes, against two for diploid oysters. Classically, triploid oysters are recognized as having reduced gametogenic activity and could be viewed as an adjunct tool in studies of reproductive biology and related physiological aspects such as growth, glycogen utilization, and survival [2–4]. In the ecotoxicological field, oysters like mussels are widely used as bioindicators in monitoring programs in coastal areas, especially in relation to their strong capacities of heavy metal accumulation [5].

Numerous field or experimental studies have shown the key role of metallothioneins (MTs) in sequestration and detoxification of toxic metals (e.g., cadmium [Cd], mercury [Hg]), as well as their important role in the metabolism of essential elements (e.g., zinc [Zn], copper [Cu]). These cytosolic proteins belong to a family of low molecular mass and cysteine-rich proteins, in which biosynthesis can be induced by intracellular metal accumulation. Nevertheless, many other important functions have been reported, such as free radical scavenging or immune response [6].

In this paper, we investigated whether the presence of an additional set of chromosomes in the Pacific oyster *Crassostrea gigas* could be at the origin of modifications in MT biosynthesis in response to metal exposure. Indeed, a long-standing assumption has been that each additional gene copy would contribute equally to expression levels after polyploidization. This occurrence could induce better physiological performances (feeding rate, absorption, and growth efficiency) because of a faster potential for transcription [7]. Nevertheless, some exceptions exist, notably as a consequence of gene silencing after polyploidization [8]. The development of triploidy in aquaculture raised the question of whether this parameter can be at the origin of changes in oyster resistance under stressful metal conditions in relation to a possible influence on metal accumulation (absorption and detoxification). To our knowledge, consideration of such a genetic factor (polyploidy) as a source of variation of MT expression has not been analyzed so far for bivalves.

In *C. gigas*, three MTs have been reported to date [9,10]. The *CgMT1* and *CgMT2* genes have been characterized, whereas *CgMT3* is only available as a complementary DNA (cDNA) sequence. The *CgMT1* and *CgMT2* possess quasi-similar sequences and can be differentiated by their molecular organization. Indeed, whereas *CgMT1* encodes for a classical αβ domain organization, *CgMT2* presents an additional β domain. However, the differential expression level of each MT is not yet known. Moreover, nothing is known about the expression of *CgMT3*.

In this context, we have set up an experimental study with juvenile diploid and triploid oysters to investigate Cd and Zn effects on MT biosynthesis in gills. Cadmium and Zn were selected as toxic and essential elements, respectively; they are often present simultaneously in the environment, notably in manufacturing wastes from extraction and treatment of Zn ores. These two metals were added to the water individually and in combination to study their potential interactive effects.

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Two complementary approaches were used to quantify MT biosynthesis: global protein determination with the HgII saturation assay, and differential expression of the three MT genes by quantitative real-time polymerase chain reaction (PCR). In addition, metal concentrations in the gills were determined after a two-week exposure period of diploid and triploid oysters.

MATERIALS AND METHODS

Experimental metal exposures

Juvenile diploid and triploid *C. gigas* oysters (12 months old, 5.7 ± 0.1 cm shell length) were obtained from an oyster farmer in Arcachon Bay, on the French Atlantic coast, in September 2003. Triploidy in oysters was originally achieved by crossing diploid females and tetraploid males. A total of 288 individuals were acclimated for 7 d before the experiment began in tanks containing aerated artificial seawater prepared with Instant Ocean® synthetic sea salt (Mentor, OH, USA) in dechlorinated tap water and adjusted to 25 ± 0.5‰. Subsequently, diploid and triploid oysters were transferred into four polypropylene tanks (60 L) at a density of 36 animals of each population per tank. In these tanks, the oysters were exposed for 14 d to 0.133 μM Cd (15 μg/L), 15.3 μM Zn (1 mg/L), or a combination of both metals (0.133 μM Cd + 15.3 μM Zn) adjusted in water from stock aqueous solutions $CdCl_2$ and $ZnCl_2$ (0.266 mM for Cd and 15.3 mM for Zn). These exposure levels are appropriate for significant metal accumulation after 14 d of exposure. The fourth tank contained untreated individuals (controls). Experiments were performed at 20 ± 0.5°C, pH 8.5 ± 0.3, with 9.5 mg/L of dissolved O_2 under a 12:12 h light:dark photoperiod. Water in each tank was renewed every 2 d to maintain contamination pressure throughout the experiment and to avoid changes in the physicochemical parameters. Metal concentrations were measured in water samples collected at the end of each renewal cycle (n = 7 for each condition). Results showed a very small decrease, close to 4%, with no significant variations during the 14 d of the experiment: 14.4 ± 0.2 μg Cd/L and 0.96 ± 0.02 mg Zn/L. Same values were obtained when the two metals were added simultaneously. On alternate days, the oysters were fed with a restricted quantity of fresh green marine algae (~0.05 g from a culture of *Isochrysis galbana*) to minimize metal transfers via the trophic exposure route.

Flow cytometry analysis of hemocyte DNA content

Flow cytometry measurements of hemocyte DNA content were performed to validate ploidy status of exposed populations. After 14 d of exposure, the hemolymph of 30 randomly selected individuals for each treatment (15 diploid and 15 triploid oysters) was withdrawn and processed individually for analysis. Hemolymph (0.5–1 ml) was collected from the adductor muscle sinus with a sterile 1-ml syringe equipped with a needle (0.9 × 25 mm) and filtered (80 μm) to eliminate aggregates or large pieces of debris. Hemolymph samples were conserved on ice to prevent hemocyte aggregation [11]. Hemocytes were washed in sterile seawater by centrifugation (200 *g*, 10 min). After hemocyte collection, oysters were frozen at −80°C under nitrogen atmosphere to ensure anoxic conditions and kept for biochemical and genetic analysis and metal determination performed two months later. The hemocyte samples were treated with solutions of trypsin (30 mg/L) and ribonuclease A (100 mg/L) and stained with propidium iodide for a minimum of 1 h according to the method described by Vindelov and Christensen [12]. Red fluorescence signals emitted by the propidium iodide–stained hemocyte nuclei were collected with a flow cytometer at 617 nm (Becton Dickinson Immunocytometry Systems FACScalibur, San Jose, CA, USA). Chicken blood cells were used as an internal control in each sample for calibration (reference peaks) and to estimate average DNA content of oyster hemocytes. Light emission was analyzed on 10,000 events per sample. For fluorescence-level analysis, frequency distribution histograms were established with Cell Quest software (Becton Dickinson). A relative mean fluorescence intensity was calculated by the software from these histograms for selected cell populations and expressed in a range of 1 to 10^4 arbitrary units then used to estimate hemocyte DNA. Most oysters analyzed per exposure condition were found to be diploid or triploid, as expected. The few mosaic individuals found in the triploid population (4%) were discarded from our analysis.

Metal determination

Of all the bivalve organs, the gills have proved to be the most suitable for analysis because they represent the main barrier involved in metal uptake via the direct exposure route, with a large exchange area in contact with the surrounding environment [13]. Gills from three oysters used for ploidy determination were dissected after thawing, dried on absorbent paper and weighed (wet wt). Samples were digested in 3 ml of concentrated nitric acid added to a pressurized medium (borosilicate glass tube) at 95°C for 3 h. The resulting digestate was diluted to 20 ml with ultrapure water (MilliQ Plus®, Millipore, Saint Quentin-Yvelines, France) and analyzed by atomic absorption spectrophotometry [14]. The Cd determinations were performed with a polarized Zeeman atomic absorption spectrophotometer (Varian AA400; Victoria, Australia) equipped with a graphite tube atomizer (model GTA 96, Varian). To avoid interference, the appropriate diluted digestates were analyzed in a tube atomizer with a mixture of $Pd+Mg(NO_3)_2$. The detection limit was 0.1 μg Cd/L (3× the standard deviation of 10 reagent blanks). Zinc concentrations were carried out on the same samples by flame atomic absorption spectrophotometry (Varian AA220FS). The detection limit was 5 μg Zn/L. The analytical methods were simultaneously validated for each sample series by the analysis of standard biological reference materials (TORT-2, lobster hepatopancreas; DOLT-2, dogfish liver; National Research Council of Canada, Ottawa, ON). Percent recovery of metals was 98 ± 2% for Cd and 100 ± 2% for Zn (n = 8).

The results are expressed as average metal concentrations accumulated in oyster gills (in nmol Cd/g [wet wt] ± SE and μmol Zn/g [wet wt] ± SE) for three samples from each population and metal exposure.

Mercury saturation assay

The levels of total MT proteins in gills from diploid and triploid oysters were determined by mercury saturation assay as previously described with the use of cold inorganic mercury [14,15]. The analysis of MTs was conducted on three replicates (three gills from three different oysters) per exposure condition for each oyster population, with the saturation assay repeated twice per sample. This technique is based on the quantification of Hg bound to the saturated MT. The denaturation of non-MT proteins was performed with trichloroacetic acid, and the excess Hg not bound to the MT was removed by scavenging

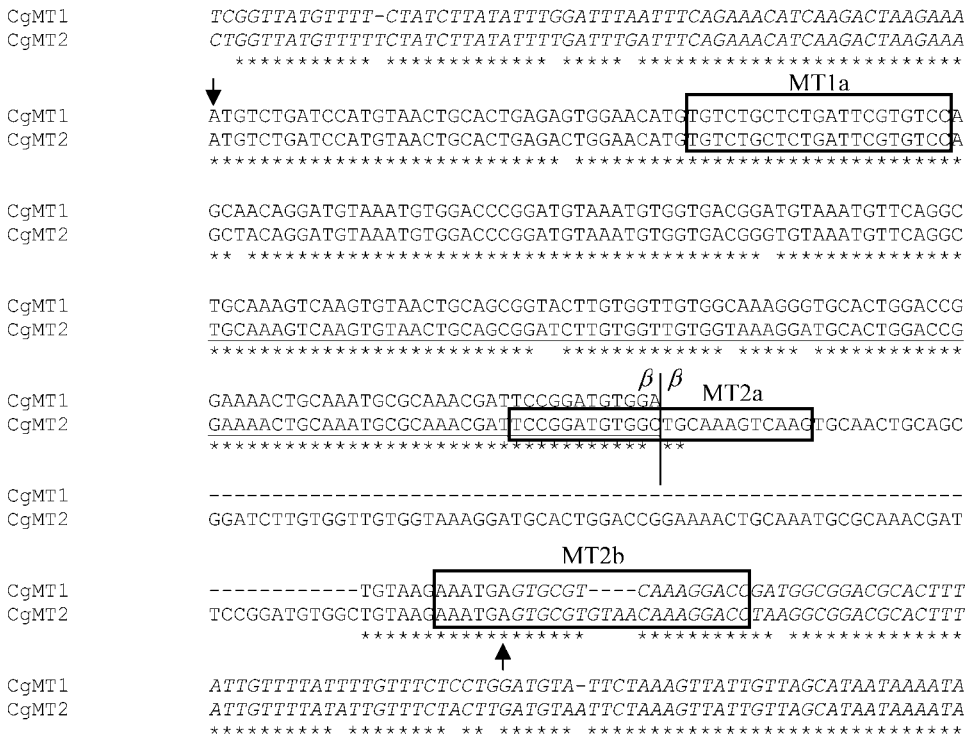

Fig. 1. Alignment of the *CgMT1* and *CgMT2* complementary DNA sequences from *Crassostrea gigas*. Position of the metallothionein (MT)1a, MT2a, and MT2b primers are framed. Sequence of the first β domain of *CgMT2* is underlined. Initiation and stop codons are indicated with arrows. The 5′ and 3′ untranslated sequences are in italic. Asterisks represent identical nucleotides at the same position in *CgMT1* and *CgMT2*.

with lyophilized pig hemoglobin (Sigma-Aldrich, St. Louis, MO, USA) at 0.05 g Hb/ml in 30 mM Tris-HCl buffer (pH 8.2 at 20°C) [15]. The final supernatant was then recovered and used for Hg determination by flameless atomic absorption spectrometry (AMA 254, Altec, Prague, Czech Republic). The detection limit was estimated at 1 ng Hg.

Reference samples or blanks (200 μl Tris-HCl homogenization buffer + 200 μl trichloracetic $HgCl_2$ solution + 400 μl pig hemoglobin solution) were prepared to monitor Hg complexation efficiency of the hemoglobin. Under our experimental conditions, an average burden of 1.2 ng Hg was measured in these reference samples, compared with the 10,000 ng initially added (0.01%). The mean of the three blank values measured in each analytical run was deducted from the Hg burdens measured in each replicate tissue sample.

At the same time, a standard of purified rabbit liver MT (Sigma) was used to check the validity of the saturation assay by determining the ratio between the binding sites measured after Hg saturation and the potential binding sites indicated by the supplier and previously verified by Hg, Cd, Zn, and Cu determinations on purified MT solution (10 μg MT/ml in the homogenization buffer). In our experimental conditions, the average value of the recovery percentage was 100.17 ± 4.6%. MT concentrations in oyster gills were expressed in nanomoles of Hg binding sites per gram (nmol Hg sites/g [wet wt] ± SE). Because the exact number of Hg binding sites per MT molecule is unknown for this species, MT concentrations cannot be expressed as nanomoles of MT per gram (wet wt).

Total RNA extraction

Total RNAs were extracted from 40 mg of gills with the Absolutely RNA reverse transcriptase PCR (RT-PCR) Miniprep kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's instructions. The quality of all RNAs produced was evaluated by electrophoresis on a 1% agarose–formaldehyde gel, and concentration was determined by spectrophotometry. For each exposure condition, samples were carried out in triplicate. Each sample was obtained by mixing gills from two individuals.

Reverse transcription of RNA

First-strand complementary DNA (cDNA) was synthesized from 5 μg total RNAs with the Stratascript First-Strand Synthesis System (Stratagene). Briefly, RNAs were adjusted to 38 μl with diethyl pyrocarbonate–treated water. After addition of 2 μl of oligo-desoxynucleoside triphosphates (-dNTPs) and 1 μl of random primers, the reaction was incubated at 65°C for 5 min. Then 40 units of RNase block ribonuclease inhibitor, 2 μl of 100 mM dNTP, 5 μl of 10× first-strand buffer, and 50 units of Stratascript reverse transcriptase were added. The reaction was incubated for 1 h at 42°C in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). The cDNA mixture was kept at −20°C until required for real-time PCR analysis.

Primer design

To determine specific primer pairs, the nucleotide sequences from *CgMT1* (AJ242657) and *CgMT2* (AJ297818) were aligned. Indeed, these two genes possessed nearly the same 5′ and 3′ untranslated regions and the same αβ domain sequences, but *CgMT2* was characterized by the occurrence of an additional β domain. From the alignment, the specific primer pair MT2a–MT2b was deduced to amplify *CgMT2*, where MT2a was chosen at the ββ domain junction (Fig. 1). The primer pair MT1a–MT2b allows the amplification of both *CgMT1* and *CgMT2*. Indeed, PCR products with this primer pair on plasmids containing *CgMT1* and *CgMT2* cDNA, used independently or mixed in equimolar quantities, possessed an

Table 1. Specific primers used in quantitative real-time polymerase chain reaction

Name	Sequence
MT1a	5′-TGTCTGCTCTGATTCGTGTCC-3′
MT2a	5′-TCCGGATGTGGCTGCAAAGTCAAG-3′
MT2b	5′-GGTCCTTTGTTACACGCACTCATTT-3′
MT3a	5′-GCTCTGCTCAATGTACTAGCG-3′
MT3b	5′-GTCCACACAAATCGTTGAAACC-3′

intensity ratio roughly equal to 1.5, meaning an equimolar amount of amplified fragments. Quantification of the *CgMT1* gene expression was therefore deduced by subtracting the quantification corresponding to the *CgMT2* gene expression (MT2a–MT2b primer pair) from the quantification obtained with the MT1a–MT2b primer pair. Specific primers (MT3a–MT3b) were determined from the *CgMT3* sequence (AJ295157). All the primers used in this study are reported in Table 1.

Real-time PCR

Real-time PCR were performed in a LightCycler (Roche, Meylan, France) according to the manufacturer's instructions (one cycle at 95°C for 10 min; 50 amplification cycles at 95°C for 5 s, 60°C for 5 s, and 72°C for 20 s). Each 20-μl reaction contained 2 μl of reverse transcribed product template; 1 μl of master mix, including the SYBR Green I® fluorescent dye (Roche), enabling the PCR amplification to be monitored; and the primer pair MT1a–MT2b, MT2a–MT2b, or MT3a–MT3b at a final concentration of 300 nM for each primer. To quantify transcript levels, a standard reference was obtained by cloning the *CgMT1* PCR product from *C. gigas* in the pGEMT vector. After transformation of the *Escherichia coli* JM109 strain, a large amount of plasmid was obtained and quantified by spectrophotometry. Dilutions of this plasmid were used as standard reference in real-time PCR with the MT1a–MT2b primer pair.

Reaction specificity was determined for each reaction from the dissociation curve of the PCR product. This dissociation curve was obtained by following the SYBR Green fluorescence level during a gradual heating of the PCR products from 60 to 95°C. The MT mRNA quantifications were expressed in number of copies (μg total RNAs ± SE).

Statistical analysis

Because diploid and triploid oysters were placed in the same tank for each treatment, they could not be considered true replicates. In this context, parametric analyses are violated (normality and homogeneity of variances). Significant differences between metal concentrations, MT concentrations, and number of mRNA copies were determined with the nonparametric Mann–Whitney *U* test (Statistica 5.1, StatSoft, Tulsa, OK, USA). Results were considered statistically significant at $p < 0.05$. Interindividual variability for each experimental condition was defined by the mean ± SE.

RESULTS

Oyster growth, as reflected in mean soft tissue weights and mean shell lengths, was not significant and did not differ among treatments during the exposure period.

Cadmium bioaccumulation

Mean concentrations of Cd measured in the oyster gills are shown in Figure 2A. Results revealed no significant effect ($p > 0.05$) of the ploidy status on Cd bioaccumulation for each exposure condition. The Cd background levels in controls were not significantly different ($p > 0.05$) between oyster populations: 3.2 ± 0.3 and 2.5 ± 0.3 nmol Cd/g (wet wt) in diploid and triploid oysters, respectively. After exposure to Cd alone, metal bioaccumulation presented a 20- and 23-fold increase in diploid and triploid oysters, respectively. Similarly, in combined Cd and Zn exposure, increases ranged from 15- to 23-fold in diploid and triploid oysters, respectively.

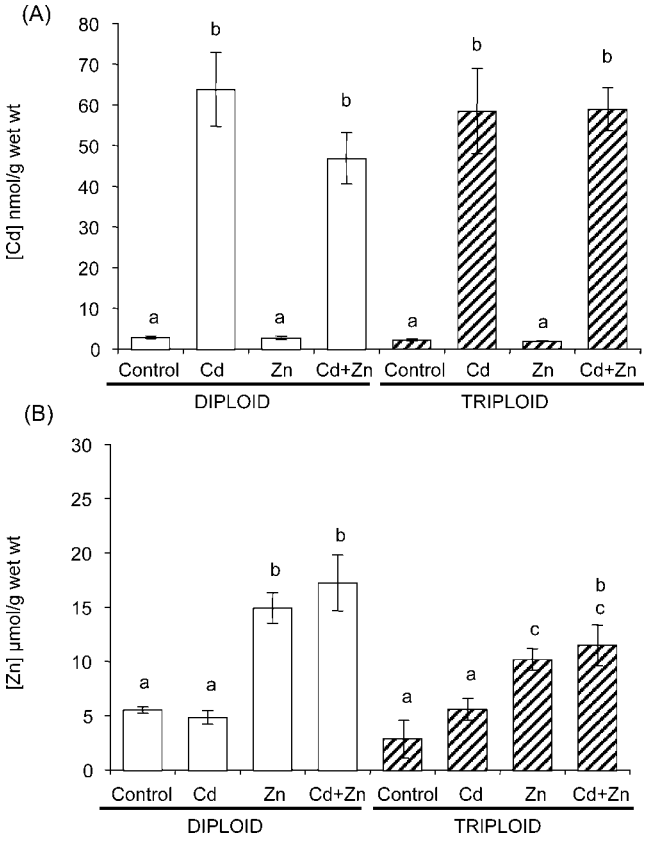

Fig. 2. Mean levels of (**A**) cadmium (nmol Cd/g [wet wt] ± SE; $n = 3$) and (**B**) zinc (μmol Zn/g [wet wt] ± SE; $n = 3$) in the gills of diploid and triploid oysters in control, cadmium, or zinc treatments or a combination treatment (0.133 μM Cd and 15.3 μM Zn) after 14 d (values with the same letter are not statistically different, $p > 0.05$).

Zinc bioaccumulation

The Zn background levels in gills of controls from the two populations also were not significantly different ($p > 0.05$): 5.6 ± 0.3 and 3.0 ± 1.7 μmol Zn/g (wet wt) in diploid and triploid oysters, respectively (Fig. 2B). However, under Zn exposure, diploid oysters were found to accumulate Zn with 30% greater efficiency than triploid oysters ($p < 0.05$), accumulating 15 ± 1.4 μmol/g compared with 10.3 ± 1 μmol/g. The same holds true for combined Zn and Cd treatment because Zn concentrations reached 17.3 ± 2.6 μmol/g and 11.6 ± 1.9 μmol/g for diploid and triploid oysters, respectively. However, accumulation factors for diploid and triploid oysters for both Zn treatments were observed to be close: a three-fold increase was measured in a single metal exposure for both populations, with a 3.5-fold increase under Cd+Zn exposure compared with respective control oysters.

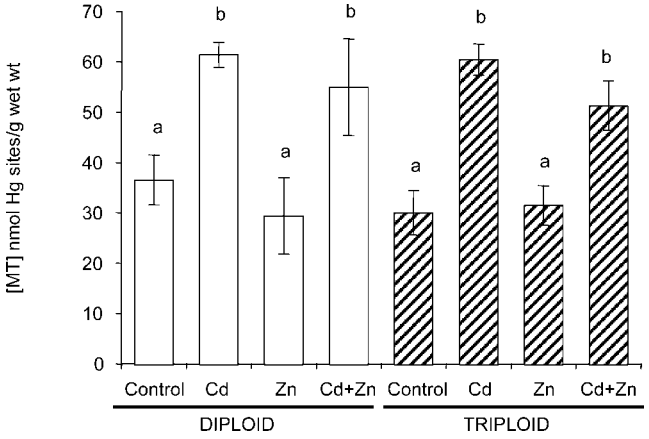

Fig. 3. Mean levels of total soluble metallothionein (MT) proteins (nmol Hg sites/g [wet wt] ± SE; $n = 3$) measured by mercury saturation assay in the gills of diploid and triploid oysters in control, cadmium, or zinc treatments or a combination treatment (0.133 μM Cd and 15.3 μM Zn) after 14 d (values annotated by the same letter are not statistically different, $p > 0.05$).

Total metallothionein proteins

The results revealed a significant effect of Cd exposure on MT concentrations measured in the gills (Fig. 3). However, no effect of ploidy was observed on MT concentration ($p > 0.05$). Untreated diploid and triploid oysters exhibited comparable basal levels in MT proteins with 36.7 ± 8 and 30.3 ± 7.6 nmol Hg sites/g (wet wt), respectively. For both oyster populations, MT concentrations increased similarly under single Cd or combined Cd and Zn exposures ($p < 0.05$). After 14 d of exposure, the total MT protein quantities were about twofold higher than control levels. In contrast, exposure to Zn alone did not significantly change the MT levels in the gills of diploid and triploid oysters compared with their control values.

Expression of metallothionein mRNA

Diploid oysters. Basal expression levels of *CgMT1*, *CgMT2*, and *CgMT3* genes were quite variable. *CgMT2* was the most abundant MT in unexposed *C. gigas*, with about 5×10^7 copies/μg of total RNA (Fig. 4A). By comparison, *CgMT1* was present at only 0.7×10^7 copies/μg of total RNA, whereas *CgMT3* showed only 30 copies/μg of total RNA (Fig. 4B) and appeared to be anecdotal compared with *CgMT1* and *CgMT2*.

The expression levels of *CgMT1* and *CgMT2* were significantly up-regulated after Cd exposure ($p < 0.05$), with *CgMT1* and *CgMT2* mRNAs increasing sevenfold relative to controls. The same genetic response was observed after joint exposure to Cd and Zn. In this condition, *CgMT1* and *CgMT2* presented a sevenfold and a fivefold increase, respectively. In contrast, no significant changes in the expression levels of *CgMT1* and *CgMT2* were observed between untreated and Zn-contaminated oysters.

Analysis of *CgMT3* gene expression revealed that the number of copies of this MT remained unchanged ($p > 0.05$) after Cd exposure (Fig. 4B). However, a significant increase was observed after 14 d of Zn treatment alone. Indeed, *CgMT3* ranged from 30 copies in the control to about 360 copies in the treated individuals. Interestingly, no increase in *CgMT3* gene expression was obtained in oysters exposed to Cd and Zn together.

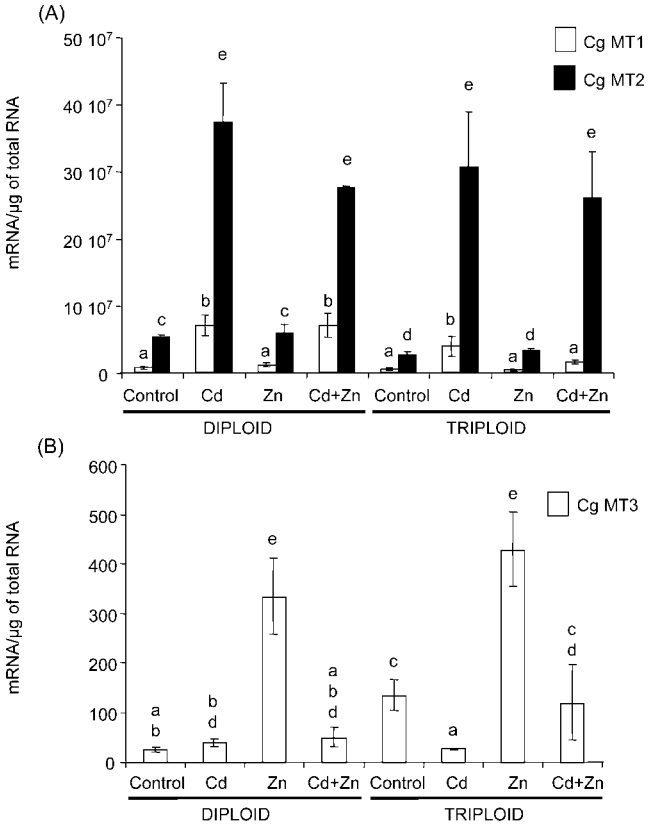

Fig. 4. Mean mRNA levels of (**A**) *CgMT1, CgMT2,* and (**B**) *CgMT3* (number of copies/μg of total RNA ± SE; $n = 3$) measured by quantitative real-time polymerase chain reaction in the gills of diploid and triploid oysters in control, cadmium, or zinc treatments or a combination treatment (0.133 μM Cd and 15.3 μM Zn) after 14 d (values annotated by the same letter are not statistically different, $p > 0.05$).

Triploid oysters. When compared to diploid oysters, the basal expression of *CgMT1* in triploids appeared comparable (0.7×10^7 copies/μg of total RNA, $p > 0.05$). However, in controls, the number of *CgMT2* copies in triploids (2.7×10^7 copies/μg of total RNA), was roughly half that in diploids, whereas *CgMT3* was five times more abundant in triploid than in diploid oysters.

Expression levels of the *CgMT1* and *CgMT2* genes increased in Cd-treated individuals compared with controls ($p < 0.05$), with a sevenfold and 11-fold increase, respectively (Fig. 4A). Similarly, a threefold and a ninefold increase in expression was observed for these two genes after Cd+Zn treatment. As in diploid oysters, induction of *CgMT1* and *CgMT2* expression did not occur after Zn exposure. It is to be noted that the induced expression levels for *CgMT1* and *CgMT2* were similar after treatment with Cd alone or Cd+Zn in diploid and triploid oysters.

Again, the *CgMT3* gene expression level remained unchanged ($p > 0.05$) after Cd and Zn exposure, whereas a decrease was observed when Cd was used alone. Exposure to Zn alone led to a significant threefold increase in expression of the *CgMT3* gene in triploid oysters, which corresponded to the same final level as in diploid oysters.

DISCUSSION

As in vertebrates, one of the response capacities of oysters to heavy metal exposure is MT protein biosynthesis through MT gene expression. Several studies have shown that hetero-

zygosity in triploid oysters could explain their growth performances, via a significant increase in feeding rates and in absorption/excretion processes [2,16,17]. In this context, significant differences could be expected in metal bioaccumulation and MT biosynthesis between diploid and triploid oysters. Nevertheless, our results revealed similar responses in terms of MT gene expression, MT protein synthesis, and Cd accumulation in gills. Only Zn bioaccumulation showed higher significance levels in diploid oysters (Fig. 2B).

We report for the first time the differential expression levels of MT isoform genes from *C. gigas*. *CgMT2* appeared to be more expressed, *CgMT1* was present at a lower copy number, and *CgMT3* seemed to be trivial compared with the other MTs. The MT genes exhibited similar levels of expression whatever the ploidy, suggesting a dosage regulation of these gene expressions in *C. gigas*. Such regulation has been reported in diploid, triploid, and tetraploid corn (*Zea mays*), for which the analysis of 18 gene expression levels evidenced that most genes were expressed equally regardless of the chromosome copy number [18]. In the same way, some genes investigated in the silkworm of *Bombyx mori* showed the same expression levels in diploid and triploid strains [19]. From these studies, a dosage compensation of MT expression can be hypothesized in *C. gigas*. In addition, another mechanism that could explain our results would be a silencing of expression of the additional copy of the MT allele (parental imprinting) [8,20].

The same order of expression level (i.e., *CgMT2* > *CgMT1* ≫ *CgMT3*) was conserved after induction by Cd. That *CgMT2* was more expressed than *CgMT1* could be related to its molecular organization. Indeed, *CgMT1* possesses a classical organization with one α domain and one β domain, which are known to bind four and three metal ions (Cd or Zn) in mammals, respectively [21]. In contrast, *CgMT2* exhibits an additional β domain and could potentially bind three additional ions. In this context, a previous study evidenced that *E. coli* BL21 transformed with a periplasmic-addressed *CgMT2* construct appeared to grow faster than those transformed with *CgMT1* when Cu^{2+} or Cd^{2+} were added to the culture medium [9]. From this analysis, *CgMT2* has been hypothesized to have a greater detoxification capacity than *CgMT1*.

The expression level of *C. gigas CgMT3* appeared to be induced only in response to Zn exposure; Cd and Zn mixture had no effect. The MT isoforms induced by specific metals have been reported in invertebrates. Cadmium-specific and Cu-specific MTs have been described in the crab *Callinectes sapidus* [22,23] and in the periwinkle *Littorina brevicula* [24]. However, no metal-specific role could be hypothesized for *CgMT3* because of its low level of gene expression. The total copy number of *CgMT3* remains extremely low compared with *CgMT1* and *CgMT2*, whereas after Zn exposure, it increased to 334 and 429 in diploid and triploid oysters, respectively ($p > 0.05$). This very low expression of *CgMT3* suggests that this MT has probably no significant physiological functions under metal exposure or could be only expressed in particular developmental stages in oysters.

Zinc did not induce *CgMT1* or *CgMT2* expression. This result disagrees with a model of MT gene induction by metals [25]. Indeed, in this model, heavy metals displace Zn bound to MTs, leading to an increased intracellular Zn concentration. Zinc is then able to activate the transcription factor MTF-1, which in turn induces MT transcription by binding to the metal regulatory elements present in the promoter region of MT genes. Thus, our results mean that in *C. gigas* this model of MT expression is not valid and that alternative posttranslational mechanisms of MTF-1 exist. This could explain, for instance, why in mammals, MTF-1 binds to metal regulatory elements boxes not only during Zn exposure, but also in response to some oxidative stresses (such as those generated by H_2O_2 or *t*-butylhydroquinone) [26]. The MT induction by a Cd-specific pathway also cannot be excluded. Indeed, recent studies on *Crassostrea virginica* suggest that MT induction by Cd could follow a specific cellular response not mediated by pathways involving Zn [27]. In this way, Cd-mediated metal regulatory element activation independent of MTF-1 and Zn has been reported in cells lacking functional MTF-1 [28]. Such observations have been reinforced by the description of a signal transduction pathway involving an upstream regulatory factor in Cd induction [26].

The MT protein concentrations followed the pattern of *CgMT1* and *CgMT2* gene expressions. Levels increased about twofold after exposure to Cd alone or in combination with Zn. However, this increase is less than the mRNA increase (about sevenfold) observed under the same conditions of treatment, suggesting that MT synthesis, in this species, is regulated at the transcriptional level as well as the translational level. Discrepancies between mRNA and protein levels were also previously reported in Antarctic fish [29,30], or blue mussels (*Mytilus edulis*) [31].

The results of this study showed comparable response in gill metal bioaccumulation and genetic/protein MT response in diploid and triploid oysters. This suggests that in *C. gigas*, the occurrence of a third set of chromosomes in triploid individuals is not subjected to modifications in MT isoform gene expression and MT levels. This result indicates that under the metal exposure conditions used during our experiment (15 μg Cd/L and 1 mg Zn/L, 14 d of exposure), triploid juvenile oysters have the same capacities to respond to Cd, Zn, or exposure to both as diploid oysters.

Acknowledgement—The authors thank Francis Maleyran for providing the oysters used in this experiment. V. Marie was supported by a grant from the Aquitaine Region.

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