Metallothionein gene identification and expression in the cockle (*Cerastoderma edule*) under parasitism (trematodes) and cadmium contaminations

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Abstract – Among benthic organisms, bivalves are often used as bioindicators of environmental pollution because of their high bioaccumulation capacities for heavy metals leading to metallothioneins (MT) induction. MT are small cytosolic metal-binding proteins involved in metal homeostasis and detoxification in living organisms. These proteins can also be induced by a wide range of factors, such as hormones, physical stress, parasitism. MT quantification in relation to parasitism is rarely reported in literature, while parasites are omnipresent and have deleterious impacts on bivalves. Moreover, only a few number of MT genes have been characterized in molluscs. This study describes the partial sequence of the MT gene (*Cemt1*) in the edible cockle *Cerastoderma edule*. The cockle's MT cDNA was sequenced and showed over 80% homology to several other bivalve MT sequences. This sequence was then used to determine MT specific primers which can be used in quantitative real time PCR. MT protein and gene expression levels were quantified for individuals selected under different conditions: free from or infected by the digenean trematode *Himasthla elongata*, and under cadmium exposure at 15 μg Cd L^{-1}. Results evidenced that MT concentrations were significantly increased by both treatments; parasite infection and Cd exposure. Moreover, congruent results between MT protein and gene expression levels were obtained.

Key words: Trematodes / Cockles / Cadmium / Metallothionein / Gene quantification

Résumé – Identification d'un gène de métallothionéine chez la coque (*Cerastoderma edule*) et analyse de son expression lors de contaminations par le cadmium et par des parasites (trématodes). Parmi les organismes benthiques, les bivalves sont souvent utilisés comme bioindicateurs des pollutions environnementales, du fait de leur importante capacité de bioaccumulation des métaux lourds conduisant à l'induction des métallothionéines (MT). Les MT sont de petites protéines cytosoliques capables de fixer les métaux et qui sont impliquées dans l'homéostasie et la détoxication de ces métaux chez les organismes. Ces protéines peuvent être induites par un grand nombre de facteurs tels que les hormones, les stress physiques, le parasitisme. La quantification des MT en relation avec le parasitisme est rarement reportée dans la littérature, bien que le parasitisme soit omniprésent et très délétère chez les bivalves. De plus, seulement un petit nombre de gènes de MT ont été identifiés chez les mollusques. Cette étude décrit la caractérisation de la séquence partielle d'un gène de MT (*Cemt1*) chez la coque *Cerastoderma edule*, qui présente plus de 80 % d'homologie avec de nombreuses séquences de MT de bivalves. A partir de cette séquence, des amorces spécifiques, utilisables en PCR quantitative en temps réel, ont été déterminées. Le niveau d'expression du gène *Cemt1*, ainsi que la quantité de protéine MT ont été analysés chez des individus soumis à différents paramètres : sans parasites ou infestés par le trématode digène *Himasthla elongata*, et lors d'expositions au cadmium à 15 μg Cd L^{-1}. Des résultats concordants ont été obtenus lors de la quantification des protéines MT et de l'analyse de l'expression du gène *Cemt1*. Ceux-ci mettent en évidence que les concentrations en MT augmentent significativement par les deux types de traitement (infestation parasitaire et exposition au cadmium).

1 Introduction

Coastal marine ecosystems are often contaminated by pollutants, especially metals, as a consequence of new industrial and agricultural technologies related to human activities.

Benthic invertebrates are frequently used as bioindicators for marine monitoring because of their relatively rapid response to anthropogenic and natural stress (Cheggour et al. 2001; Griscom et al. 2002; Lecoeur et al. 2004). More specifically, bivalves are chosen as sentinel organism to detect marine pollution essentially due to their large water filtration capabilities, their capacity to concentrate microparticles and their

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sedentary lifestyle (Cheggour et al. 2001; Griscom and Fisher 2004). Moreover, they are known to provide a space- and time-integrated measure of the environment dysfunction by a wide range of stress, and heavy metals in particular. Effects of heavy metals contaminations can be observed at a cellular scale. Heavy metals enter into cells by different ways like membrane transport or passive diffusion (Delnomdedieu et al. 1992). In cells, they generate oxidative stress (Stohs and Bagchi 1995) leading to DNA damage or lipid peroxydation, notably in the mitochondria, prior to cell apoptosis (Konigsberg et al. 2001). Different mechanisms are involved in metal detoxification including active efflux via pumps, which extrude heavy metals out of the cell and synthesis of metal-binding proteins, the metallothioneins (MT). These cytoplasmic proteins are involved in the homeostasis of essential metals (copper, zinc) and in the detoxification of toxic metals (cadmium, mercury). MT are able to sequester seven metal ions per MT molecule in mammals (Chan et al. 2002). In aquatic organisms MT synthesis is stimulated by Cd exposure (Baudrimont et al. 1999; Gonzalez et al. 2006; Marie et al. 2006). However, several confounding factors unrelated to metal contamination can affect the induction of MT and therefore influence metal uptake and accumulation: size (or age), sex, sexual maturity, handling, starvation, anoxia, freezing, hormones, second messengers, physical stress, and parasitism in target hosts (Baudrimont et al. 1997; Amiard et al. 2006; Baudrimont and de Montaudouin 2007; Baudrimont et al. 2006). These factors interfere with the mechanisms that regulate MT biosynthesis, making the validity of MT as biomarkers of metal pollution more difficult to interpret. Concerning parasitism as a stress factor, few data have been reported in the literature (Sures 2004; Baudrimont and de Montaudouin 2007; Baudrimont et al. 2006), while trematode parasites are omnipresent in European mollusc populations (Lauckner 1983; de Montaudouin et al. 2000; Desclaux et al. 2002, 2004). Moreover, these parasites are known to cause a large amount of damage to molluscs on an individual and a population scales, leading sometimes to host population mortality (Jensen and Mouritsen 1992; Desclaux et al. 2002; de Montaudouin et al. 2003; Desclaux et al. 2004; Thieltges 2006).

In the present study, we used the edible cockle (*Cerastoderma edule*) as a test organism. This species has been previously used as a bioindicator of metal contamination (Cheggour et al. 2001). Moreover, this exploited bivalve is also known to be frequently infected by a large number of trematodes (de Montaudouin et al. 2000; Desclaux et al. 2002; Russell-Pinto et al. 2006) such as *Labratrema minimus* which utilize cockles as primary host and *Himasthla elongata* which utilize it as second intermediate host. Our aim was to determine the cockle MT gene sequence and quantify its expression by real-time PCR under two forcing variables: digenean parasites (*L. minimus*, *H. elongata*) and Cd exposure.

2 Materials and methods

2.1 Sampling site and procedure

Cockles (*Cerastoderma edule*) were collected from the Banc d'Arguin (44° 40′N, 1° 10′W), an oceanic sandy area

of Arcachon Bay. This site is known to have a high specific richness of macroparasites in molluscs (de Montaudouin et al. 2000; Desclaux et al. 2002; Desclaux et al. 2006). Two digenean species were selected to perform experiments. *Labratrema minimus* is amongst the most deleterious species and uses the cockles as first intermediate host where it evolves into sporocysts, a germinative stage invading most tissues (Bowers 1969). The mean prevalence of *L. minimus* at Arguin is often less than 5% (Desclaux et al. 2002). Consequently, more than 1000 cockles (shell length >20 mm) were collected by hand to obtain a sufficient number of infected cockles. Another species, *Himasthla elongata*, which uses cockles as 2nd intermediate host, was utilized for two reasons. Firstly, this parasite can be manipulated in order to experimentally infect cockles (Wegeberg et al. 1999). Secondly, due to its less deleterious effect on the cockle, we can submit parasitized cockles to Cd exposure under laboratory conditions without any mortality. Collected cockles were transported to the laboratory where they were maintained at 15 °C, in an open sea water flow.

2.2 Cockle treatment

2.2.1 Cockles as first intermediate host

Cockles were individually placed in a 30-ml dish with filtered sea water at 20 °C during a period of 12–24 h. *Labratrema minimus* infected cockles shed cercariae larvae, allowing the separation of infected cockles from cockles free of this parasite. Nevertheless, cockles which do not emit cercariae are not necessarily parasite free (Curtis and Hubbard 1990). Therefore a larger number of unparasitized cockles were kept (more than 900) to be certain to obtain a control treatment.

Cockles infected by *Labratrema minimus* and uninfected cockles were transferred into synthetic sea water (instant ocean salts: 30‰), over 3 days, to stabilize their condition before starting the experiment. For the experiment, four experimental units (EUs) each containing 30 individuals were set up. On the first day of the experiment, half of the healthy and parasitized cockle's EUs were contaminated with cadmium at 15 μg L^{-1}. Throughout the experiment, several parameters were measured daily: temperature (15 °C ± 0.5), salinity (30‰ ± 0.5), dissolved oxygen (9 ± 0.5 mg L^{-1}), pH (8.5), photoperiod (12 h-light per day), nutrition (bivalves were fed twice a week with cultivated phytoplankton) and Cd concentration in the water just before daily adjustments with a stock solution of CdCl$_2$ (15.2 ± 0.3 μg L^{-1} in contaminated EU and 0.2 ± 0.4 μg L^{-1} in uncontaminated EU). Dead cockles were taken out of the EUs, frozen (−80 °C) and replaced by a new marked cockle to prevent eventual bacteria contamination and to maintain a constant cockle biomass. After 5 days of metal exposure, cockles were used to determine the MT gene expression by quantitative real time PCR analysis with specific primers that were determined in the present study. Prior to analysis, the cockles were squeezed between two glass slides under a stereomicroscope to confirm the absence of *L. minimus*.

2.2.2 Cockles as second intermediate host

Cockles were collected at the Banc d'Arguin and stabilized as previously described. Each cockle was infected by 160 cercariae of *Himasthla elongata* as decribed by Wegeberg et al. (1999). Cockles were considered infected after 24 hours of contact with cercariae. Four individuals were introduced into each EU, with or without Cd contamination (15 μg L^{-1}), for 7 days. For each exposure condition, samples were carried out in triplicate (=3 EUs). During one week, several abiotic parameters were measured daily: salinity (30.3‰ ± 0.3), water temperature (10.7 °C ± 0.2), pH (8.3 ± 0.04), dissolved oxygen (9.73 ± 0.3 mg L^{-1}), and Cd concentration in the water (15.1±0.2 μg L^{-1} in the contaminated EUs and 0.1±0.5 μg L^{-1} in the uncontaminated EUs). The photoperiod was 12 h per day and cockles in each EU were fed twice with diatoms (*Thalassiosira weissflogii*). After 7 days exposure, cockles were used to determine Cd bioaccumulation, MT protein and MT gene expression levels in the gills, in triplicate. Prior to analysis, cockles were autopsied to evaluate infection success (number of *H. elongata* settled in tissues) and to make sure that cockles were free of *L. minimus*.

2.3 Cadmium gills concentration

Bioaccumulation of Cd was determined in the gills. This organ is often considered as the most suitable tissue in many species (*Ruditapes philippinarum, Corbicula fluminea, Crassostrea gigas*) (Achard-Joris et al. 2006; Amiard et al. 2006; Marie et al. 2006). Moreover, as they are a respiratory epithelium in direct contact with the water, gills constitute an important site for metal uptake and storage where MT sequester a significant proportion of accumulated metals (Gundacker 1999; Lecoeur et al. 2004). In addition, gills are particularly appropriate in this experiment because either they are free of parasites when using the cockle as a second intermediate host (*Himasthla elongata*) or the presence of parasites using the cockle as first intermediate host (*Labratrema minimus*) is easy to detect.

After dissection, three biological samples per experimental condition were treated with 3 ml of pure nitric acid (Fluka; Buchs, Switzerland) in tubes at 100 °C for 3 h. After a six-fold dilution of the digestates with ultrapure water (MilliQ, Bedford MA, USA), Cd concentrations were determined by electrothermic atomic absorption spectrophotometry with Zeeman correction, using a graphite furnace tube (EAAS Thermoptec M6Solaar). Samples of 20 μl were mixed before atomisation with 4 μg of Pd (analyte modifier) and 3 μg of Mg(NO$_3$)$_2$ (matrix modifier). The detection limit was 0.1 μg L^{-1} Cd. The validity of the method was checked periodically by the measurement of certified biological reference materials (Tort-2: lobster hepatopancreas and Dolt-2: dogfish liver from National Research Council of Canada, Ottawa). Values were consistently within the certified ranges (data not shown). For Cd determination in EUs, water samples were diluted, acidified at 2% HNO$_3$ and mixed with Pd and Mg(NO$_3$)$_2$.

2.4 Metallothionein quantification

The concentration of total MT protein was determined by the mercury-saturation assay in gills, using cold inorganic mercury (Dutton et al. 1993; Baudrimont et al. 2003). MT analysis was conducted on 3 replicates per exposure condition, the saturation assay being repeated twice per sample. This technique is based on the quantification of Hg bound to the saturated MT. The denaturation of non-MT proteins was performed using trichloroacetic acid and the excess Hg not bound to the MT was removed by scavenging with lyophilised beef hemoglobin (Sigma) prepared in 30 mM Tris-HCl buffer (pH 8.2 at 20 °C). The final supernatant was then quantitatively recovered and used for Hg determination by flameless atomic absorption spectrometry (AMA 254, Altec, Prague, Czech Republic). The detection limit was estimated at 1 ng Hg. Owing to the fact that the exact quantity of Hg binding sites per MT molecule is unknown for this species, MT concentrations cannot be directly expressed in nmol MT g^{-1} (wet weight), but in nmol Hg g^{-1} (wet weight).

2.5 Total RNA purification

Total RNAs were extracted from 40 mg of fresh tissue using the Absolutely RNA RT-PCR Miniprep kit (Stratagene), 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. For each exposure condition, samples were carried out in triplicate.

2.6 Reverse transcription of RNAs

First-strand cDNA was synthesized from 5 μg total RNA using the Stratascript First-Strand Synthesis System (Stratagene) according to the manufacturer's instructions. The cDNA mixture was conserved at −20 °C until it was used in a real-time PCR reaction.

2.7 Primer design

MT gene sequences from bivalves (*Crassostrea angulata, C. gigas, C. virginica, Ostrea edulis, Mytilus galloprovinciallis, Perna viridis*), available in databases, were aligned using the ClustalW software. From this alignment, two sense primers named MTa (5'ATGTCTGATCCTTGTAACTG 3') and MTb (5'CACATTTACAGGTTTCTGGTCC 3') and one reverse primer named MTc (5'AACTACTTTACAGCCTGAACA 3') were deduced in conserved regions.

2.8 Real-time PCR

Real-time PCR reactions were performed in a LightCycler (Roche) following the manufacturer instructions (one cycle at 95 °C for 10 min, and 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

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ATG TCT GAT CCT TGT AAC TGC ACT GAG ACT GGA ACA TGT GTC TGC TCT GAT TCG   54
 M   S   D   P   C   N   C   T   E   T   G   T   C   V   C   S   D   S    18


TGT CCA GCA ACA GGA TGT AAA TGT GGA CCC GGA TGT AAA TGT GGT GAC GGG TGT  108
 C   P   A   T   G   C   K   C   G   P   G   C   K   C   G   D   G   C    36


AAA TGT TCA GGC TGT AAA GTA GTT                                          132
 K   C   S   G   C   K   V   V                                            44
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Fig. 1. Partial nucleotide sequence of this MT gene (*Cemt1)*. The resulting amino acids sequence is indicated and the primer sequences of MTa and MTc indicated in bold case.

2 μl of reverse transcribed product template, 1 μl of master mix including the SyberGreen I fluorescent dye (Roche), enabling the monitoring of the PCR amplification, and the gene-specific primer pair at a final concentration of 300 nM for each primer. Gene-specific primer pairs CeMT1 (5' CTGATC-CTTGTAACTGCACTGAG 3') and CeMT2 (5' ACAGC-CTGAACATTTACACCCGT 3') were determined using the LightCycler probe design software (version 1.0, Roche).

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 and quantified as previously described (Marie et al. 2006).

2.9 Statistical analysis

The effect of parasitism and Cd exposure (fixed variables) on gene and protein expressions (dependant variables) were tested using a crossed two-ways ANOVA. Prior to analysis, homogeneity of variance was verified and data were $\log(x + 1)$ transformed when necessary. A posteriori test of Tukey was applied to compare means. Analysis were performed using Statistica software.

3 Results and discussion

3.1 Isolation of MT gene sequence

In a first attempt to isolate an MT gene from *C. edule*, a cDNA was amplified using primers MTa and MTc deduced from conserved MT sequence alignments of various bivalves available in databases. This isolated product was then cloned and sequenced. The resulting sequence corresponded to a partial cDNA of 132 bp which was called *cemt1* (accession number EF397700). This cDNA encoded for a 44 aa protein (Fig. 1) presenting extensive homologies with several MT gene sequences from other bivalves at a protein level as well as a nucleotide level. Indeed, these homologies ranged from 84% with *C. virginica* to 97% with *C. gigas*. Homologies were significantly lower when the *Cemt1* sequence was compared to MT sequences from other invertebrates. Indeed, *Cemt1* exhibited homologies around 56% with the crustaceans *Carcinus maenas* and *Artemia franciscana* to 70% with the fruit fly *Drosophila melanogaster*. The *Cemt1* sequence exhibited a high content of cysteine residues arranged in typical Cys-X$_{(1–3)}$-Cys motifs and no aromatic

Table 1. Results of the ANOVA testing the effect of parasite and cadmium contamination on *Cerastoderma edule* MT gene expression and/ MT protein concentration. In bold: significant effects.

Parasite	Variable	Source of variation	p
Labratrema minimus	Gene expression	Parasitism (a)	**<0.05**
		Cd contamination (b)	**<0.001**
		(a) × (b)	**<0.05**
Himasthla elongata	Gene expression	Parasitism (a)	> 0.05
		Cd contamination (b)	> 0.05
		(a) × (b)	> 0.05
	MT concentration	Parasitism (a)	**<0.05**
		Cd contamination (b)	**<0.05**
		(a) x (b)	> 0.05

amino acids residues. Moreover, no Cys-Cys motifs, classically found in the α domains of mammals MT or in other invertebrates such as the crabs *Cancer pagurus* (Overnell et al. 1988) and *Carcinus maenas* (Pedersen et al. 1994), were evidenced in *C. edule*. This finding is in agreement with previously reported MT amino acids sequences from bivalves where only Cys-X$_{(1–3)}$-Cys were observed (Tanguy et al. 2001). The *Cemt1* sequence represented about 65% of the total coding sequence (Fig. 2).

3.2 Cemt1 gene expression in cockles as first intermediate host

From the obtained sequence, specific primers were determined and gene expression levels were investigated by quantitative real time RT-PCR in the gills from cockles free of parasites or parasitized by the digenean *L. minimus* (Fig. 3). There was a significant effect of Cd contamination ($p < 0.05$) and parasite infection ($p < 0.001$) on gene expression (Table 1). In unparasitized cockles, MT gene expression was high ($>2 \times 10^6$ MT copies by μg of total RNA) and was not affected by Cd contamination ($p > 0.05$). This result could be explained by the fact that the cockles were sampled during high gametogenesis activity leading to an important MT background in these bivalves. Important concentration of MT in unparasitized cockles was already observed at the protein level and was explained by the gonadal maturation phase in cockles (Baudrimont et al. 2006). On the contrary, parasitized cockles presented a very low basal expression. However, Cd contamination lead to a 9-fold increase in MT gene expression ($p < 0.05$) in these individuals. This observation was already reported at the protein level for the same bivalve parasite species

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M.galloprovincialis    MPGPCNCIETNVCICGTGCSGKCCQCGDACKCA-SGCGCSGCKVVCRCS--GTCACG-CD
P.viridis              MPSPCNCIETQVCICGTGCSGEGCRCGDACKCS-XGCGCSGCKVVCKCQP-GECACG-KQ
B.thermophilus         ---PCNCVETNVCICDTGCSGDGCRCGDACKCSGADCKCSGCKVVCKCSA-GSCECG-KG
M.edulis               MPAPCNCIETNVCICDTGCSGDGCRCGDACKCSGADCKCSGCKVVCKCS--GSCECG-KG
T.granosa              ---PCNCVKGGDCTCSSSCSGDSCRCGDSCKCA-SGCACSNCRVKCSCS--GSCACG-KG
C.virginica            MSDPCNCTETGTCVCSNSCPATGCKCGPGCKCG-DECKCAGCKVKCNCSSEGGCKCGDEK
C.edule                MSDPCNCTETGTCVCSDSCPATGCKCGPGCKCG-DGCKCSGCKVV--------------
C.angulata             MSDPCNCIETGTCVCSESCPATGCKCGLGCKCG-DGCKCSGCKVKCNCS--GSCGCG-KG
C.gigas                MSDPCNCTESGTCVCSDSCPATGCKCGPGCKCG-DGCKCSGCKVKCNCS--GTCGCG-KG
O.edulis               MSDPCNCKTTGNCLCSDTCPSTGCNCASGCKCG-AGCKCPGCQIVCKCN--STCACG-VG
                       ****      * *.   *..  *.*.  .***.    * *..*::
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M.galloprovincialis    CTGPINCKCESG--CSCK------
P.viridis              CTGPDTCKCDSS--CSCK------
B.thermophilus         CTGPSTCRCAPG--CSCK------
M.edulis               CTGPSTCKCAPG--CSCK------
T.granosa              CKGPATCECAPGS-CSCK------
C.virginica            CTGPATCKCE--SGCSCKK-----
C.edule                ----------------------
C.angulata             CTGPENCKCANDSGCVCKVKCNCS
C.gigas                CTGPENCKCANDSGCGCKK-----
O.edulis               CTGPDTCKCAADSGCSCK------
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Fig. 2. Clustal W multiple sequence alignment of the amino acid sequences, of the protein CeMT1 with the MT gene from *Mytilus galloprovincialis* (AY566247), *Perna viridis* (AF092971), *Bathymodiolus thermophilus* (AJ517188), *Mytilus edulis* (AJ577131), *Tegillarca granosa* (AY568678), *Crassostrea virginica* (X59862), *Crassostrea angulata* (AF349907), *Crassostrea gigas* (AJ242657) and *Ostrea edulis* (AJ306365). Identical residues are marked with *, equivalent amino acid are indicated by . or :.
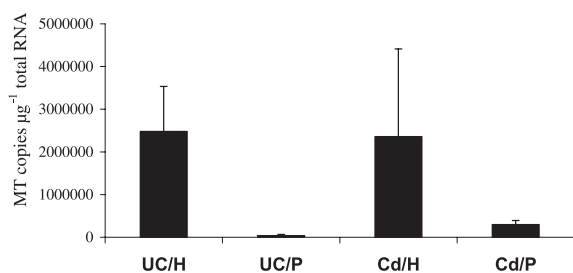

Fig. 3. Expression of MT from cockle gills (*C. edule*) at the gene level, under several conditions, after 5 days of experiments (mean ± SE, $n = 3$). UC = uncontaminated cockles; Cd = cockles contaminated by Cd at 15 μg L^{-1}; P = parasitized cockles by *Labratrema minimus*; H = healthy cockles.

(Baudrimont and de Montaudouin 2007) but with a lower MT concentration increase (×3). Parasite infection could lead to cellular necrosis of tissues inducing cytokins synthesis and activate γ interferon, both mechanisms that could stimulate MT synthesis (Roesijadi 1992). On the other hand, parasitism is associated with a dramatic decrease in MT gene expression, compared with unparasitized cockles and independently of Cd contamination (78-fold decrease factor, $p < 0.05$). This result is due to the castrating effect of *L. minimus* on cockles (Bowers 1969).

Fig. 4. a: MT and Cd concentrations in the gills of cockles (*C. edule*), under several conditions, after 7 days of experiments (mean ± SE, $n = 3$). **b:** Expression of MT from gills of cockles (*C. edule*) at gene and protein levels, under several conditions, after 7 days of experiments (mean ± SE, $n = 3$). UC = uncontaminated cockles; Cd = cockles contaminated by Cd at 15 μg L^{-1}; P = parasitized cockles by *Himasthla elongata*; H = healthy cockles.

3.3 MT protein and gene expression levels in cockles as second intermediate host

After the partial gene sequence and the use of specific primers in the determination of MT gene copies expression, another one-week experiment was performed to compare MT induction at protein (MT) and gene levels (Fig. 4). Another digenean trematode, *Himasthla elongata*, was used. The
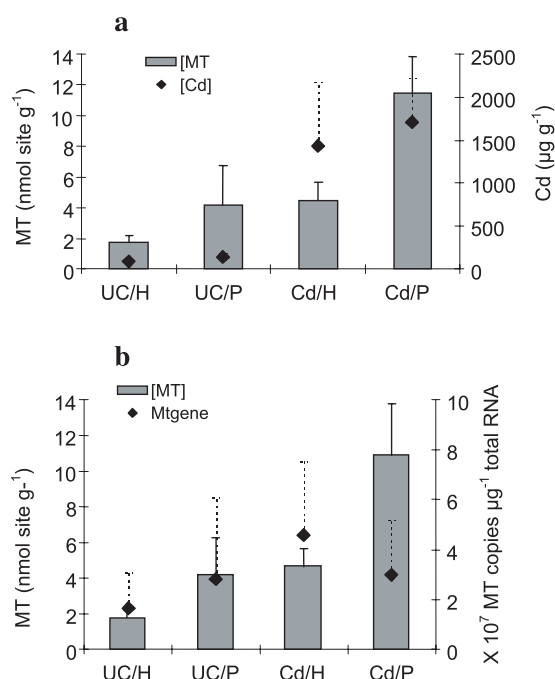
advantage of this parasite is that infestation can be experimentally performed, allowing to know the exact date of infection (=stress) (Wegeberg 1998; Baudrimont and de Montaudouin 2007). Cercariae larvae use penetrating glands to perforate

Table 2. Amplification factors of MT gene expression and MT proteins levels under the different conditions, in comparison with the control: uncontaminated healthy cockles (UC/H).

Exposure conditions	Gene	Protein
Parasitism	1.9	2.3
Cd exposure	3	2.4
Parasitism + Cd exposure	1.9	5.8

host tissues where they transform into metacercariae, which is a latent stage of the parasite (Haas 2000). The mean parasite abundance in cockles after experimental infestation was 63.8 ± 18.4 metacercariae per cockle ($n = 6$).

MT protein levels were quantified by mercury saturation assay in gills as well as the Cd bioaccumulation in this tissue (Fig. 4a). MT concentrations were significantly affected ($p < 0.05$) by both treatments, parasite infection and Cd exposure (Table 1). Results showed a good correlation between Cd uptake and MT concentration in individuals. In unparasitized cockles, Cd exposure resulted in a 2.4-fold increase of MT protein content. The same olds true for parasitized individuals, where a 2.5-fold increase was observed after Cd exposure. In the same way, *H. elongata* lead to a significant increase in MT proteins. This finding is in disagreement with recent reports that evidenced the alteration of the MT protective effect in parasitized individuals after Cd exposure (Baudrimont and de Montaudouin 2007). However, these studies were conducted at the whole organism level and the observed effects in gills could have been hidden. Thus, our results evidenced that MT responses should be investigated at the organ level.

No significant effect of Cd and parasitism was observed on MT gene expression ($p > 0.05$), due to a high inter-individual variability (Table 1). However, observed tendencies showed congruent results between MT protein and gene expression levels (Fig. 4b). Indeed, in uncontaminated cockles, parasitism resulted in a 2.3-fold increase in MT protein level and a 1.9-fold overexpression of the gene *Cemt1* (Table 2). In the same way, a 3-fold increase in expression level was evidenced between uncontaminated and contaminated healthy cockles, which was similar to the increase in MT protein (2.4-fold). The only difference concerned the level of MT gene expression in parasitized individuals with or without Cd exposure. Indeed, the expression level remained unchanged between these two conditions, while MT protein concentrations appeared more important after Cd exposure (5.8-fold compared to controls). In fact, in the case of double stress (parasitism and Cd exposure) a decrease in the MT gene expression level ($\times 1.9$) was observed. These discrepancies observed between MT protein levels and the regulation of MT gene expression could be explained by a time delay between gene expression and protein accumulation, suggesting that MT synthesis in *C. edule* is regulated at the transcriptional level as well as the translational level. Such discrepancies have already been reported in mussels (Lemoine and Laulier 2003) or more recently, in different tissues of the zebrafish *Danio rerio* after Cd exposure (Gonzalez et al. 2006). Nevertheless, in all cases, these results showed that MT gene expression and MT protein concentration give complementary results and should both be investigated during Cd contamination studies.

Results concerning *H. elongata* impact were opposite to what was observed with *L. minimus*, where MT gene expression was inhibited in parasitized cockles (Fig. 3). The major difference between these two digenean species is that *H. elongata* does not castrate or even impair reproductive cycle of its host, and its effects on MT synthesis are consequently not masked by cockle reproductive status.

4 Conclusion

This study determined for the first time the partial sequence of *Cerastoderma edule* MT gene, *cemt1*. Results evidenced a good relationship between MT protein and gene expression levels in the gills of cockles, except in the case of double stress; parasitism and Cd exposure. However, depending upon the parasite species some physiological parameters like the reproductive cycle could hide the genetic response of MT during Cd exposure. Finally, this study again highlights the controversial question about the use of MT as a biomarker of metal contamination (Cd) in the environment. Effectively, the high variability of MT gene induction with Cd, reproductive event and parasitism omnipresence could induce false signals of the biomarker. The advantage to use the competitive RT-PCR to determine such responses is that it is a sensitive, qualitative and/or quantitative method, requiring very small tissue samples. However, our results evidenced that MT gene expression and MT protein concentrations give complementary results and therefore could not be investigated separately during Cd contamination studies.

Now, our future prospects are (1) to complete the sequence of *Cemt1*, (2) to find the other MT isoforms (by building and screening a cDNA library with *Cemt1* as probe), (3) to find the MT gene sequence of the parasites, to determine its implication in the MT response at the protein level and its involvement during heavy metal contamination.

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