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Functional characterization of four metallothionein genes in *Daphnia pulex* exposed to environmental stressors

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Abstract

We characterized the metallothionein genes (*Mt1*, *Mt2*, *Mt3*, and *Mt4*) in *Daphnia pulex* on both molecular and ecotoxicological level. We therefore conducted a bioinformatical analysis of the gene location and predicted protein sequence, and screened the upstream flanking region for regulatory elements. The number of these elements and their positions relative to the start codon varied strongly among the four genes and even among two gene duplicates (*Mt1A* and *Mt1B*), suggesting different roles of the four proteins in the organisms' response to stress. We subsequently conducted a chronic 16-day exposure of *D. pulex* to different environmental stressors (at sublethal levels causing approximately 50% reduction in reproduction). Based on prior knowledge, we exposed them to the metals Cd, Cu, and Ni, the moulting hormone hydroxyecdysone (20E), and the oxidative stressors cyanobacteria (*Microcystis aeruginosa*), and paraquat (Pq). We then compared mRNA expression levels of the four *Mt* genes under these stress conditions with control conditions in "The Chosen One" clone (TCO), for which the full genome was sequenced and annotated. All together, the mRNA expression results under the different stress regimes indicate that different *Mt* genes may play different and various roles in the response of *D. pulex* to stress and that some (but not all) of the differences among the four genes could be related to the pattern of regulatory elements in their upstream flanking region.

Keywords

Metallothionein; mRNA expression; *Daphnia*; Environmental toxicology

1. Introduction

The rapid evolution of molecular techniques has initiated a new approach in environmental science and risk assessment to link molecular and ecotoxicological responses. The interest in environmental genomics, an emerging field that combines higher-throughput molecular studies of natural populations with current ecotoxicological approaches, fuels the further development of molecular tools with an ecological perspective. Thereby, it offers more insights into the specific biological responses of organisms to pollutants (Chen et al., 1999;

Schwarzenberger et al., 2009; Shaw et al., 2007; Poynton et al., 2007). Moreover, a well-founded identification and understanding of underlying molecular mechanisms will lead to a more effective risk assessment (Amiard et al., 2006; Watanabe et al., 2007).

Here, we apply molecular tools to the currently best studied stress response systems in organisms exposed to metals: the metal-binding proteins or metallothioneins (MTs) (Nordberg, 1998; Thirumoorthy et al., 2007). These proteins have an unusual structure characterized by a lack of secondary structure in the absence of metal ions, high metal content, high cysteine content and the absence of histidine or aromatic residues. The cysteines are arranged in metal-thiolate clusters to form specific metal binding sites. In contrast, the primary structure of MTs is exceedingly variable, solely conserved in highly related species (Shaw et al., 2007). Thus, classification of MTs is not straightforward. Moreover several paralogs often exist within the genome of one species, complicating the task even further (Janssens et al., 2009; Nordberg, 1998).

MTs are important in metalloregulatory processes due to their binding with metals, primarily with metals of group 1B and 2B of the Table of Mendeljev. MTs are involved in several cellular processes: metal transport and storage, (de)activation of zinc-regulated proteins and scavenging of free radicals. However, MTs are primarily known for their detoxification function in metal poisoning. MTs bind certain free metal ions or metals bound to certain ligands. Consequently, metal poisoning is avoided in the cell (Janssens et al., 2009). MT proteins are encoded by *Mt* genes, whose expression is strongly regulated by certain environmental factors, such as exposure to metals, hormones, hypoxia and oxidative stress (Kägi, 1991). Due to the strong correlation between *Mt* expression and metal concentration in the environments, MT levels are used to predict or diagnose metal exposure in a wide variety of freshwater ecosystems. Moreover, MTs are highly specific and have differential sensitivity to metals (Haq et al., 2003). Yet, little is known about the potential correlation of MT regulations and functions with other environmental stressors. This deficiency could impact their use as biomarkers, particularly if these stressors influence metallothioneins in a similar manner as metals. Until more is known, care should be taken when implementing metallothioneins as biomarkers for metals.

Metallothionein stress response systems have already been investigated in several invertebrate organisms: *Orchesella cincta*, *Caenorhabditis elegans* and *Lumbricus rubellus* (Janssens et al., 2009; Moilanen et al., 1999; Stürzenbaum et al., 2004). Yet, much variability still remains in studies concerning the sensitivity and response of metallothioneins to environmental stressors (Amiard et al., 2006). In addition, the majority of these studies focus on the response of metallothionein to acute exposures of metals. Often, these exposures are in contrast to those in the natural environment, where metals are often present in lower concentrations for prolonged time periods. Under these circumstances, pollutants affect the organism more gradually and strong effects are not immediately observed. Furthermore, chronically exposed organisms can acclimate or even adapt to the presence of metals (Klerks and Weis, 1987; Lopes et al., 2006, 2004; Ward and Robinson, 2005). Thus, to fully understand the metallothionein response system, it is crucial to study on both acute and chronic responses.

In *Daphnia pulex*, three *Mt* genes were discovered by Shaw et al. (2007) by investigating cadmium stress. *D. pulex*, commonly known as the waterflea, is routinely studied in ecology, ecotoxicology and evolutionary biology because of the diversity of traits observed among ecomorphs within the species complex. Its ubiquitous presence in lentic freshwater ecosystems and its central role in food webs have contributed to their widespread use in research. The *Daphnia* system is now widely recognized and used in regulatory risk assessments and was recently recognized by the National Institutes of Health (NIH) as a

model for biomedical research because of its ability to link molecular responses to environmental factors (NIH, 2011). Its mode of reproduction, called cyclical parthenogenesis (Innes and Herbert, 1988) is ideally suited to such studies by replicating clonal isolates across environmental gradients. Furthermore, recent efforts by the *Daphnia* Genomics Consortium resulted in a range of molecular tools available for this model species in addition to the fully sequenced genome (Colbourne et al., 2011).

Hence, we focus our study on the metallothionein stress response system in this sentinel and surrogate species, by measuring gene transcription levels in conventional ecotoxicological experiments; the biosynthesis of metallothioneins is mostly dependent on the activation cis-regulatory elements such as metal and oxidative responsive elements and thus transcriptionally regulated (Haq et al., 2003; Miles et al., 2000).

We investigate the metallothionein stress response system at three levels. First, we characterize the different metallothioneins in *D. pulex*. More specifically, we compare the protein sequences of these different loci as well as screen their promoter region for the presence of responsive elements. Second, we study the organismal response of *Daphnia* chronically exposed to different types of stressors and will determine the median effect concentration on reproduction (EC50). This value is the concentration at which reproduction declines by 50% in the isolates exposed to the treatment compared to the same clonal isolates under a benign control condition. Third, we study the mRNA expression of the metallothioneins in organisms exposed to those different types of stressors at the EC50 level focusing on longer-term (16-day) exposures with quantitative reverse transcription PCR. Finally, mRNA expression levels of these loci are correlated with the discovered cis-regulatory elements and with their sequence similarity (bioinformatically characterized) to achieve a better mechanistic understanding of the stress response system. We specifically focus on mRNA expression for a number of reasons. First, expression of metallothioneins is primarily controlled at the level of transcriptional regulation (Roelofs et al., 2007; Isson and Kille, 1997). The expression is mostly dependent on conserved cis-acting regulatory elements and transcription factors induced by environmental factors (Amiard et al., 2006; Haq et al., 2003; Miles et al., 2000). Second, mRNA expression has a much shorter half-life than proteins (Amiard et al., 2006), which are mostly used in biomarker detection: using mRNA levels thereby allows for a more accurate detection of sudden changes in the environment. Furthermore, contrary to the majority of studies on metallothioneins, we focus on chronic exposure to low concentrations instead of acute exposures to high concentrations as the latter are less environmentally relevant. Thus, this study is one of few that compare chronic results to the current known effects occurring at acute doses reported in the literature.

We choose to study six different types of stressors, based on reported experiments in the literature. We expose *D. pulex* to three metals cadmium, copper and nickel (Roelofs et al., 2007; Shaw et al., 2007). In addition, *D. pulex* is exposed to paraquat, a broad spectrum herbicide known to induce the production of reactive oxygen species (ROS) (Suntres, 2002) and increased levels of antioxidant enzymes in *Daphnia magna* Straus after sublethal exposure (Barata et al., 2005). Haq et al. (2003) reported the presence of antioxidant response elements in the promoter region of metallothionein genes and subsequent induction of metallothionein expression following ROS exposure. Furthermore, we expose *D. pulex* to 20-hydroxyecdysone (20E), a steroid hormone commonly found in arthropods (Lafont and Dinan, 2003). Roelofs et al. (2007) demonstrated an inhibition of metallothionein expression after 20E exposure. 20E affects metallothionein expression through interaction with the 20-hydroxyecdysone responsive element in the upstream promoter region of the gene. Finally, we also expose *Daphnia* to *Microcystis aeruginosa*, a toxic cyanobacterium with low food quality and capable of producing several toxins such as microcystin. *Microcystis* exposure

has been linked to oxidative stress in a variety of organisms (Ding et al., 1998; Li et al., 2003; Pinho et al., 2003) In addition, both Chen et al. (2005) and Wiegand et al. (2002) observed oxidative stress damage in *D. magna* upon exposure to *Microcystis*.

2. Material and methods

2.1. Bioinformatical characterization

The *D. pulex* draft genome sequence version 1.1 is available online at <http://wfleabase.org> (Colbourne et al., 2005) and was screened for the presence of metallothionein genes through the functional search window. For all genes, scaffold location and nucleotide position were noted as along with the protein sequence. Afterwards all protein sequences were aligned with the MAFT version 6 program using E-INS-I strategy (Katoh, 2010). Similar to Shaw et al. (2007), the scoring matrix for the protein sequences was Blosom62 and the alignment parameters included a gap opening penalty of 3 and a gap extension penalty (offset value) of 0.15. This alignment was then used to construct a neighbor-joining tree including 1000 bootstrap pseudo-replicates with a poisson correction as well as pairwise deletion of the alignment gaps by MEGA version 6. (Tamura et al., 2007) Metallothionein sequences of other species were obtained through NCBI database based on Shaw et al. (2007).

Two approaches were used to gain the maximum amount of information from the metallothionein sequences. First, a transcription element search system (TESS; available online on <http://www.cbil.upenn.edu/cgi-bin/tess/tess>) was utilized to discover transcription elements and their corresponding transcription factors. Therefore, the promoter region of each *Mtm* gene was downloaded from <http://wfleabase.org>. The promoter region was arbitrarily chosen as 3000 basepairs upstream of the predicted transcriptional starting position of the annotated gene model (except for *Mt4* gene model which begins at the translational start site). Based on Egg et al. (2009), the string scoring setting parameters were modified. Additional information on the transcription factors was obtained with a subprogram of TESS, 'query for transcription factor info'. Second, the promoter regions were screened for the presence of some specific regulatory elements using GenePalette (Rebeiz and Posakony, 2004). The choice of regulatory elements was based on Roelofs et al. (2007). These included regulatory elements and their corresponding transcription factors known for metallothionein induction, stress response, cell and moult cycle regulation (Table 1). When the TESS results revealed a high number of potential binding sites for heat shock TBFs, an additional query for the heat shock regulatory element consensus sequence was conducted (Amin et al., 1988). This conserved consensus sequence, which in addition to the longer length (10 bp), increases the reliability of the query compared to shorter motifs queried by TESS.

The results of both approaches were combined in a visual representation of the genes with the software GenePalette. This software allows easy representation of genes with promoter, intron and exon regions and marks key features such as regulatory elements on the sequences. Such a type of visual representation simplifies comparison between different genes. For the TESS approach, only transcription factors with a *p*-value better than 0.05 were retained for further study and imported into Gene Palette. Transcription factors with a *p*-value between 0.05 and 0.10 were retained if the log likelihood value was greater than 10. In addition, binding sites with an unknown transcription factor, a transcription factor with an unknown function or transcription factor with an irrelevant function in the test organism (e.g. TBF for leaf formation) were omitted.

Finally, the *Daphnia* genome (available online: <http://wfleabase.org>) was screened for the presence of genes to generate such TBFs as TESS is a mathematical software and thus can generate TBFs only present in specific organisms.

2.2. Ecotoxicological experiments

2.2.1. Animals—*D. pulex* used in this study were obtained from isoclonal laboratory cultures of an isolate obtained from the laboratory of Shaw (School of Public and Environmental Affairs, Bloomington, IN, USA; Shaw et al., 2007). Animals were cultured in no N, no P COMBO medium (Shaw et al., 2007) in 4 L polyethylene aquaria in a thermostatic room (20 ± 1 °C) under a constant photoperiod (16:8 light–dark). They were fed daily with *Ankistrodesmus falcatus* at a rate of 1.5 mg C L^{-1} . The medium was renewed three times a week.

Neonates (<24 h old) from these cultures were isolated for the chronic toxicity tests and the batch exposure experiments.

2.2.2. Chronic toxicity tests—Chronic toxicity tests (21 days) were performed in 25 mL polyethylene vessels, each containing one neonate according to the OECD protocol for toxicity tests with *Daphnia*. All treatments consisted of 10 replicates. In each treatment, animals were monitored daily for survival and reproduction. Observations consisted of noting (1) whether the animal had reproduced and (2) the number of juveniles from each brood. Reproduction was finally quantified as the total number of juveniles per surviving female after 21 days of exposure. If the animal reproduced, neonates were counted and removed from the vessel. During the experiment, pH of old and new media was monitored at regular intervals. Samples of the medium and maintenance stock solutions were taken for concentration analysis of the toxic component. These samples were stored in the dark at 4 °C in polyethylene tubes after filtering them with a $0.45 \mu\text{m}$ Acrodisc Syringe Filter.

The experimental design of the copper toxicity test consisted of a geometric range from 5 to $160 \mu\text{g Cu L}^{-1}$. Maintenance stocks were made with $\text{CuCl}_2 \cdot \text{H}_2\text{O}$ (analytical grade, VWR International, Haasrode, Belgium). Solutions were added to COMBO medium at least 24 h prior to use. Samples for concentration analysis were acidified with 1% (v/v) HNO_3 (Normatom™ Ultrapure 65% HNO_3 , VWR, Leuven, Belgium). Copper concentrations were determined with a graphite furnace AAS (GF-AAS, SpectrAA800 with Zeeman background correction, Varian, Mulgrave, Australia).

Test concentrations in the chronic 20-hydroxyecdysone toxicity test covered a geometric range from 10 to $160 \mu\text{g 20E L}^{-1}$. Test solutions were prepared with 20E (93% 20-hydroxyecdysone, Sigma–Aldrich, Bornem, Belgium) dissolved in a small amount of ethanol. To ensure effects in treatments were not due to effect of ethanol, the same amount of ethanol was added to the medium of the control treatment. 20E concentrations in samples were determined through a cell transfection assay protocol developed by the Laboratory of Agrozoology (Prof. Dr. ir. G. Smagghe), Ghent University (Mosallanejad et al., 2008).

The chronic nickel toxicity test covered the following range of nickel concentrations: 62.5, 88.39, 125, 176.78 and $250 \mu\text{g Ni L}^{-1}$. All test solutions were prepared prior to use with culture media from stocks made with $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (analytical grade, Merck, Darmstadt, Germany). Nickel concentrations in samples of media and stocks were determined with graphite furnace AAS (GF-AAS, Spec-trAA800 with Zeeman background correction, Varian, Mulgrave, Australia).

The test design for the chronic paraquat toxicity test consisted of a geometric range of paraquat concentrations from 10 to $160 \mu\text{g paraquat L}^{-1}$. All test solutions were prepared prior to use with culture media from stocks made with paraquat (analytical standard, paraquat dichloride or pestanal, Fluka, Sigma–Aldrich, Bornem, Belgium). As paraquat absorbs easily to glass, all solutions were prepared and maintained in polyethylene beakers.

Samples of the medium and paraquat stock solution for paraquat concentration analysis were filtered with a 0.45 µm Acrodisc Syringe Filter and analyzed by Fytolab (Ghent, Belgium).

Analysis of the population characteristics and calculation of the EC50 for reproduction for all toxicity tests was performed with the software package Statistica 6 (Statsoft, Tulsa, OK, USA). The final EC50 calculation was based on the log logistic model proposed by Van Ewijk and Hoekstra (1993).

2.2.3. Batch exposure experiments—These batch exposures provided the source material for further expression analysis and consisted of three replicates per treatment. Therefore neonates (<24 h) were exposed to EC50 concentrations of toxicant for reproduction for a period of 16 days in 2 L polyethylene aquaria. These EC50 concentrations were determined from the chronic toxicant exposures (Table 5). An exposure period of 16 days was chosen following standard guidelines for chronic or life-cycle toxicity tests with *Daphnia sp.*, recommending minimal test durations of 14 days for *Daphnia* species other than *D. magna* (OECD, 1984) or covering at least three brood releases ASTM (1997). The latter, often used in ecotoxicity literature, is also commonly referred to as the three-brood test for cladoceran species (e.g. Cowgill and Milazzo, 1991). Offspring of the animals was discarded and not used as starting material for RNA extraction.

During the experiment, pH of old and new media was monitored at regular intervals. Samples of the medium and maintenance stock solutions were taken for concentration analysis of the toxic component. These samples were stored in the dark at 4°C in polyethylene tubes after filtering them with a 0.45 µm Acrodisc Syringe Filter. Medium was renewed three times a week. Survival and reproduction were monitored during the change outs in an identical manner as during the chronic reproduction tests. Density of the animals was closely monitored to ensure that densities of all aquaria were never greater than 20–25 adults L⁻¹.

Microcystis aeruginosa treatment consisted of diet containing 50% *M. aeruginosa* and 50% *A. falcatus* in a final feeding suspension of 1.5 mg CL⁻¹.

As immediate mRNA extraction after 16 days of exposure was necessary (due to the instability of the RNA), not all batch exposure experiments could be started at the same time as it would make immediate mRNA extraction impossible. To exclude the effect of time, an additional control was started alongside exposures differing in time. Moreover, the goal of this study was to compare the metallothionein mRNA expression in organisms exposed to a certain stressor and non-exposed organisms. Hence, the comparison between stressors was not necessary and experiments did not need to be set up on the same time.

2.3. mRNA expression analysis

2.3.1. mRNA Extraction—After 16 days of exposure, animals were isolated and total RNA was immediately extracted using an RNeasykit and Qiashredder (Qiagen, Venlo, Netherlands) following manufacturer's protocol. DNA contamination was removed by a DNase treatment (Qiagen, Venlo, Netherlands). Prior to cDNA transcription, RNA quality and quantity were determined with a nanodrop spectrophotometer (low quality: min. phenolate ion contamination: 260/230 <2.1, min. protein contamination: 260/280 < 1.9). Subsequently aliquots for reverse transcriptase were prepared and stored separately. Both these aliquots and RNA samples were stored at -80°C.

2.3.2. Reverse transcription—These aliquots were used to reverse transcribe 1 µg of RNA to cDNA with the MessageAmp™ II mRNA Amplification kit (Applied Biosystems, Carlsbad, CA, USA) following manufacturer's protocol. Only the first strand cDNA

synthesis was executed. Again sample quality and yield were determined with the nanodrop spectrophotometer. Samples with low yield (<2000 ng/μL) were reverse transcribed again. Finally samples were stored in nuclease free water at -20°C at final concentration of 2000 ng/μL until qPCR analysis. Quantitative real-time PCR was used to discover significant differences in metallothionein expression after chronic exposure to different stressors.

2.3.3. Primer design—Primers for all four metallothionein genes were developed and ordered with PrimerQuest (IDT technologies, Coralville, IA, USA) and are listed in Table 2. Primers were developed based on the transcribed sequences available in <http://wfleabase.org> (ID numbers for *Mt1a*: JGIV11_290503, *Mt1b*: JGIV11_290505, *Mt2*: JGIV11_290507, *Mt3*: JGIV11_290508, *Mt4*: JGIV11_312222). In addition, primers were screened with Primer Quest for the lowest primer penalty possible to avoid primer dimerization and self-hybridization. As *Mt1a* and *Mt1b* differ by only one nucleotide, both were amplified with the same primer as no distinction can be made with a normal qPCR analysis. The specificity of primer pairs was validated through a specific BLAST sequence similarity search. Here, the available BLAST program on <http://wfleabase.org> was used with default settings. Primers were selected only if the BLAST return resulted in a cut-off E-value solely for the gene of interest.

Glyceraldehyde-3-phosphate dehydrogenase was added as a reference gene, i.e. a gene whose expression remains constant independent of the treatment. Primers for the reference gene were developed by Dr. Florian Leese (Spanier et al., 2010). Furthermore, cDNA concentrations were accurately quantified with an Oligreen ssDNA Assay Kit (Invitrogen, Carlsbad, CA, USA) on a fluorescence spectrophotometer.

2.3.4. qPCR design—RT qPCR was performed on the Mx3000P qPCR system of Stratagene. Each run, i.e. a 96-well plate, included a standard curve for each gene as well as a melting curve for each sample. The standard curve consisted of dilutions of a single cDNA sample. Each sample had three biological replicates; each biological replicate had an additional technical replicate. In each run, positive controls and, no primer and no template controls were included to control for false negatives and positive results as well as DNA contamination.

SYBR Green Super mix (Quanta Biosciences, Gaithersburg, MD, USA) was used and contained all necessary PCR components besides the cDNA primers. This master mix was scaled to 25 μL total reaction volume according manufacturer's protocol. Mastermix, primers and H₂O were assembled in a reaction cocktail prior to dispensing the adequate volume in each well with an automated repeater pipette. Finally, 1 μL cDNA was added in each well and plates were vortexed to homogenize the solution. Amplification consisted of 40 cycles (30 s at 95°C, 30 s at 60°C, 35 s at 72°C) preceded by 3 minutes at 95 °C.

2.3.5. Data analysis—Following quality assurance tests by analyzing melting curves of samples primed by shared primer pairs and from positive and negative controls, raw fluorescence data were extracted from the MxPro Software and exported to the statistical software R. In this statistical environment, the data were analyzed with the qPCR package for R developed by Ritz and Spiess (2008). This package contains most relevant and current qPCR models and analysis functions. Based on statistical parameters such as log likelihood ratio and AIC criterion, the most appropriate model was selected. Next, the amplification efficiency and the cycle of quantification (C_q-value) were determined.

Normalization of the mRNA expression values of the gene of interest to the mRNA expression values of the reference gene was conducted according to Vandesompele et al. (2002) to eliminate expression variability due to variation in cDNA concentration. No

additional reference gene was chosen here. Instead expression ratios were also normalized to the cDNA concentration as quantified by Oligreen ssDNA Assay Kit. Hence, cDNA concentrations were accurately quantified and the expression of the reference gene could be verified. This method resulted in a relative mRNA expression for each gene of interest under each treatment. The relative expression was interpreted as the amount of increase or decrease in mRNA expression of the sample under the treatment compared to the control. (e.g. relative expression of 1 means expression is equal in control and treatment, relative expression of 2 means expression is two times higher in the treatment than in the control) (Pfaffl, 2010).

Finally, normalized expression ratios were analyzed statistically with either parametric or non-parametric analysis of variance methods, depending on whether data assumptions for parametric methods were fulfilled or not. In addition, expression patterns were compared with each other in a similar manner to identify potential gene clusters.

3. Results

3.1. Bioinformatical analysis of MT genes

Characterization of the different metallothioneins consisted of three parts: location of the genes within the *D. pulex* genome, multiple alignments of the protein sequences for phylogenetic reconstruction (Fig. 1) and characterization of the upstream promoter regions (Tables 3 and 4). In *D. pulex*, five different metallothioneins are identified. Shaw et al. (2007) reported discovering three *D. Pulex* metallothioneins (*Mt1*, *Mt2* and *Mt3*). We report two additional loci, a gene duplicate of *Mt1* differing by only one nucleotide (thymine replacing cytosine) at position 24 of the first exon, and a fourth gene, *Mt4*. *Mt2*, *Mt3* and *Mt4* are located on scaffold 9, while two other loci are located on separate scaffolds (*Mt1A*: scaffold 36, *Mt1B*: scaffold 549).

All promoter regions contained various binding sites for different heat shock transcription factors based on the Transcription Element Search Software (TESS) (Table 3). The heat shock transcription factors targeting to these binding sites (HSF1, HSF2 and HSTF) were characterized in *Drosophila melanogaster* (Westwood and Wu, 1993; Wiederracht et al., 1987; Wu, 1995), *Homo sapiens* (Rabindran et al., 1991), *Mus musculus* (Sarge et al., 1991) and *Saccharomyces cerevisiae* (Tamai et al., 1994). A subsequent query in the *D. pulex* genome revealed two genes encoding heat shock transcription factors (JGIV11_318386 and JGIV11_213340). The proteins encoded by these two genes contained the same DNA binding domain as the transcription factors predicted by TESS (IPR000232). In addition, the first gene (JGIV11_318386) is an ortholog gene of those transcription factors predicted by TESS. Furthermore, the consensus sequence for heat shock regulatory elements was present in all *Mts*, excluding *Mt1* (Table 4).

Next, binding sites for activator proteins, AP-1 and AP-4, were present in all *Mts*, except *Mt3*. Neither anti-oxidant responsive elements nor DNA replication elements were detected in any of the promoter regions of the *Mt* genes (Table 4). In addition both *Mt1* variants demonstrated similar regulatory elements, as well as transcription factors (Table 3) up to 1500 basepairs upstream of the promoter region (Fig. 2). Differences for these two variants manifested in the upstream region from 1500 to 3000 basepairs, where regulatory elements were observed for *Mt1A* but none were found for *Mt1B*. Finally, initiator elements were present in all *Mtn* genes across the entire length of the upstream region, except in *Mtn2* where only two initiator elements are located quite far upstream for the actual gene. Final summary of both approaches is visualized in Fig. 2.

3.2. Ecotoxicological experiments

3.2.1. Chronic toxicity tests—During all toxicity tests, pH did not show significant increases or decreases ($\text{pH}: 7.45 \pm 0.19$). In the copper toxicity tests, no animals lived long enough to reproduce when exposed to concentrations higher than $5 \mu\text{g Cu L}^{-1}$ (Fig. 3). From the concentration response curve for nickel toxicity an EC_{50} of $150.48 \mu\text{g L}^{-1}$ was estimated, as for both 125 and $180 \mu\text{g L}^{-1}$ treatments the response approximated the EC_{50} . In addition, we compared our results with a previously executed nickel toxicity test by Shaw (unpublished) who estimated an EC_{50} of $125 \mu\text{g L}^{-1}$ for identical exposure conditions for the same clone. Therefore, we chose $125 \mu\text{g L}^{-1}$ as concentration for our batch exposures.

No chronic toxic test was conducted with *M. aeruginosa* as well as for cadmium as chronic toxicity tests for reproduction had already been executed with the same *D. pulex* clone (Shaw, unpublished; Shaw et al., 2007).

3.2.2. Chronic batch exposure experiments—In all batch exposures excluding the *M. aeruginosa* exposure, organisms showed similar population characteristics and phenotypic effects at EC_{50} concentrations as in the respective chronic toxicity studies and the effect on reproduction approximated the EC_{50} level (Table 5). For all batch exposures, pH was monitored and medium sampled for concentration analysis ($\text{pH}: 6.93 \pm 0.06$, Table 5).

3.3. mRNA expression analysis

First, we verified the specificity of the primers as well as the overall reaction through the melt curves. Neither cross creations nor primer dimerizations were present, only a specific amplification product for the gene of interest. Second, we compared the results of the relative expressions normalized with the reference gene to the relative expressions normalized with the absolute cDNA concentration. This revealed no significant differences between the two types of normalization ($p = 0.40$). Thus, we will report further the relative expression with a normalization based on the reference gene.

The relative expression normalized with the reference gene resulted overall in few significant differences between control and exposed organisms (Fig. 4). *Mt1* mRNA expression was only influenced by paraquat, where exposure to this stressor resulted in a downregulation of *Mt1* (ANOVA: $p_{\text{Cd}} = 0.21$, $p_{\text{Cu}} = 0.50$, $p_{\text{Ni}} = 0.75$, $p_{20\text{E}} = 0.33$, $p_{\text{MC}} = 0.63$, $p_{\text{P}} = 0.03$). *Mt2* mRNA expression demonstrated no influence of any stressor (ANOVA: $p_{\text{Cd}} = 0.85$, $p_{\text{Cu}} = 0.58$, $p_{\text{Ni}} = 0.46$, $p_{20\text{E}} = 0.17$, $p_{\text{MC}} = 0.11$, $p_{\text{P}} = 0.42$). Next, both 20E and paraquat exposure resulted in a significant increase in *Mt3* mRNA expression compared to the control treatment, while copper exposure resulted in a significant decrease. (ANOVA: $p_{\text{Cd}} = 0.09$, $p_{\text{Cu}} < 0.01$, $p_{\text{Ni}} = 0.19$, $p_{20\text{E}} = 0.01$, $p_{\text{mc}} = 0.07$, $p_{\text{P}} = 0.04$). In *Mt4* mRNA expression only 20E caused significant upregulation after exposure (ANOVA: $p_{\text{Cd}} = 0.79$, $p_{\text{Cu}} = 0.49$, $p_{\text{Ni}} = 0.61$, $p_{20\text{E}} = 0.02$, $p_{\text{mc}} = 0.07$, $p_{\text{P}} = 0.06$).

4. Discussion

4.1. Bioinformatical analysis of MT genes

Our online search of the *D. pulex* draft genome sequence assembly version 1.1 resulted in finding 5 *Mt* genes (*Mt1A*, *Mt1B*, *Mt2*, *Mt3* and *Mt4*). Based on protein sequence analysis, the neighbor-joining distance tree retains the previously reported monophyletic grouping of *Daphnia Mts* (Shaw et al., 2007), yet with the addition of *Mt4* and *Mt1A* (Fig. 1). Promoter region analysis revealed some distinct features for genes sharing phylogenetic histories (Tables 3 and 4); we can still conclude that genes within the cluster will most likely have expression patterns more similar to each other than to other genes.

When observing the promoter analysis in more detail, we notice that the results of TESS in Table 3 included several binding sites for activator proteins. Such binding sites are also present in the upstream regions of *Mt* genes in *Helix pomatia* (Egg et al., 2009), suggesting potential similarities in promoter regions between the *Mt* gene in *D. pulex* and its homolog in *H. pomatia*. In addition, other binding sites such as MREs are also present in *O. Cincta* (Roelofs et al., 2007). If this promoter region is conserved between these organisms, the extent of this conservation and its potential influence on gene expression will need to be investigated. The conservation of such binding sites throughout evolution has already been studied by Seetharam et al. (2010). They compared 40 orthologous groups of C₂H₂ zinc-finger genes, among which MTF1 which binds to MREs in bilaterians including *Daphnia*. In addition with a previous study (Knight and Shimeld, 2001), Seetharam et al. (2010) demonstrate that these 40 orthologous groups represent the gene set present in a common bilaterian ancestor. Furthermore, this potential conservation might be interesting to implement as a biomarker.

No anti-oxidant responsive elements were found, although literature on the role of metallothioneins in oxidative stress is well documented and extensive (Levadoux-Martin et al., 2001; Mosleh et al., 2005). Several explanations for this apparent contradiction are possible. First, metallothioneins may be indirectly responsive to oxidative stress through other discovered factors or elements, e.g. heat shock TBFs which can be stimulated during exposure to oxidative stress (Feder and Hofmann, 1999). Secondly, the *Mt* genes may contain anti-oxidant responsive elements with a yet unknown consensus sequence. This aspect is very interesting for further investigation.

The high number of regulatory elements observed with the TESS software required further validation as TESS is only a computational tool and is based on a relative short regulatory motif (5–8 bp). The presence of two genes encoding heat shock transcription factors, one of which is likely orthologous to the predicted TBFs by TESS, confirmed the likelihood of these findings. Furthermore, an additional query for the heat shock regulatory element consensus sequence also revealed regulatory elements in *Mt2*, *Mt3* and *Mt4*. Although all these results indicate the high likelihood of heat shock TBF binding sites, in vitro validation is needed confirm whether all these regulatory elements are effective binding sites.

The presence of metal regulatory elements in all *Mt* genes suggested a potential influence of metals on these genes, which is in agreement with the general knowledge that *Mt* genes are known for their function in metal binding and detoxification (Janssens et al., 2009; Nordberg, 1998).

From the results of the TESS software and the specific regulatory element search, a representation in GenePalette was constructed (Fig. 2). It could be stated that *Mt* genes have clearly distinct promoter regions and will potentially be differentially responsive to the same stressors. In addition, the gene grouping indicated by the phylogenetic analysis (Fig. 1) suggests more similar expression patterns within a grouping than across groups. In conclusion, the distinct and complex patterns of these genes in relation to each other add further complexity to the characterization of this stress response system.

4.2. Ecotoxicological experiments

4.2.1. Chronic toxicity tests—Significant effects on the reproduction and survival of *D. Pulex* was observed from all six tested exposures. The 20-hydroxyecdysone toxicity response of *D. Pulex* (i.e. effect on mortality and survival with increasing concentrations) was comparable to the response observed by Bodar et al. (1990) in *D. magna*. Moreover, the concentrations significantly affecting the reproduction of the organisms were well above the observed no effect and lowest effect concentrations determined by Peterson et al. (2001).

Results of chronic experiments with *D. magna* by Münzinger and Monicelli (1991) demonstrated a similar trend in response as the chronic nickel exposure performed in this study. Barata et al. (2005) have investigated acute toxicity of paraquat in *D. magna*. Yet, no publications investigating the chronic effect of paraquat on reproduction were available, which makes comparison difficult.

Next, we chose to determine the median effect concentration for reproduction (EC50) and implement this concentration in the batch exposures for further mRNA expression study, for which the results are discussed in the next two paragraphs (see Sections 2.2 and 2.3). This median effect concentration was chosen at such concentrations, as to elicit a specific response, avoiding the non-specific stress response often associated with high concentrations of toxicant. Previous studies have already demonstrated toxicant-specific mRNA expression patterns after exposure to sublethal stress (Poynton et al., 2008, 2007; Shaw et al., 2007). No chronic toxicity test was executed for *M. aeruginosa*, as a previous study (Shaw, unpublished) already revealed a median effect on reproduction when animals were fed on a diet consisting of 50% of *M. aeruginosa* and 50% of *A. falcatus*.

The environmental relevance of the exposure concentrations used for mRNA expression studies was determined by comparing them with environmental concentrations that may be expected to occur in reality, according to (i) regional (away from industrial point sources) and local (close to industrial point sources) exposure analyses in the context of recent EU risk assessments, or (ii) examples of concentrations at contaminated sites reported in the open literature (Table 5). The comparison shows that for cadmium, copper, paraquat, and *Microcystis* the exposure concentrations were within environmentally realistic ranges, while the nickel exposure concentration was only slightly above (Table 5). A similar comparison for 20E was not possible, but 20E was chosen as a model for juvenile hormone analogues.

4.2.2. Chronic batch exposure experiments—In all treatments, excluding the *Microcystis* treatment, reproduction of the exposed animals approximated the median effect, confirming the results of the chronic toxicity test. The absence of effect in the *M. aeruginosa* treatment might be explained by contamination of the *M. aeruginosa* culture with *A. falcatus*. The contamination would implicate a lower percentage of diet consisting of *Microcystis*, resulting in a less toxic treatment than expected.

4.2.3. mRNA expression analysis—Prior to evaluating the mRNA expression analysis, we will evaluate the mRNA expression of the reference gene. We concluded that relative mRNA expression values normalized with the reference gene were not significantly different from relative mRNA expression values normalized with the oligreen measurements. These results confirm the choice of our reference gene and allow us to further use these relative expression values to formulate correct conclusions. This means more specifically that the mRNA expression of the reference gene does not vary under the different treatments as its relative expression is proportional to the concentrations of the cDNA used in the qPCR. Thus it can be used to normalize the samples for differences in cDNA concentrations and differences in plate variability of the qPCR as each sample had a reference sample on the same plate.

The mRNA expression analysis revealed no significant influence of cadmium or nickel on the different isoforms after a chronic exposure period even though *Mt* genes possess metal responsive elements (MREs). However, as metallothioneins are known to preferably bind metals from groups IA and IIB, these results are not surprising for nickel (nickel belongs to group VIIIB). Moreover, several studies have already postulated the differential sensitivity of metallothioneins to different metals (Amiard et al., 2006; Shaw et al., 2007). Hence, it is crucial to study all the different isoforms in an organism to observe a correct stress response.

The lack of response after cadmium exposure contrasts with the results of Shaw et al. (2007) where a significant upregulation of metallothioneins after acute cadmium exposure was observed in one locus. Here, the exposure was chronic, which might lead to the hypothesis that cadmium only induces metallothionein expression after either acute period of time or at high concentration of cadmium. The first case would indicate a time-response relationship between metallothionein expression and metal exposure concentration while the latter suggests a dose-response relationship between the two. Dose response relationships between metallothioneins and cadmium have been observed by Onosaka and Cherian (1981) and others as reviewed by Amiard et al. (2006). In addition, the organisms might have acclimated in their metallothionein response to the cadmium after a chronic exposure period which would also lead to no differential expression. Acclimation to metals has already been demonstrated by Muyssen and Janssen (2004). To verify these hypotheses, further research will be needed to evaluate the metallothionein mRNA expression levels over time. Hence, a distinction can be made between a constant response throughout the whole time period or a changing response as the exposure period lasts as a result of potential acclimation on the metallothionein response level.

Another explanation can be found in standard biomarker studies using metallothioneins. In these studies, protein levels are used as indicator or biomarker for metal pollution. In contrast to mRNA, proteins have a much longer half-life (weeks compared to hours) (Amiard et al., 2006). Thus, in this case, we might not see a response anymore after a 16-day exposure since the mRNA that was expressed upon initial cadmium stress is already degraded. However, the proteins encoded by this mRNA might still present after 16 days and might still help the organism in coping with the cadmium stress. Additionally, we only studied expression at the level of transcription. Thus, differences in protein levels and mRNA levels might not only be caused by differences in turnover but also by differences in translation and post translational modifications. Although regulation occurs mainly at the level of transcription through interaction with regulatory elements (Amiard et al., 2006; Roelofs et al., 2007), we cannot fully discard this possibility. At present there is no indication for translational or post translational regulation neither in literature nor in our study. All these aspects might be important features for choosing the appropriate type of biomarker in risk assessment. Proteins could be seen as biomarkers with the ability to detect pollution long after its initial introduction in the ecosystem. In contrast, mRNA levels have the ability to detect pollution almost immediately after its introduction in the ecosystem. Hence, the choice of biomarker depends on the question asked.

Furthermore, Amiard et al. (2006) stated in his review that metallothionein induction after Cd-exposure in different Crustaceae species occurred within a day after exposure. Barka et al. (2001) observed induction after 1 day of exposure and no induction after 2–14 days of exposure in *Tigriopus brevicornus*. Hence, the most likely hypothesis is that the induction of cadmium occurs early on in the exposure period and is not observed after chronic periods of exposure. This of course has important implications in risk assessment where metallothionein induction is used as biomarker and needs to be considerate when choosing if either the mRNA encoding metallothionein or the protein itself will be measured. Furthermore, this research illustrates that care should be taking when extrapolating acute effects to environmentally relevant concentrations over a chronic period.

In contrast, exposure to copper resulted in a significant downregulation of *Mt3* expression. While *Mt3* is the gene containing the most MREs in its promoter region (Table 4), this reasoning contrasts with the general mechanistic basis of the metallothionein response system. Indeed, MREs are known to stimulate expression and to inhibit or decrease expression (Haq et al., 2003). The downregulation of metallothioneins after exposure to metals has already been observed by Woo et al. (2006). They observed a downregulation

after exposure to nickel and chrome. They postulated the inhibition of a specific Cd metallothionein by nickel or chrome and the potential induction of other Ni- or Cr-specific *Mtn* genes. This inhibition–induction phenomenon was already observed in heat shock proteins by Franzellitti and Fabbri (2005). Consequently, this could also be the case here. *Mt3*, downregulated by copper, is known as a Cd-specific metallothionein. The lack of a coupled induction of Cu-specific *Mts* such as *Mt2* or *Mt4* can again be caused by a potential acclimation to copper after chronic exposure. An early induction and then subsequent long half life of proteins as explained above might again explain the observation of no differential expression after 16 days. Furthermore, differences in expression might be explained by differences in concentration-response relationships among each gene and the stressor. Wan et al. (1993) have observed such differences for four different metallothionein loci in rats. Although no literature is available on such studies in neither invertebrates nor aquatic organisms, the possibility of such differences cannot be excluded.

Exposure to paraquat affected two of the four *Mt* genes, while no REs for oxidative stress were found in the upstream regions of any of the *Mts*. However, bioinformatical analysis did reveal potential binding sites for heat shock transcription factors that are known defence mechanisms against oxidative stress (Banzet et al., 2002). Another hypothesis is that paraquat will probably have more effects than just oxidative stress. Most likely, some of these other effects of paraquat will influence the expression of *Mt*.

Bioinformatical analysis of the stressor 20E revealed distinct patterns of 20-hydroxyecdysone regulatory elements (HEREs) in different genes. Both *Mt3* and *Mt4* were upregulated after exposure, while *Mt4* did not possess any HEREs. Furthermore, *Mt2* was not influenced by 20E yet it contained the most HEREs of all loci. Roelofs et al. (2007) studied the effect of 20E on metallothionein promoter alleles in *O. cincta*. In that study, the promoter alleles were slightly induced at low levels of 20E and inhibited at high levels of 20E. Here, we observed induction for two genes at low levels of 20E. Similar to our study, Roelofs et al. (2007) could not explain the induction of their genes as not all of the upregulated genes contained HEREs in their promoter region. At present, this induction of *Mt3* and *Mt4* cannot be fully explained. Yet, it does reveal a significant interaction between the metallothioneins and 20E, which can potentially be an interaction with the ecdysteroids and the ecdysis.

All genes, excluding *Mt2*, were responsive to at least one of the stressors of this experiment. These results might be explained by the bioinformatical analysis (Table 5), where *Mt2* was the gene with the smallest amount of initiator and the only one in which initiator elements were absent in the first 1500 bp.

Finally, we have compared the mRNA expression patterns of the *Mts* with each other. As predicted by the grouping in the phylogenetic tree (Fig. 1), *Mt2* and *Mt4* mRNA expression patterns were more similar to each other than to any of the other genes. In addition, these genes are in close proximity of each other in the genome. Several studies have already concluded that genes in close proximity of each other are very often similarly influenced (Taylor et al., 1998; Sproul et al., 2005; Buechler et al., 2004). These results suggest similar functional roles for *Mt2* and *Mt4* in stress response.

5. Conclusion

This research has resulted in a broader characterization of *Mts* in *D. pulex* at chronic exposure concentrations by studying the level of transcription. In conclusion, metallothioneins can be seen as multifunctional stress proteins. While some genes have similar expression patterns that can be attributed to a high similarity in regulatory sequence,

others demonstrate various and distinct expression patterns most likely due to a complex pattern of regulatory elements. Further research on both ecotoxicological and molecular level is needed to comprehend the full extent of their role in acute and chronic stress responses at low concentration levels which are relevant in the environment. Furthermore, it can be stated that investigating genes on both an ecotoxicological and molecular level leads to a more integrative approach in the characterization of genes on an environmental relevant level and can be seen as a very interesting synergy.

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References

- Amiard JC, Amiard-Triquet C, Barka S, Pellerin J, Rainbowd PS. Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. *Aquat. Toxicol.* 2006; 76:160–202. [PubMed: 16289342]
- Amin J, Ananthan J, Voellmy R. Key features of heat shock regulatory elements. *Mol. Cell. Biol.* 1988; 8:3761–3769. [PubMed: 3146692]
- American Society for Testing and Materials (ASTM). Standard guide for conducting three-brood, renewal toxicity tests with *Ceriodaphnia dubia*. E1295-9. Annual Book of ASTM Standards, vol. 11.05, Biological Effects and Environmental Fate; Biotechnology; Pesticides. 1997 Section 11, Water and Environmental Technology. American Society for Testing and Materials, West.
- Banzet N, Richaud C, Deveaux Y, Kazmaier M, Gagnon J, Triantaphylidis M. Accumulation of small heat shock proteins, including mitochondrial HSP22, induced by oxidative stress and adaptive response in tomato cells. *Plant J.* 2002; 14:519–527.
- Barata C, Varo I, Navarro JC, Arun S, Porte C. Antioxidant enzyme activities and lipid peroxidation in the freshwater cladoceran *Daphnia magna* exposed to redox cycling compounds. *Comp. Biochem. Physiol.* 2005; 140 C:175–186.
- Barka S, Pavillon JF, Amiard JC. Influence of different essential and nonessential metals on MTLP levels in the copepod *Tigriopus brevicornis*. *Comp. Biochem. Physiol.* 2001; 128C:479–493.
- Bodar CWM, Voogt PA, Zandee DI. Ecdysteroids in *Daphnia magna*: their role in moulting and reproduction and their levels upon exposure to cadmium. *Aquat. Toxicol.* 1990; 17:339–350.
- Buechler C, Bodzioch M, Bared SM, Sigruener A, Boettcher A, Lapicka-Bodzioch K, Aslanidis C, Duong CQ, Grandl M, Langmann T, Dembinska-Kiec A, Schmitz G. Expression pattern and raft association of NIPSNAP3 and NIP-SNAP4, highly homologous proteins encoded by genes in close proximity to the ATP-binding cassette transporter A1. *Genomics.* 2004; 83:1116–1124. [PubMed: 15177564]
- Chen CY, Sillett KB, Folt CL, Whittemore SL, Barchowsky A. Molecular and demographic measures of arsenic stress in *Daphnia pulex*. *Hydrobiologia.* 1999; 401:229–238.
- Chen W, Song L, Ou D, Gan N. Chronic toxicity and responses of several important enzymes in *Daphnia magna* on exposure to sublethal microcystin-LR. *Environ. Toxicol.* 2005; 20:323–330. [PubMed: 15892060]
- Colbourne JK, Pfrender ME, Gilbert D, Thomas WK, Tucker A, Oakley TH, Tokishita S, Aerts A, Arnold GJ, Basu MK, Bauer DJ, Cáceres CE, Carmel L, Casola C, Choi J, Detter JC, Dong Q, Dusheyko S, Eads BD, Fröhlich T, Geiler-Samerotte KA, Gerlach D, Hatcher P, Jogdeo S, Krijgsveld J, Kriventseva EV, Kültz D, Laforsch C, Lindquist E, Lopez J, Manak JR, Muller J, Pangilinan J, Patwardhan RP, Pitluck S, Pritham EJ, Rechtsteiner A, Rho M, Rogozin IB, Sakarya O, Salamov A, Schaack S, Shapiro H, Shiga Y, Skalizky C, Smith Z, Souvorov A, Sung W, Tang

- Z, Tsuchiya D, Tu H, Vos H, Wang M, Wolf YI, Yamagata H, Yamada T, Ye Y, Shaw JR, Andrews J, Crease TJ, Tang H, Lucas SM, Robertson HM, Bork P, Koonin EV, Zdobnov EM, Grigoriev IV, Lynch M, Boore JL. The ecoresponsive genome of *Daphnia pulex*. *Science*. 2011; 331:555–561. [PubMed: 21292972]
- Colbourne JK, Singan VR, Gilbert D. wFleabase: the *Daphnia* genome database. *BMC Bioinform*. 2005; 6:45.
- Cowgill UM, Milazzo DP. The sensitivity of *Ceriodaphnia dubia* and *Daphnia magna* to seven chemicals utilizing the three-brood test. *Arch. Environ. Contam. Toxicol*. 1991; 20:211–217.
- Ding WX, Shen HM, Zhu HG, Ong CN. Studies on oxidative damage induced by cyanobacteria extract in primary cultured rat hepatocytes. *Environ. Res*. 1998; 78:12–18. [PubMed: 9630440]
- Egg M, Höckner M, Brandstätter A, Schuler D, Dallinger R. Structural and bioinformatic analysis of the Roman snail Cd-metallothionein gene uncovers molecular adaptation towards plasticity in coping with multifarious environmental stress. *Mol. Ecol*. 2009; 18:2426–2443. [PubMed: 19457198]
- EU. Paraquat Volume 3 Annex B. Addendum to the Draft Assessment Report and Proposed Decision of the United Kingdom prepared in the context of the possible inclusion of the above active substance in Annex I of Council Directive 91/414/EEC. 2000 European Commission Peer Review Programme.
- EU. Institute for Health and Consumer Protection, Toxicology and Chemical Substances, European Chemicals Bureau, European Commission. Ispra, Italy: Joint Research Centre; 2007. Risk assessment cadmium metal, cadmium oxide.
- EU. Risk assessment nickel and nickel compounds. Ispra, Italy: Institute for Health and Consumer Protection, European Commission, Joint Research Centre; 2008a.
- EU. Voluntary risk assessment copper and copper compounds. Brussels, Belgium: Final report submitted to the European Chemicals Agency (Helsinki, Finland) by the European Copper Institute; 2008.
- Feder ME, Hofmann GE. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu. Rev. Physiol*. 1999; 61:243–282. [PubMed: 10099689]
- Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*. 1985; 39:783–791.
- Franzellitti S, Fabbri E. Differential HSP70 gene expression in the Mediterranean mussel exposed to various stressors. *Biochem. Biophys. Res. Commun*. 2005; 336:1157–1163. [PubMed: 16168387]
- Haq F, Mahoney M, Koropatnick J. Signalling events for metallothionein induction. *Mutat. Res*. 2003; 533:211–226. [PubMed: 14643422]
- Innes DJ, Herbert PDN. The origin and genetic basis of obligate parthenogenesis in *Daphnia pulex*. *Evolution*. 1988; 42:1024–1035.
- Isson PE, Kille P. Functional comparison of the metal-regulated transcriptional control regions of metallothionein genes from cadmium-sensitive and tolerant fish species. *Biochim. Biophys. Acta*. 1997; 1350:325–334. [PubMed: 9061029]
- Janssens TKS, Roelofs D, Van Straalen NM. Molecular mechanisms of heavy metal tolerance and evolution in invertebrates. *Insect Sci*. 2009; 16:3–18.
- Kägi JH. Overview of metallothionein. *Methods Enzymol*. 1991; 205:613–626. [PubMed: 1779825]
- Katoh K. Parallelization of MATF multiple sequence alignment program. *Bioinformatics*. 2010; 26:1899–1900. [PubMed: 20427515]
- Klerks PL, Weis JS. Genetic adaptation to heavy metals in aquatic organisms: a review. *Environ. Pollut*. 1987; 45:173–205. [PubMed: 15092750]
- Knight RD, Shimeld SM. Identification of conserved C2H2 zinc-finger gene families in the Bilateria. *Genome Biol*. 2001; 2:1–8.
- Lafont R, Dinan L. Practical uses for ecdysteroids in mammals including humans: an update. *J. Insect Sci*. 2003; 3:7. [PubMed: 15844229]
- Lawton LA, Codd GA. Cyanobacterial (blue-green algal) toxins and their significance in UK and European waters. *J. Inst. Water Environ*. 1991; 5:460–465.

- Levadoux-Martin M, Hesketh JE, Beattie JH, Wallace HM. Influence of metallothionein-1 localization on its function. *Biochem. J.* 2001; 355:473–479. [PubMed: 11284736]
- Li XY, Liu YD, Song LR, Liu JT. Responses of antioxidant systems in the hepatocytes of common carp (*Cyprinus carpio* L.) to the toxicity of microcystin-LR. *Toxicol.* 2003; 42:85–89. [PubMed: 12893065]
- Lopes I, Baird DJ, Ribeiro R. Genetic adaptation to metal stress by natural populations of *Daphnia longispina*. *Ecotoxicol. Environ. Saf.* 2006; 63:275–285. [PubMed: 16677911]
- Lopes I, Baird DJ, Ribeiro R. Genetic determination of tolerance to lethal and sublethal copper concentrations in field populations of *Daphnia longispina*. *Arch. Environ. Contam. Toxicol.* 2004; 46:43–51. [PubMed: 15025163]
- Marie V, Baudrimont M, Boudou A. Cadmium and zinc bioaccumulation and metallothionein response in two freshwater bivalves (*Corbicula fluminea* and *Dreissena polymorpha*) transplanted along a polymetallic gradient. *Chemosphere.* 2006; 65:609–617. [PubMed: 16545425]
- Miles AT, Hawksworth GM, Beattie JH, Rodilla V. Induction, regulation, degradation, and biological significance of mammalian metallothioneins. *Crit. Rev. Biochem. Mol. Biol.* 2000; 35:35–70. [PubMed: 10755665]
- Moilanen LH, Fukushima T, Freedman JH. Regulation of metallothionein gene transcription identification of upstream regulatory elements and transcription factors responsible for cell-specific expression of the metallothionein genes from *Caenorhabditis elegans*. *J. Biol. Chem.* 1999; 274:29655–29665. [PubMed: 10514435]
- Mosallanejad H, Soin T, Swevers L, Iatrou K, Nakagawa Y, Smagghe G. Non-steroidal ecdysteroid agonist chromafenozide: gene induction activity, cell proliferation inhibition and larvicidal activity. *Pest. Biochem. Physiol.* 2008; 92:70–76.
- Mosleh YY, Paris-Palacio S, Couderchet M, Biagianti-Risbourg S, Vernet G. Metallothionein induction, antioxidative responses, glycogen and growth changes in *Tubifex tubifex* (Oligochaete) exposed to the fungicide, fenhexamid. *Environ. Pollut.* 2005; 135:73–82. [PubMed: 15701394]
- Münzinger A, Monicelli F. A comparison of the sensitivity of three *Daphnia magna* populations under chronic heavy metal stress. *Ecotoxicol. Environ. Saf.* 1991; 22:24–31. [PubMed: 1914992]
- Muyssen BTA, Janssen CR. Multi-generation acclimation and tolerance in *Daphnia magna* Straus. *Environ. Pollut.* 2004; 130:309–316. [PubMed: 15182964]
- Institutes of Health. <http://www.nih.gov/science/models/> (consulted on 31.3.2011)
- Nordberg M. Metallothioneins: historical review and state of knowledge. *Talanta.* 1998; 46:243–253. [PubMed: 18967148]
- OECD (Organization for Economic Cooperation and Development). Guideline for the testing of chemicals: *Daphnia* sp. acute immobilization test and reproduction test, No. 1984:202.
- Onosaka S, Cherian MG. The induced synthesis of metallothionein in various tissues of rat in response to metals. I. Effect of repeated injection of cadmium salts. *Toxicology.* 1981; 22:91–101. [PubMed: 7324075]
- Peterson JK, Kashian DR, Dodson SI. Methoprene and 20-OH-ecdysone affect male production in *Daphnia pulex*. *Environ. Toxicol. Chem.* 2001; 20:582–588. [PubMed: 11349860]
- Philbey AW, Morton AG. Paraquat poisoning in sheep from contaminated water. *Aust. Vet. J.* 2008; 79:2001.
- Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 2010; 29:45.
- Pinho GL, Moura C, Yunes JS, Luquet CM, Bianchini A, Monserrat JM. Toxic effects of microcystins in the hepatopancreas of the estuarine crab *Chasmagnathus granulatus* (Decapoda, Grapsidae). *Comp. Biochem. Physiol. C: Toxicol. Pharmacol.* 2003; 135:459–468. [PubMed: 12965191]
- Poynton HC, Varshavsky JR, Chang B, Caviglioglio G, Chan S, Holman PS, Loguinov AV, Bauer DJ, Komachi K, Theil EC, Perkins EJ, Hughes O, Vulpe AD. *Daphnia magna* aecotoxicogenomics provides mechanistic insights into metal toxicity. *Environ. Sci. Technol.* 2007; 41:1044–1050. [PubMed: 17328222]
- Poynton HC, Zuzow R, Loguinov AV, Perkins EJ, Vulpe AD. Gene expression profiling in *Daphnia magna*, part II: validation of a copper specific gene expression signature with effluent from two copper mines in California. *Environ. Sci. Technol.* 2008; 42:6257–6263. [PubMed: 18767696]

- Rabindran SK, Giorgi G, Clos J, Wu C. Molecular cloning and expression of a human heat shock factor, HSF1. *Proc. Natl. Acad. Sci. U.S.A.* 1991; 88:6906–6910. [PubMed: 1871105]
- Rebeiz M, Posakony JW. GenePalette: a universal software tool for genome sequence visualization and analysis. *Dev. Biol.* 2004; 271:431–438. [PubMed: 15223345]
- Ritz C, Spiess A. qpcR: an R package for sigmoidal model selection in quantitative real-time polymerase chain reaction analysis. *Bioinformatics.* 2008; 24:1549–1551. [PubMed: 18482995]
- Roelofs D, Marien J, Van Straalen NM. Differential gene expression profiles associated with heavy metal tolerance in the soil insect *Orchesella cincta*. *Insect Biochem. Mol. Biol.* 2007; 37:287–295. [PubMed: 17368192]
- Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 1987; 4:406–425. [PubMed: 3447015]
- Sarge KD, Zimarino V, Holm K, Wu C, Morimoto RI. Cloning and characterization of two mouse heat shock factors with distinct inducible and constitutive DNA-binding ability. *Genes Dev.* 1991; 5:1902–1911. [PubMed: 1717345]
- Schwarzenberger A, Courts C, Von Elert E. Target gene approaches: gene expression in *Daphnia magna* exposed to predator-borne kairomones or to microcystin-producing and microcystin-free *Microcystis aeruginosa*. *BMC Genomics.* 2009; 10:527. [PubMed: 19917101]
- Seetharam A, Bai Y, Stuart GW. A survey of well conserved families of C₂H₂ zinc-finger genes in *Daphnia*. *BMC Genomics.* 2010; 11:276. [PubMed: 20433734]
- Shaw JR, Colbourne JK, Davey JC, Glaholt SP, Hampton TH, Chen CY, Folt CL, Hamilton JW. Gene response profiles for *Daphnia pulex* exposed to the environmental stressor cadmium reveals novel crustacean metallothioneins. *BMC Genomics.* 2007; 8:477. [PubMed: 18154678]
- Spanier KI, Leese F, Mayer C, Colbourne JK, Gilbert D, Pfrender ME, Tollrian R. Predator-induced defences in *Daphnia pulex*: selection and evaluation of internal reference genes for gene expression studies with real-time PCR. *BMC Mol. Biol.* 2010; 11:50. [PubMed: 20587017]
- Sproul D, Gilbert N, Bickmore WA. The role of chromatin structure in regulating the expression of clustered genes. *Nat. Rev. Genet.* 2005; 6:775–781. [PubMed: 16160692]
- Stürzenbaum SR, Georgiev O, Morgan AJ, Kille P. Cadmium detoxification in earthworms: from genes to cells. *Environ. Sci. Technol.* 2004; 38:6283–6289. [PubMed: 15597883]
- Suntres ZE. Role of antioxidants in paraquat toxicity. *Toxicology.* 2002; 180:65–77. [PubMed: 12324200]
- Tamai KT, Liu X, Silar P, Sosinowski T, Thiele DJ. Heat shock transcription factor activates yeast metallothionein gene expression in response to heat and glucose starvation via distinct signaling pathways. *Mol. Cell. Biol.* 1994; 14:8155–8165. [PubMed: 7969152]
- Tamura K, Dudley J, Nei M, Kumar S. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 2007; 24:1596–1599. [PubMed: 17488738]
- Taylor PR, Nash JT, Theodoridis E, Bygrave AE, Walport MJ, Botto M. A targeted disruption of the murine complement factor B gene resulting in loss of expression of three genes in close proximity, Factor B, C2, and D17H6S45. *J. Biol. Chem.* 1998; 273:1699–1704. [PubMed: 9430715]
- Thirumoorthy N, Manisenthil Kumar KT, Shyam Sundar A, Panayappan L, Chatterjee M. Metallothionein: an overview. *World J. Gastroenterol.* 2007; 13:993–996. [PubMed: 17373731]
- Van Ewijk JA, Hoekstra PH. The bounded effect concentration as an alternative to the NOEC. *Sci. Total Environ.* 1993; 134:705–711.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 2002; 3:1–11.
- Wan M, Hunziker PE, Kägi JHR. Induction of metallothionein synthesis by cadmium and zinc in cultured rabbit kidney cells (RK-13). *Biochem. J.* 1993; 292:609–615. [PubMed: 8503893]
- Ward TJ, Robinson WE. Evolution of cadmium resistance in *Daphnia magna*. *Environ. Toxicol. Chem.* 2005; 24:2341–2349.
- Watanabe H, Takahashi E, Nakamura Y, Oda S, Tatarazako N, Iguchi T. Development of a *daphnia magna* DNA microarray for evaluating the toxicity of environmental chemicals. *Environ. Toxicol. Chem.* 2007; 26:669–676. [PubMed: 17447551]

- Westwood JT, Wu C. Activation of *Drosophila* heat shock factor: conformational change associated with a monomer-to-trimer transition. *Mol. Cell. Biol.* 1993; 13:3481–3486. [PubMed: 8497263]
- Wiegand C, Peuthert A, Pflugmacher S, Carmeli S. Effects of microcin SF608 and microcystin-LR, two cyanobacterial compounds produced by *Microcystis* sp., on aquatic organisms. *Environ. Toxicol.* 2002; 17:400–406. [PubMed: 12203963]
- Wiederracht G, Shuey DJ, Kibbe WA, Parker CS. The *Saccharomyces* and *Drosophila* heat shock transcription factors are identical in size and DNA binding properties. *Cell.* 1987; 48:507–515. [PubMed: 3100052]
- Woo S, Yum S, Jung JH, Shim WJ, Lee CH, Lee TK. Heavy metalinduced differential gene expression of metallothionein in Javanese Medaka, *Oryzias javanicus* . *Mar. Biotechnol.* (NY). 2006; 8:645–662.
- Wu C. Heat shock transcription factors: structure and regulation. *Annu. Rev. Cell. Dev. Biol.* 1995; 11:441–469. [PubMed: 8689565]
- Yeh HC, Chen IM, Chen P, Wang WH. Heavy metal concentrations of the soldier crab (*Mictyris brevidactylus*) along the inshore area of Changhua, Taiwan. *Environ. Monit. Assess.* 2009; 153:103–109. [PubMed: 18600467]
- Zuckerandl, E.; Pauling, L. Evolutionary divergence and convergence in proteins. In: Bryson, V.; Vogel, HJ., editors. *Evolving Genes and Proteins*. New York: Academic Press; 1965. p. 97-166.

Fig. 1.

Phylogenetic tree based on multiple alignments of all protein sequences of 5 different isoforms of metallothionein in *Daphnia pulex* and 18 metallothionein sequences of different species (based on Shaw et al., 2007). The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All ambiguous positions were removed for each sequence pair. There were a total of 79 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2007).

Fig. 2.

Representation of all *Mt* genes in *Daphnia pulex* and their 1500 bp upstream promoter region with regulatory elements search using TESS software. Exons are represented by rectangular blocks, using white blocks for non coding exon sequences and blue blocks for coding exon sequences, all connected by triangles representing intron sequences as determined by <http://wflabase.org>. Reverse regulatory elements are represented upside down. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 3. Concentration response curves for the four chronic toxicity tests, fitted to the log logistic model (Van Ewijk and Hoekstra, 1993).

Fig. 4. mRNA expression ratios for all *Mt* genes in control (Ctr Cd, CtrCu, Ctr, Ctr P), cadmium (Cd), copper (Cu), nickel (Ni), 20E, *M. aeruginosa* and paraquat (P) treatments. Stars represent significant differences ($\alpha = 0.05$) between the treatment and its corresponding control group.

Table 1

Regulatory elements and their consensus sequence.

Regulatory element	Abbreviation	Consensus sequence
Initiator	Inr	TCAKTY
Metal regulatory element	MRE	TGCRCNC
Antioxidant responsive element	ARE	TGACNNNGC
DNA replication-related element	DRE	TATCGATA
20-hydroxyecdysone responsive element	HERE	KNTCANTNNMM
Heat shock regulatory element	HSRE	GAANNTTCNG

Table 2

Primer sequences for genes of interest (bp = base pairs, T_m = melting temperature).

Metallothionein1	Sequence
Forward Primer	TGGTGGTGAATGTAAATGCAGCGG
Reverse Primer	TGTAGTCGTTTACTTGCAGCAGGC
Metallothionein2	Sequence
Forward Primer	TGTGTTTCGTTGTCAAAGTGGGTGC
Reverse Primer	AGTCTCTCCTTGTGACGAAGCTGT
Metallothionein3	Sequence
Forward Primer	AAATTCCGCTTGTATCTGCGCCAC
Reverse Primer	TTCGAATTATTTGGCCGGACGGTG
Metallothionein4	Sequence
Forward Primer	AGTCTGTTCTCATTGCCAAGGTCC
Reverse Primer	CCACAACGGCAATTATTAGGGCCA

Table 3

Transcription binding factors (TBF), discovered with the TESS software in the *D.pulex* genome, ranked on their *p*-value with the regulatory element sequence (RE), Number of REs present and log likelihood ratio (t_{s-a}). TBF represented are heat shock factors (HSF), heat shock transcription factors (HSTF), ecdysone-responsive key regulator (BR-C_Z4), activator proteins (AP).

<i>Mt/A</i>	TBF	RE	Number of REs	<i>p</i> -Value	t_{s-a}
<i>Mt1A</i>	HSF1	CTTCT	4	1.23e-03	6
	HSTF	ATTCT	5	2.42e-03	6
	BR-C_Z4	TTAGTAAACATAA	1	1.48e-02	15
	AP1	GATGACTCCGA	1	6.50e-02	12
<i>Mt1B</i>	TBF	RE	Number of REs	<i>p</i> -Value	t_{s-a}
	HSF1	TTTCT	3	6.94e-07	6
	HSTF	ATTCT	6	4.45e-06	6
	BR-C_Z4	TTAGTAAACATAA	1	1.04e-02	15
	AP1	GGTGACGTACT	1	5.02e-02	13
<i>Mt2</i>	HSF2	AGATAATTCT	2	7.28e-02	10
	TBF	RE	Number of REs	<i>p</i> -Value	t_{s-a}
	AP-4	ATCAGCTGGA	1	6.64e-02	13
	HSF1	CTTCT	2	9.30e-02	10
	HSTF	GAATCTTC	6	6.04e-02	6
	TBF	RE	Number of REs	<i>p</i> -Value	t_{s-a}
<i>Mt3</i>	HSF1	CTTCT	2	4.58e-03	6
	HSTF	ATTCT	8	7.95e-07	6
<i>Mt4</i>	TBF	RE	Number of REs	<i>p</i> -Value	t_{s-a}
	HSF1	GAATCTTC	1	3.94e-03	12
	HSTF	CGAAA	5	8.54e-03	6
	AP-4	ATCAGCTGGA	1	6.64e-02	12
	TBF	RE	Number of REs	<i>p</i> -Value	t_{s-a}

Table 4

Responsive elements, discovered with the Perl script, their abbreviation and the number of times they are present in the promoter region of metallothionein genes in *D. pulex*.

Consensus	Metal responsive element (MRE) TGCRCNC	Initiator (Inr) TCAKTY	20-Hydroxyecdysone responsive element (HERE) KNTCANTNNMM	Heat shock responsive element (HSRE) GAANNTTCNG
<i>Mt1A</i>	1	4	0	0
<i>Mt1B</i>	1	5	2	0
<i>Mt2</i>	1	2	2	2
<i>Mt3</i>	4	4	1	1
<i>Mt4</i>	1	5	0	2

Table 5

Summary of batch exposure experiments. For each stressor, estimated EC50 (based on chronic toxicity tests), concentration (conc.) administered and measured in aquaria, response (i.e. reproduction relative to reproduction in control treatment per initial female) are represented. Stars (*) denote estimated EC50 from previous data, which were not determined again in this study. Environmental concentrations with their references are also given.

Toxicant	Estimated EC50 (μgL^{-1})	Conc. Administered (μgL^{-1})	Conc. measured (μgL^{-1})	Response (reproduction) (%)	Environmental conc. (μgL^{-1})	Reference
Copper	5.11 $\pm$ 0.51	5	6.25 $\pm$ 1.62	59.12 $\pm$ 0.65	0.5–4.7 ^a 3.2–22.5 ^b 27–1700 ^d	EU (2008b) EU (2008b) Lopes et al. (2004)
20E	27.76 $\pm$ 16.26	28	28.02 $\pm$ 0.50	49.04 $\pm$ 0.14	NA 1.1–4.8 ^a	NA EU (2008a)
Nickel	150.48 $\pm$ 29.54	125	121.43 $\pm$ 5.93	55.99 $\pm$ 3.00	0.5–99.7 ^c 22.6–74.4 ^d	EU (2008a) Yeh et al. (2009)
Paraquat	50.73 $\pm$ 28.32	51	45.73 $\pm$ 1.51	63.06 $\pm$ 1.58	18 45e03–400e03 ^d	EU (2000) Philbey and Morton (2008)
Cadmium	0.50*	0.5	0.45 $\pm$ 0.03	47.18 $\pm$ 2.57	0.04–0.31 ^a 1–19.8 ^b	EU (2007) EU (2007)
<i>M. aeruginosa</i>	50% of diet*	2.0e5 cells mL ⁻¹	1.98e5 $\pm$ 10cells mL ⁻¹	99.28 $\pm$ 0.61	5.5–28 ^c 0–100%	Marie et al. (2006) Lawton and Codd (1991)

^aRegional (i.e. away from industrial point sources) predicted environmental concentration (PEC) (measured data).

^bLocal (i.e. close to industrial point sources) predicted environmental concentration (PEC) (measured data).

^cLocal (i.e. close to industrial point sources) predicted environmental concentration (PEC) (calculated estimates).

^dLocal concentrations at contaminated site (measured data).