Early and late response of *Nematostella vectensis* transcriptome to heavy metals

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Abstract

Environmental contamination from heavy metals poses a global concern for the marine environment, as heavy metals are passed up the food chain and persist in the environment long after the pollution source is contained. Cnidarians play an important role in shaping marine ecosystems, but environmental pollution profoundly affects their vitality. Among the cnidarians, the sea anemone *Nematostella vectensis* is an advantageous model for addressing questions in molecular ecology and toxicology as it tolerates extreme environments and its genome has been published. Here, we employed a transcriptome-wide RNA-Seq approach to analyse *N. vectensis* molecular defence mechanisms against four heavy metals: Hg, Cu, Cd and Zn. Altogether, more than 4800 transcripts showed significant changes in gene expression. Hg had the greatest impact on up-regulating transcripts, followed by Cu, Zn and Cd. We identified, for the first time in Cnidaria, co-up-regulation of immediate-early transcription factors such as *Egr1*, *API* and *NF-κB*. Time-course analysis of these genes revealed their early expression as rapidly as one hour after exposure to heavy metals, suggesting that they may complement or substitute for the roles of the metal-mediating *Mtf1* transcription factor. We further characterized the regulation of a large array of stress-response gene families, including Hsp, ABC, CYP members and phytochelatin synthase, that may regulate synthesis of the metal-binding phytochelatins instead of the metallothioneins that are absent from Cnidaria genome. This study provides mechanistic insight into heavy metal toxicity in *N. vectensis* and sheds light on ancestral stress adaptations.

Keywords: cnidaria, heavy metal, *Nematostella vectensis*, transcriptome

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Introduction

Cnidarians, such as coral, sea anemone, jellyfish and hydra, are distributed worldwide and play important roles in the marine environment. They are the main reef builders and act as both predators and prey in the marine ecosystem (Pandolfi *et al.* 2003). Dating back about 700 million years, they are some of the simplest animals at the tissue level of organization and are considered as a sister group to the Bilateria (Putnam *et al.* 2007; Park *et al.* 2012). The cnidarians and specifically the anthozoans (sea anemone and coral) are exposed to anthropogenic disturbances, which, together with natural environmental fluctuations, have already caused a deterioration estimated at more than one-third of coral reef populations worldwide (Hughes *et al.* 2003; Hoegh-Guldberg *et al.* 2007). Among the anthropogenic pollutants in the marine environment are heavy metals that are discharged into the sea through sewage or by industrial, urban and agricultural run-off and accumulate in the sediment sometimes reaching concentrations as high as tens of mg/kg sediment (Sabdono 2009; Pan & Wang 2012). Heavy metals do not decay but represent a persistent form of toxicity that is passed up the...
Effects of heavy metals on sea anemones and corals

et al.

chemicals (Goldstone to sense, transform and eliminate potentially toxic components of the metal-stress pathway in metazoan are absent in Cnidaria. Metal stress in metazoan is mediated by the metal transcription factor-1 (Mtf1), a metal-sensing protein that is highly conserved from insects to mammals, responds to heavy metal exposure and mediates gene expression (Günther et al. 2012). Its main targets are the metallothioneins, a group of small cysteine-rich proteins involved in heavy metal detoxification. Mtf1 was identified in N. vectensis and Hydra but not in Acropora (Shinzato et al. 2012). Furthermore, the metallothioneins, which are found in diverse eukaryotes, were not found in coral, sea anemone or hydra genomes, suggesting that they might be absent in all cnidarians and that the metal signal is transduced through different pathways (Andersen et al. 1988; Reitzel et al. 2008; Shinzato et al. 2012).

The starlet sea anemone N. vectensis inhabits estuarine habitats and is widely distributed in the eastern Pacific, western Atlantic, northern English Channel and western North Sea (Hand & Uhlinger 1994). It has emerged as a cnidarian model system for developmental and genomic studies with the advantages of having a published genome and being easy to grow in laboratory conditions (Darling et al. 2005; Putnam et al. 2007).

Moreover, because it can tolerate a wide range of anthropogenic and environmental changes, for example in salinity (2–54 ppt), temperature (1–28 °C) and various dissolved oxygen and metal concentrations (Sheader et al. 1997; Harter & Matthews 2005), it was suggested as a potentially useful cnidarian model for addressing questions in molecular ecology and toxicology (Goldstone 2008; Reitzel et al. 2008; Ambrosone et al. 2014). Although the defence module of N. vectensis has been characterized bioinformatically, it has yet to be tested comprehensively with heavy metals (Harter & Matthews 2005; Reitzel et al. 2014; Tarrant et al. 2014).

In this study, we examined the effects of heavy metals on the defence mechanisms of N. vectensis. We chose an unbiased approach in which we compared, using RNA-Seq, the gene expression profiles of N. vectensis following the addition of four heavy metals (Hg, Cd, Cu and Zn). We identified a common set of immediate-early transcription factors that were up-regulated after treatment with each of the metals and may transform the environmental stress signals to a large specific array of defence genes that were characterized in this study.

Materials and methods

N. vectensis culture

The study was performed on an established laboratory population of Nematostella vectensis that was originally received from StarletDerma, which commercially cultivates the same strain that its genome was published (Putnam et al. 2007). Sea anemones were raised at 18 °C in 12.5 ppt artificial sea water (Red Sea, Israel) in the dark. The anemones were fed 5 days a week with freshly hatched Artemia brine shrimps with weekly medium changes, as previously described (Darling et al. 2005).

Metal treatments

Experiments were carried out in triplicate groups, each containing five sea anemones aged 2–4 months. For the transcriptome experiments, groups were exposed for 24 h to mercury (HgCl₂), cadmium (CdCl₂), zinc (ZnCl₂) and copper (CuCl₂), each at a concentration of 10μg/L (0.3–0.7 μM), and compared to four untreated groups of sea anemones that served as controls. The anemones were collected into RNA Save (Biological Industries, Israel) and kept for up to a week at 4 °C or for longer periods at ~80 °C prior to RNA extraction. For qPCR experiments, anemone groups were exposed to each of the four metals at the concentrations of 100 μg/L for 1–24 h.
RNA extraction

Total RNA was extracted from groups each containing five anemones with the aid of a Tri-reagent kit (Sigma) according to the manufacturer’s instructions. The extracted RNA was further purified using the RNA Clean & Concentrator™-5 kit (Zymo Research), and genomic DNA residues were removed by DNase (Ambion). RNA concentrations were determined in a NanoDrop 2000c spectrophotometer (Thermo Scientific), and RNA quality was tested in an Agilent 2100 bioanalyzer. The RNA samples were kept at −80 °C until used.

RNA sequencing and bioinformatic analysis

Samples were prepared for multiplex sequencing using Illumina TruSeq Kits (Illumina) according to the manufacturer’s instructions. The 16 samples (triplicates of the four metal treatments and four replicates of the control) were sequenced using 50-bp single-end reads in two lanes on the Illumina HiSeq2000 lane and TruSeq v3 flow chamber at the Life Sciences and Engineering Infrastructure Center at the Technion, Haifa. Bioinformatic analysis was carried out at the Bioinformatics Core Facility at the National Institute for Biotechnology in the Negev at Ben-Gurion University of the Negev, Beer Sheba. Mapping of 50-bp single-end reads (fastq files) to the N. vectensis genome was performed using the STAR aligner (Dobin et al. 2013). N. vectensis genome sequence and its annotation (fasta and gtf files, respectively) were downloaded from Ensembl. Splice junction loci from the gtf file were used to guide STAR in aligning the reads to annotated exons. Intron maximum and minimum sizes were set to 50 000 bp and 10 bp, respectively, and a maximum of 49-bp overhang across splice junctions was allowed. Read alignments were further reported only if they had fewer than two mismatches and the read mapped to a unique location. Raw read counts per transcript were obtained using HTSeq-count and were submitted to DESeq R package for normalization and differential expression analysis (Anders & Huber 2010). As we used sublethal doses of heavy metals, Cd and Zn treatments affected a relatively small number of differentially expressed transcripts in comparison with hundreds in Cu treatment and over 2200 transcripts in Hg treatment at the FDR < 0.05 cut-off. Therefore, in order not to lose insights into potentially interesting pathways, we decided to use transcripts with P-value < 0.05 for all further analyses acknowledging that this retains some false positives and requires further validation for candidate genes. Hence, transcripts were considered differentially expressed between a metal and control if they had P-value < 0.05 and if at least two of the samples in either the metal or the control had reads more than 5. N. vectensis protein sequences and annotations were retrieved from UniProt. Proteins designated as ‘predicted protein’ were annotated using BLAST2GO through BLASTP VS. REFSEQ (considering up to top 20 hits, e-value cut-off 10⁻³).

Differentially expressed up-regulated and down-regulated transcripts were tested for gene ontology (GO) enrichment using DAVID with the whole genome as background (Huang et al. 2008, 2009). Unique transcripts and transcripts common to each of the heavy metals (P < 0.05) were analysed by Venn diagrams using VENNY software (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

Protein interactions of Hg-regulated transcripts were examined by STRING software (version 9.1) (Jensen et al. 2009), which is capable of inferring protein–protein interactions from homologues of N. vectensis proteins, using default parameters of medium confidence (0.4). The resulting String protein interaction network was imported to CYTOSCAPE software (version 2.8.3) (Lopes et al. 2010) to test first-neighbour interactions of selected proteins (a function not presently available in the STRING software), using default parameters. The resulting first-neighbour interaction networks were transferred back to String for visualization and analysis using a medium confidence score as a threshold.

Heat maps were used to visualize transcript fold change in log 2 scale of each metal vs. control and were generated by EXPANDER software suite (version 6.1) (http://acgt.cs.tau.ac.il/expander/).

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems). cDNA was synthesized with random primers using a Verso cDNA Kit (Thermo Scientific), and primers were selected from the exon–exon junctions using PRIMER EXPRESS Version 3.0 software (Applied Biosystems) (Table S1, Supporting information). cDNA amplification was quantitatively assessed with SYBR Green dye using Power SYBR Green PCR Master Mix (Applied Biosystems). Each cDNA sample (diluted to 1/10) was quantified in triplicate for candidate genes and for β-actin (Table S1, Supporting information) as an internal control. The constant expression of the β-actin level was verified across treatments by qPCR analysis of reactions loaded with equal amounts of RNA. Reactions were carried out with 5 μL Fast SYBR® Green Master Mix (Applied Biosystems), 0.5 μL of 10 μM primers and ~25 ng cDNA template in a 10 μL volume, using reagent samples without cDNA as negative controls. The thermal profile was 95 °C for 20 s, 40
amplification cycles of 95 °C for 3 s and 60 °C for 30 s and dissociation cycle of 95 °C for 15 s and 60 °C for 1 min and then brought back to 95 °C. The specificity of the amplified products was determined by the presence of a single peak in the melting curve. To quantify the expression of the different genes, we used the quantitation-comparative \( C_T (\Delta \Delta C_T) \) programme. To calculate delta cycle threshold (\( \Delta C \)) values, the \( C_T \) value of the actin gene was subtracted from the \( C_T \) values of the genes of interest. Delta delta \( C_T (\Delta \Delta C) \) values were calculated by subtracting the \( dC \) values from control samples (without heavy metal treatment), and fold changes were calculated using the 2^{-\Delta \Delta C} method (Livak & Schmittgen 2001). Results are presented as the means and standard error of at least three biological replicates. Statistical analysis, comparing treatment to control, was performed using \( t \)-test.

### Results

**RNA-Seq data**

To study the defence mechanisms activated by *Nematostella vectensis*, we carried out a preliminary experiment to find a sublethal dose that did not cause mortality for at least 96 h of metal exposure (data not shown). Thereafter, anemones were exposed for 24 h to the heavy metal Hg, Cu, Cd or Zn (100 \( \mu \)g/L) and were analysed in comparison with untreated (control) anemones by next-generation sequencing using Illumina HiSeq 2000. On average, 15.22 million sequence reads were obtained for each sample and were mapped to the exons of the reference genome ([http://metazoa.ensembl.org/Nematostella_vectensis/Info/Index](http://metazoa.ensembl.org/Nematostella_vectensis/Info/Index)). We further analysed only differentially expressed transcripts (see Materials and methods). Within the combined metal treatments, more than 4800 transcripts showed significant changes in gene expression, with Hg having the greatest impact on gene expression (Table S2, Supporting information).

**Gene ontology analysis**

To determine whether gene transcripts with related functions were significantly enriched in the four studied metals, we analysed the enrichment of the GO biological processes. Figure 1 demonstrates selected GO terms fold enrichment of FDR < 0.05, a cut-off representing only two metal treatments (for the complete results of the four metals, see Table S3, Supporting information). Common to Hg and Cu were down-regulation of DNA synthesis and processes related to cellular organization and involving, for example, chromatin and nucleosome assembly and microtubule processes. Decrease in microtubule activity was related to cytoskeleton organization and cell division, which was consistent with our finding of DNA synthesis reduction. However, decrease in microtubule movement was also associated with functions related to ciliary or flagellar motility and might imply down-regulation of ciliogenesis or spermatogenesis. Up-regulated processes were prominent mostly after Hg treatment and included regulation of cell death, transmembrane transport and proteolysis (Fig. 1).

**Gene expression regulated by metal exposure**

We next asked whether the processes affected were similar between the different metals, or whether the responses were metal specific. Venn analysis demonstrated that about 80% of the up-regulated transcripts (and about 60% of the down-transcripts) of the Cu-treated anemones were also regulated in the Hg-treated anemones (Fig. 2), despite the fact that Hg is a highly toxic, nonessential metal, whereas Cu participates in numerous processes in the cell.

A total of 14 up-regulated and 3 down-regulated transcripts were shared by anemones treated with any of the four metals (Table 1, Fig. 3). Among the commonly up-regulated transcripts were several early response transcription factors, including early growth response protein 1 (Egr1) known to respond to external stress stimuli, c-fos-like transcription factor that mediates extracellular signals and regulates gene expression, and c-jun, which may interact with c-fos-like protein to form the activator protein 1 (AP-1) (Eferl & Wagner 2003). Additional genes included a progesterone receptor membrane component (PGRMC), a heme-binding protein, suggested to be involved in activation of cytochrome P450 (CYP450) enzymes (Hughes et al. 2007), and a B-cell translocation 1 (Btg1)-like gene that plays a role in cell proliferation (Rouault et al. 1992).

To identify the pathways in which the common transcripts participate, we used the String database, which describes interactions between proteins (both experimentally determined protein–protein interactions and indirect interactions based on literature), in a variety of organisms. We looked for the common up-regulated transcripts’ first-neighbour interactions using a combination of CYTOSCAPE and STRING software and visualized the results using Hg stress as this metal has the strongest impact on gene expression. When visualizing the known interactions of genes up-regulated under Hg stress in String, a common network emerged centred on c-fos-like, c-jun and Egr1 (Fig. 4). The complete list of up-regulated genes and their expression patterns in the four metal treatments are shown in Table S4 and Fig S1, Supporting information. This analysis revealed differential regulation in transcription factors such as Elk1, which together with the transcription factor serum...
response factor (SRF) may bind the serum response element (SRE) of c-fos-like or Egr1 and activate their expression. Also found were megakaryoblastic leukaemia myocardin-like 1 (Mkl1), which serves as a transcriptional coactivator of SRF, and cyclic-AMP response-element-binding protein (CREB) and the CREB-binding protein, which may act together with c-fos. Also detected were MafG (a bzip-Maf transcription factor) and C/EBPγ (CCAAT/enhancer-binding protein gamma), both of which can also dimerize with c-fos. Several MAP kinases, such as Erk5 and MAK6, which may phosphorylate the above transcription factors, were also found in the network. In addition, the predominant allele of NF-κB, which contains cysteine in the DNA recognition loop (Sullivan et al. 2009), was found to be associated in the network with Egr1 and c-jun (Fig. 4a). Further examination revealed that most of the NF-κB pathway components were also up-regulated in Hg-treated anemones, including the Toll receptor, its adaptor Myd88 (v1g82163), its kinase activator IKK
Of the three transcripts that were down-regulated in all metal treatments, one was related to microtubule processes as indicated by its conserved tubulin tyrosine ligase-like 10 (TTL) domain, which is involved in tubulin modification (Ikegami & Setou 2010). The other two transcripts were related to ion transportation: one was sodium/phosphate cotransporter 2B of the solute carrier 34 (SLC34) family, which plays an important role in Pi homeostasis (Murer et al. 2004), and the other was an anoctamin-like needed for transepithelial ion transport (Pedemonte & Galietta 2014) (Table 1 and Fig. 3).

The complete list of transcripts and their expression patterns in the four metal-treated anemones are shown in Table S4 and Fig S1, Supporting information.

Time-course of transcripts detected in metal-treated anemones

To validate the results obtained by RNA-Seq, we measured the expression of selected transcripts at different times after exposure to heavy metals using qPCR. Groups of anemones in three biological replicates were treated with the metal pollutants at the concentrations of 100 μg/L. We examined their time of response processes as indicated by its conserved tubulin tyrosine ligase-like 10 (TTL) domain, which is involved in tubulin modification (Ikegami & Setou 2010). The other two transcripts were related to ion transportation: one was sodium/phosphate cotransporter 2B of the solute carrier 34 (SLC34) family, which plays an important role in Pi homeostasis (Murer et al. 2004), and the other was an anoctamin-like needed for transepithelial ion transport (Pedemonte & Galietta 2014) (Table 1 and Fig. 3). The complete list of transcripts and their expression patterns in the four metal-treated anemones are shown in Table S4 and Fig S1, Supporting information.

Fig. 4 String protein interaction map of the first neighbours in the Hg-treated anemones of commonly up-regulated. The nodes coloured in red represent transcription factors or co transcription factors, and the yellow nodes represent kinases or phosphatases. All other node colours are from STRING software and are used only for visualization. The different node size reflects whether there is structural information of the node in the STRING software. Strong associations are represented by thicker lines. The JGI accession numbers are near the nodes, and the description of the nodes can be found in Table S4, Supporting information. The sequence names of several noteworthy nodes are shown.

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Table 1 Common up-regulated and down-regulated transcripts found in all four metal-treated N. vectensis

<table>
<thead>
<tr>
<th>JGI no.</th>
<th>Sequence description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up-regulated</td>
<td></td>
</tr>
<tr>
<td>v1g238589</td>
<td>c-jun</td>
</tr>
<tr>
<td>v1g232694</td>
<td>c-fos-like</td>
</tr>
<tr>
<td>v1g244653</td>
<td>Pleckstrin homology domain</td>
</tr>
<tr>
<td>v1g167250</td>
<td>Unknown</td>
</tr>
<tr>
<td>v1g28119</td>
<td>btg1-like</td>
</tr>
<tr>
<td>v1g80458</td>
<td>Progesterone receptor membrane component (PGRMC)</td>
</tr>
<tr>
<td>v1g81300</td>
<td>Dual-specificity protein phosphatase 1 (DUSP1)</td>
</tr>
<tr>
<td>v1g246364</td>
<td>Immediate-early response (IER) domain</td>
</tr>
<tr>
<td>v1g231776</td>
<td>Unknown</td>
</tr>
<tr>
<td>v1g39805</td>
<td>Egr1</td>
</tr>
<tr>
<td>v1g171367</td>
<td>Adipor-like receptor</td>
</tr>
<tr>
<td>v1g232819</td>
<td>Unknown</td>
</tr>
<tr>
<td>v1g220809</td>
<td>Ankycorbin isoform 2</td>
</tr>
<tr>
<td>v1g110773</td>
<td>sixdkey-partial</td>
</tr>
<tr>
<td>Down-regulated</td>
<td></td>
</tr>
<tr>
<td>v1g32113</td>
<td>Sodium/phosphate cotransporter 2B</td>
</tr>
<tr>
<td>v1g248849</td>
<td>Anoctamin-like</td>
</tr>
<tr>
<td>v1g107550</td>
<td>TTL domain</td>
</tr>
</tbody>
</table>

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because some of the identified transcripts were considered to be early response genes, and we expected to see their up-regulation earlier than the 24-h time point tested in the RNA-Seq experiment. We therefore tested the effect of an exposure time as short as 1 h, followed by testing after 6 h and also after 24 h, the exposure time of the original experiment (Fig. 5).

Similar trends to those observed in the RNA-Seq experiment were demonstrated by the qPCR results. Immediate-early response genes such as \textit{Egr1} and \textit{c-fos-like} showed high levels of expression after 1 h, and by 6 h and 24 h, these levels had decreased (Fig. 5). \textit{NF-kB}, which was identified in the first-neighbour analysis in the Hg-treated anemone, was also up-regulated after one hour in all metal-treated anemones, but only in Hg treatment was its level still up-regulated at 24 h (Fig. 5).

In line with the transcriptome results, \textit{Atp7A} (a copper-transporting ATPase) and one member of the cytochrome P450 (CYP) family were expressed mainly after Hg or Cu treatment (Fig. 5). Heat-shock protein 70 (\textit{Hsp70}), which in the transcriptome experiment was mostly expressed in Hg-treated anemones with a small increase in Cu-treated anemones, demonstrated a similar pattern in the qPCR experiments. Up-regulation of

Fig. 5 qPCR determination of expression levels of selected genes: \textit{Egr1} (v1g399805), \textit{Atp7a} (v1g87416), \textit{Cyp450} (v1g142057), \textit{c-fos-like} (v1g232694), \textit{Hsp70} (v1g189485), \textit{Mtf1} (v1g120836), \textit{NF-xB} (v1g174238) and \textit{Pcs1} (v1g136743), in metal-treated anemones relative to untreated controls. \textit{β-Actin} (v1g234494) was used as a normalizing gene. Experiments were performed with at least three biological replications, and the results are presented as the average fold change ± SE. Asterisks indicate significant differences from the control $(P < 0.05)$. Dashed lines at 24-h time points represent fold results of RNA-Seq experiment.
phytochelatin synthase 1 (Pcs1) was analysed here for the first time in Cnidaria, and its expression increased during the first 6 h, mainly in Hg and Cu treatments (Fig. 5) (see next section). We also tested Mtf1, a known regulator of metal pollution in metazoans. In line with the transcriptome results, qPCR revealed no significant change in its expression in any of the four metal treatments (Fig. 5).

Stress-response genes

The putative gene defensive network of N. vectensis was previously suggested, on the basis of bioinformatic analysis, to contain 379 transcripts in different families (Goldstone 2008). We analysed expression of these 379 candidate transcripts and found 131 transcripts whose expression levels were significantly changed after treatment with at least one metal (Fig. 6). Of the 248 transcripts whose expression levels did not change in our data set, most (except for three transcripts of epoxide hydrolase, eEF1-gamma and catalase) were other members of families identified in our study as being differentially expressed. The most commonly regulated genes in our study were ATP-binding cassette (ABC) efflux transporters followed by CYPs, which participate in oxidation of xenobiotic compounds, and organic anion and cation transporters of the SLC22 family. From this list, a heat map of the transcript expression levels was generated (Fig. 7). The most highly regulated genes were those of the Hsp20 group, mainly in response to Hg treatment. Their expression levels increased in some cases up to 60-fold from very low basal expression, in marked contrast to Hsp70 and Hsp90, whose basally high expression levels in the control increased even more after metal exposure. Among the ABC transporters, most of the members of the ABC A, B, D, F and G families were up-regulated, whereas family C had both up- and down-regulated members. Similarly to the latter, the CYP group showed both up- and down-regulated expression in all clans. The conjugative groups sulphotransferases (SULT) and UDP-glucuronosyl transferases (UGT) and the oxidative aldehyde dehydrogenases (ALDH) group were mostly down-regulated, while members of the oxidative flavoprotein monooxygenase (FMO) were up-regulated. Genes from the glutathione cycle were also regulated, and an interesting finding was that Pcs1, which synthesizes the nonribosomal formation of metal-binding phytochelatins (Clemens & Persöhn 2009), was up-regulated after Hg and Cu treatments (Fig. 5).

Discussion

An unbiased comparative RNA analysis was used in this study to investigate the molecular responses to heavy metal stress in the sea anemone Nematostella vectensis. We tested the effects of four heavy metals: Hg and Cd, which are nonessential metals, and Cu and Zn, which are essential metals that participate in many metabolic processes and are used by enzymes and other proteins as cofactors (Waldron et al. 2009). Interestingly, Hg had the greatest impact on gene up-regulation 24 h after exposure, followed in order by Cu, and Cd and Zn (the last two had similar effects). In addition, more regulated genes were shared with Hg by Cu (~80% up-regulated and ~60% down-regulated) than by Cd or Zn (~25% up-regulated and ~30% down-regulated), suggesting also metal-specific regulation.

Our findings are consistent with the results of Karnatnut & Pascoe (Karnatnut & Pascoe 2002), who showed that Cu is much more toxic than Cd in hydra. Similar results were found in other marine invertebrates such as Ciona intestinalis and the sea urchin Paracentrotus lividus, demonstrating that both Hg and Cu are much more toxic than Cd (Bellas et al. 2001). Viarengo & Nott (Viarengo & Nott 1993) suggested that metal toxicity could be ranked according to electronegativity, which results in differing affinities to sulphydryl (SH) groups in the order Hg>Cu>Cd>Zn.

In most organisms, the first line of defence against heavy metals is achieved by triggering overexpression of the metallothionein group of genes by the transcription
Fig. 7 Heat map of stress-response genes in the four treated metals shown in four groups: efflux transporters (a), oxidative (b), glutathione related and conjugating (c) and Hsp (d). Fold change is presented in log 2 scale. Red: up-regulated genes, green: down-regulated genes.
factor Mtf1. The metallothionein proteins were not identified in Cnidaria (Goldstone 2008; Reitzel et al. 2008; Shinzato et al. 2012), and in our study, the transcription level of Mtf1 did not change in response to metal exposure (Fig. 5); though, it is possible that Mtf1 may regulate other defence proteins by post-transcriptional modification. Instead, in this study we identified immediate-early genes (Sheng & Greenberg 1990) that were up-regulated in all four metal treatments, and we also demonstrated that large sets of defence genes are regulated by the metal-induced stress.

Immediate-early response genes

Immediate-early genes encode transcription factors or signalling pathway regulators that are rapidly induced by a variety of stimuli that activate transcription of ‘late’ responsive genes. We identified the immediate-early genes Egr1 and c-fos-like whose transcript levels were up-regulated in all four metal treatments. Using qPCR, we confirmed the rapid induction of Egr1 and c-fos-like genes only 1 h after metal treatment (Fig. 5). Egr1 and c-fos-like are induced by many environmental signals, including oxidative stress, metals, hypoxia and internal cellular stress (Eferl & Wagner 2003), but understanding of their functional role in Cnidaria is limited. Expression of c-fos-like was recently shown to be increased after injury in N. vectensis (DuBuc et al. 2014) and during metamorphosis of coral larvae (Siboni et al. 2014). While analysing network interactions of c-fos-like and c-jun, which together compose the primary activating form of AP1 (Eferl & Wagner 2003) and Egr1 on the background of Hg-up-regulated transcripts, the involvement of additional early response genes was revealed, including SRF that may activate c-fos-like and Egr1 genes by binding, together with Elk1, to their SREs (Thiel & Cibelli 2002; O’Donnell et al. 2012). Concomitantly with the detection of these immediate-early genes, we also found members of the MAP kinase pathway, which mediates gene regulation by phosphorylation. In addition, we identified the stress-responsive transcription factor NF-κB, which was up-regulated together with its activating kinase IKK. NF-κB regulates the transcriptional activation of numerous genes involved in inflammation, apoptosis and differentiation, and in Cnidaria, it was suggested to play a role in innate immunity (Miller et al. 2007; Augustin & Bosch 2010). The NF-κB pathway was recently characterized in N. vectensis and was found to be required for stinging cell development, with specific expression limited to these cells (Miller et al. 2007; Gilmore & Wolenski 2012; Wolenski et al. 2013). It will be intriguing to find out whether NF-κB expression under metal stress remains confined to these specialized cells, which would imply a novel function of mediating stress signals to the stinging cells, or broadens to other epithelial cells. Interestingly, Egr1 stimulation in a carcinoma cell line was recently shown to be coregulated with NF-κB and AP1, as the expression of these two genes was decreased when Egr1 was inhibited by siRNA and increased when Egr1 was overexpressed (Parra et al. 2011a,b).

Our results thus provide the first demonstration that in a basal metazoan such as N. vectensis, coregulation of Egr1, NF-κB and AP1 may have an important role in integrating the environmental signals and translating them into transcriptional regulation of defence-related genes.

Down-regulated transcripts

In all metal-treated anemones, we identified down-regulated transcripts that may be related mainly to DNA synthesis, ion transfer and microtubule activity as evident from the common transcripts that were down-regulated and the GO down-regulated biological processes. These processes are vital for cellular proliferation and homeostasis, but interestingly, we also found a decrease in microtubule association with cilia or flagellar mobility. The destructive effect of heavy metal on ciliogenesis and sperm development has been described in other systems (Ribeiro et al. 2002; Kruatrachue et al. 2011; Lewis & Ford 2012), but in Cnidaria, including N. vectensis, studies of the molecular mechanism of cilia and sperm development and maturation are limited (Kuznetsov et al. 2001; Hwang et al. 2008; Künzelt et al. 2010), and further basic studies are needed before molecular ecotoxicological studies can be carried out.

Defence-related genes

From the transcriptome profiling, we analysed the different groups of the defence network (Goldstone 2008) and identified members that are capable of eliminating toxicants. These defence-related genes included the group of efflux transporters, a group of oxidative, reducing, conjugating metal detoxification and antioxidant-encoding proteins, and a heat-shock protein group, all of which will be discussed in the following sections. The extensive response, mainly to Hg and Cu, suggests that N. vectensis may be useful in the future as a bioindicator species in laboratory studies.

Transporters. ATP-binding cassette (ABC) transporters are among the largest families of active transport molecules that cause intracellular molecules to flow out of the cell or into organelles such as lysosomes and peroxisomes (Dean & Annilo 2005). N. vectensis contains six
subfamilies comprising a total of 65 ABC transporters, of which 34 (representing all six subfamilies) were found to be regulated in our study. Three subfamilies (ABCA, ABCD and ABCF) have no known role in detoxification (Goldstone 2008). However, in the present study, genes of these subfamilies were up-regulated, the most prominent group being the ABCA with five identified genes that were overexpressed (Fig. 7). The ABCA subfamily was suggested to participate in cholesterol transport (Yin et al. 2010), and ABCA3 genes have been shown to be expressed in testes and were suggested to play a role in sperm maturation in vertebrates (Dean & Annilo 2005). Interestingly, the two N. vectensis ABCA3 members were found here to be up-regulated, but further studies are needed to determine whether they are involved in protection of spermatogenesis. The ABCB subfamily contains three members that were up-regulated in the present study. Two of these belong to the ABCB1 group, which encodes p-glycoproteins that play a role in drug excretion, and are associated with multidrug resistance (MDR) (Chang 2003). The largest subfamily containing MDR proteins is the ABCB group. In N. vectensis, this group has 38 members, of which 20 were found to be regulated. The ABCB proteins are involved in export of toxic compounds conjugated with glutathione, glucuronate and sulphate (Borst et al. 2007). The sixth subfamily is the ABCG with three identified genes, two of which were found to be up-regulated here. One showed similarity to the mammalian ABCG2, which belongs to the MDR group and has a role in the xenobiotic protection of stem cells (Polgar et al. 2008).

Another large group of transporters is the SLC superfamily of solute carrier membrane proteins in which the SLC22 and the organic anion transporter polypeptides (OATPs) were identified. This family controls the uptake and efflux of many critical ions and substrates (Hediger et al. 2004). Most members of this group, especially the OATPs, were found here to be down-regulated; however, owing to the lack of adequate characterization, it is questionable to suggest substrate specificity in N. vectensis.

Our finding demonstrates that during metal stress the main group of transporters activated in N. vectensis belongs to the ABC family. The efflux transporter activity provides the first line of defence (Goldstone 2008), followed by coordinated function of proteins that modify and reduce the toxicity of the xenobiotic.

Oxidative, reducing, conjugating metal detoxification and antioxidant proteins. Flavoprotein monoxygenase (FMO) and CYP function in the oxidative transformation of xenobiotics and play a role in the maintenance of homeostasis (Cashman & Zhang 2006; Goldstone 2008). In N. vectensis, more than 80 genes of the CYP family have been identified and were divided into several clans (Goldstone 2008; Nelson et al. 2013). Most of the regulated genes in our study were from clans 2 and 3, known to play a role in the oxidative transformation of xenobiotics in higher organisms (Goldstone et al. 2006). Two FMO genes were also up-regulated, but not much is known about their biochemistry in Cnidaria.

Oxidation is followed by reductive or conjugative modification of xenobiotics, and glutathione plays important roles in these modifications and exhibits high affinity for toxic metals (Forman et al. 2009). Genes involved in the glutathione pathway have been identified, with specific up-regulation of glutathione-S-transferase (GST theta), considered the most ancient of the GSTs, and microsomal GST (MAPEG), which shows high homology with the vertebrate MAPEG1 that catalyses the conjugation of GSH to a number of electrophilic compounds (Hayes et al. 2005).

Among the antioxidant defensive genes, glutathione peroxidases (GPX) and Cu/Zn superoxide dismutase (SOD) were found here to be up-regulated. Glutathione is also the substrate for Pcs1 (Clemens & Persoh 2009). We detected overexpression of Pcs1 (Fig. 5), which catalyses formation of the metal-binding peptides, the phytochelatins, from glutathione molecules. Phytochelatins were first identified in yeast and plants and were shown to participate in metal detoxification and in metal ion homeostasis (Cobbett & Goldsborough 2002). Pcs genes have since been identified in almost all eukaryotic kingdoms (Clemens & Persoh 2009) and were suggested to have different specificities from the cysteine-rich peptides metallothioneins or to play complementary roles to them (Cobbett & Goldsborough 2002; Bundy et al. 2013). In Caenorhabditis elegans, it was demonstrated that Pcs activity is critical for heavy metal tolerance and is more important for this function than metallothioneins (Vatamaniuk et al. 2001; Hall et al. 2012). Our finding that metal stress induced overexpression of Pcs1, together with the observation that metallothioneins were absent in N. vectensis and possibly in the entire Cnidaria phylum, may suggest that phytochelatins are the main compounds for metal detoxification in cnidarians. Therefore, identifying and isolating phytochelatins in the future is likely to be of great importance for understanding cnidarian tolerance to metals.

Heat-shock protein. Hsps function as chaperones and are involved in protein folding and in preventing aggregation of inappropriately folded proteins (Richter et al. 2010). They are considered as general markers of cell damage and stress and can be induced by temperature changes, heavy metals, UV radiation and other
environmental stimuli. Hsps are divided into families according to their molecular weight, and in *N. vectensis* three families, including Hsp20, 70 and 90, were identified. In Cnidaria, an increase in expression levels of Hsp90 and Hsp70 induced by different kinds of stress has been described in corals, sea anemones including *N. vectensis*, jellyfish and hydra (Schroth et al. 2005; Bridge et al. 2010; Meyer et al. 2011; Tarrant et al. 2014). We observed expression changes in most representatives of the three Hsp groups, mainly in response to Hg treatment, which elicited relatively high expression of most of the Hsp20 genes. Small Hsps serve as protective mediators against apoptosis and carcinogenesis in higher organisms and are used as biomarkers for pathological conditions (Edwards et al. 2011). The abundant synthesis of Hsp20 RNA, mainly in response to Hg treatment, compared to its low basal expression, may suggest a conserved function of first-line protection of this group in *N. vectensis* at high toxicity.

**Concluding remarks**

Transcriptomic analysis was used in this study to examine response mechanisms of *N. vectensis* to heavy metals. We found that 24 h of exposure to heavy metals induced substantial transcriptomic responses in this cnidarian, supporting its ability to cope with the pollutants applied here and probably in the wider environment. Among the regulated transcripts, we identified immediate-early response genes, including Egr1, AP1 and NF-kB, which were already up-regulated after only one hour of exposure to the tested metals. We suggest that these transcription factors play a role in the first line of protection against the polluted environment by regulating a large array of defense genes. While the Egr1 and AP1 pathways have not yet been characterized in Cnidaria, the NF-kB pathway has been characterized and was found to be related to stinging cell development in *N. vectensis* (Wolenski et al. 2013). Future studies that characterize the immediate-early genes' regulation and spatial expression, as well as their target genes, will enable us to gain a better insight into the enhanced ability of the sea anemone to cope with its changing environment. We also detected a large array of defense genes whose expression levels were regulated, and which belong to groups known to oxidize, conjugate, reduce, transport or detoxify the heavy metals. In addition, our finding that the *PcsL* transcript is up-regulated provides the first indication that phytochelatins may fulfill the role of the missing metallothioneins in Cnidaria. Altogether, the data contributed by this study reveal early and late molecular responses of *N. vectensis* to metal pollutants and provide a broad foundation for future studies.

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**Conflict of interest**

The authors declare no conflict of interest.

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R.K., R.E. M.R. and V.B. performed the research; R.E., M.R., N.S., I.P., V.C. and T.L. performed the analyses; T.L. designed the research and wrote the paper. All authors read, edited and approved the manuscript.

Data accessibility

Illumina results were deposited in the SRA database (http://www.ncbi.nlm.nih.gov/books/NBK47529/) under accession no. SRP041974 and the full transcriptomics data in the GEO database (http://www.ncbi.nlm.nih.gov/geo) under accession no. GSE58769. The raw qPCR data can be found in Dryad (http://datadryad.org/) at doi:10.5061/dryad.62nk0.
Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Primers of the qPCR.

Table S2 Differentially expressed transcripts.

Table S3 Complete GO biological function of the four metals.

Table S4 List of first-neighbor interactions of common up-regulated transcripts of up-regulated Hg treated anemones.

Fig. S1 Heat map of up-regulated transcripts in Table S4 in the four treated metals. Fold change is presented in log 2 scale. Red: up-regulated gene.