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Early transcriptional response pathways in *Daphnia magna* are coordinated in networks of crustacean specific genes

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Author contribution

LO and LDM conceived the study and the experimental design. LO coordinated the research activities, data analysis, and manuscript writing with input from all co-authors.
MJ coordinated and performed the exposures of the two natural genotypes.
MEP coordinated and performed the exposures of the inbred genotype.
JBB and OSS coordinated and performed the DE analyses.
SH, JBB and DL coordinated and performed the network analysis.
RP contributed to DE analysis, and quality checks.
KIS, and RP performed manual functional gene annotations.
MS performed functional analysis across species.
All authors contributed to manuscript editing and discussion.

Data accessibility:

The transcriptome assembly is available from NCBI Sequence Read Archive SRP059260 (2015): http://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA284518
A searchable data base of functional annotations for *D. magna* genes against other model species including *Daphnia pulex* (dpul), *Danio rerio* (drer), *Caenorhabditis elegans* (cele), *Drosophila melanogaster* (dmel), *Mus musculus* (mmus), and *Homo sapiens* (hsap) can be found here: http://merlot.lbl.gov/omid/Daphnia/deliverables-v2.2/. *D. magna* genes are also deposited in OrthoDB (Zdobnov *et al.* 2017). The scripts used to combine methods of ortholog detection are provided in supplementary material (Appendix 1).
NCBI accession numbers to RNASeq data across the 12 environmental conditions and genotypes are provided in Appendix 2.

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Abstract

Natural habitats are exposed to an increasing number of environmental stressors that cause important ecological consequences. However, the multifarious nature of environmental change, the strength and the relative timing of each stressor largely limit our understanding of biological responses to environmental change. In particular early response to unpredictable environmental change, critical to survival and fitness in later life stages, is largely uncharacterized. Here, we characterize the early transcriptional response of the keystone species *Daphnia magna* to twelve environmental perturbations, including biotic and abiotic stressors. We first perform a differential expression analysis aimed at identifying differential regulation of individual genes in response to stress. This preliminary analysis revealed that a few individual genes were responsive to environmental perturbations and they were modulated in a stressor and genotype-specific manner. Given the limited number of differentially regulated genes we were unable to identify pathways involved in stress response. Hence, to gain a better understanding of the genetic and functional foundation of tolerance to multiple environmental stressors we leveraged the correlative nature of networks and performed a weighted gene co-expression network analysis. We discovered that approximately one third of the *Daphnia* genes, enriched for metabolism, cell signalling and general stress response, drives transcriptional early response to environmental stress and it is shared among genetic backgrounds. This initial response is followed by a genotype and/or condition-specific transcriptional response with a strong genotype by environment interaction. Intriguingly, genotype and condition- specific transcriptional response is found in genes not conserved beyond crustaceans, suggesting niche-specific adaptation.

Keywords

waterflea; differential co-expression networks; differential gene expression; biotic stressors; abiotic stressors; ecoresponsive genes; ecological gene annotation

Introduction

Natural habitats are under increasing threat from human activity, with pronounced ecological consequences (Hoffmann & Sgro 2011; Hofmann & Todgham 2010). However, the multifarious nature of environmental change (Gunderson *et al.* 2016) as well as the relative timing of each stressor (Vincenzi 2014) represent major obstacles to our understanding of biological responses to environmental change. Traditionally, biological studies subject organisms to constant and severe experimental stress conditions (Hofmann & Todgham 2010; Somero 2012). However, environmental parameters fluctuate on multiple timescales, from hours to months and years. Moreover, the intensity of stress events may vary considerably, especially between biotic and abiotic perturbations. The evolutionary success of organisms resides is their capacity to keep pace with environmental conditions that

change over short as well as long periods (Vincenzi 2014), and in their ability to cope with multiple stressors, which depends on the magnitude and relative timing of each stressor. Understanding these complex dynamics is essential for assessing organismal performance under prevailing conditions and under human-induced environmental change (e.g. Boyd *et al.* 2015)

To cope with changes in the environment, organisms must respond during and immediately after environmental perturbations (Wingfield 2013). Especially for short term responses ranging from hours to days, early response is critical to survival and fitness in later life stages (Brooks *et al.* 2011). Early response to environmental perturbations requires a fine tuning of the molecular machinery regulating physiological and behavioural responses. Early response genes maximize resource utilization while maintaining structural and genetic integrity by repairing and minimizing damage to cellular structure (Huisman & Kolter 1994; Ram *et al.* 2005). Genes interacting with the environment generally return to their original expression level after an initial acclimation phase (Eng *et al.* 2010). Because a large proportion of studies does not focus on this early modulation phase (but see studies on temperature of maximum tolerance: Geerts *et al.* 2015; Hofmann & Todgham 2010; Somero 2010), the modulation of mRNA in early response to environmental stress is largely uncharacterized. Conversely, environmental perturbations are most commonly studied in the context of organisms life cycle [e.g. tadpoles (Leduc *et al.* 2015), mice (Jangiam *et al.* 2015), marine algae (Zou *et al.* 2015), and plants (Kim *et al.* 2015)] or to uncover the mode of action of lethal concentrations of toxicants [e.g. fish (Techer *et al.* 2015), frogs (Mardirosian *et al.* 2015), bivalves (Larguinho *et al.* 2014), copepods (Overjordet *et al.* 2014), snails (Khalil 2015) crustaceans (Tang *et al.* 2015), and plants (Keunen *et al.* 2015), (Fu *et al.* 2014)].

Although the response to simultaneous stressors generally leads to more complex scenarios than responses to single stressors (Holmstrup *et al.* 2010; Rejeb *et al.* 2014), the analysis of a wide range of single stressors is the first critical step in the identification of defence mechanisms pathways that may be shared among stressors, leading to a better understanding of cross-tolerance mechanisms (Perez & Brown 2014). In addition, stress responses can vary substantially between genotypes as plasticity in gene expression, i.e. variation in expression of a gene in response to stress, is known to be at least partly heritable and can be affected by natural selection (Whitehead & Crawford 2006). Understanding variation in stress response among genotypes is a prerequisite to our understanding of adaptive evolution in nature.

Here, we investigate the early transcriptional response of three genotypes of the branchiopod crustacean *Daphnia magna* to a suite of environmental perturbations, including six biotic and six abiotic stressors. These stressors and their intensity represent either biotic ecologically relevant perturbations encountered in the natural environment or abiotic perturbations found in human-impacted environments.

Daphnia are freshwater grazers renowned as ecotoxicological models (Colbourne *et al.* 2005; Miner *et al.* 2012) and central to virtually all inland lentic aquatic habitats (Miner *et al.* 2012). As filter feeders these small crustaceans are exposed to numerous environmental insults to which they respond via physiological, microevolutionary or genetic mechanisms

(e.g. Decaestecker *et al.* 2007; Latta *et al.* 2012; Orsini *et al.* 2012; Yampolsky *et al.* 2014). *Daphnia* has a parthenogenetic life cycle that allows the rearing of populations of genetically identical individuals (clones) from a single genotype, providing the advantages of isogenic model organisms while retaining the natural genetic variation (Miner *et al.* 2012). Capitalizing on the ability to maintain isoclonal lines in the laboratory, we exposed populations of three *D. magna* genotypes to twelve environmental perturbations lasting maximum 24 hours to capture the early transcriptional response to environmental perturbations via genome-wide transcriptional profiling.

We identified a few individual genes responsive to environmental perturbations and these were modulated in a genotype-specific manner. Leveraging a gene co-expression network analysis, we were able to link clusters of genes of unknown function to genes with known function and a range of environmental perturbations. Many of the identified clusters of genes were co-expressed across the environmental conditions surveyed and/or the genotypes analysed. With evidence of shared networks among conditions and/or genotypes we identified the biological roles of hundreds of genes in stress response of a key grazer of the aquatic community. Intriguingly, highly responsive networks of genes to environmental perturbations were less conserved beyond crustaceans than genes overall. This finding indicates that these genes may be broadly associated with crustacean-specific adaptation to environmental stress.

Materials and Methods

Study species

The cladoceran *D. magna* is a keystone species present in virtually all lentic ecosystems. This species has a parthenogenetic life cycle that allows the rearing of populations of genetically identical individuals (clones) from a single genotype. For the present study we used two natural and a recombinant genotype obtained from the crossing of the first two. The two natural genotypes were collected from a system of ephemeral rock pools from the northern distributional range of the species (Xinb3, South west Finland 59.833183, 23.260387) and a fish-rearing pond in Southern Germany (Iinb1, Germany, 48.206375, 11.709727), respectively. The Xinb3 genotype was the result of three generations of selfing, and the Iinb1 strain was selfed for one generation, leading to a predicted 87.5 and 50% reduction in their original level of heterozygosity, respectively. The recombinant line is an F2 laboratory strain part of a mapping panel supporting research on the genetic basis of adaptive traits in *D. magna* (Routtu *et al.* 2014). The genotypes will be hereafter referred to as: X - Xinb3, I - Iinb1 and XI – recombinant. The X and I genotypes were previously used to generate ‘omics’ resources for *D. magna*. More specifically, the X genotype was used to generate a reference genome (NCBI accession number: LRGB00000000), whereas the X and I RNA-Seq data were previously used to generate a reference transcriptome (Orsini *et al.* 2016). Here, we use the available RNA-Seq data previously for X and I as well as the newly generated RNA-Seq data for the XI genotype to investigate transcriptional response across stressors and genotypes.

Experimental design

The three genotypes were distributed for environmental exposures between two laboratories of the Stressflea consortium (ESF EUROCORES Programme EuroEEFG, Grant 09-EEFGFP-040) that studies mechanisms of adaptation to environmental stress using *Daphnia* as model species. X and I genotypes were exposed to five biotic and one abiotic perturbations: vertebrate (FI, 19 sticklebacks in 100L water) and invertebrate predation (TR, 1 adult *Triops* in 2L water), parasites (PA, 40,000 *Pasteuria ramosa* spores/mL), crowding (CR, 100 individuals/250 mL), the methylcarbamate insecticide Carbaryl (CA, 8μg/L), microcystin-producing (BX) and microcystin - free (BN) cyanobacteria. Perturbation from cyanobacteria was obtained by feeding *Daphnia* with a toxic (Cyanobacteria, strain MT50) and a non-toxic strain of *Microcystis aeruginosa* (strain CCAP 1450/1) (Lemaire *et al.* 2012). The recombinant genotype XI was exposed to five abiotic perturbations: Cadmium (CD, 6 μg/L), Lead (PB, 278 μg/L), low pH (5.5), Sodium Chloride (NaCl, 5g/L), and UV radiation (UV, 30 W, 36-inch Reptisun 5.0 UV-B fluorescent light bulbs). Overall the exposures cover a wide range of environmental perturbations that *Daphnia* is exposed to in the natural environment, including human-impacted habitats.

The exposures of the X and I genotypes were completed over two days. For each day a control (no stress imposed) was run in parallel to the environmental perturbations. Each treatment, including controls, was performed on three biological replicates and for each replica we obtained genome-wide transcription profiling. The environmental perturbations for the X and I genotypes and the UV exposures for the XI genotype were 4-hours long, whereas the remaining exposures were 24 hours long. The length of exposure varied with the environmental stress tested driven by previous pilot experiments designed to identify realistic environmental perturbations encountered in the natural environment. Prior to the exposures to environmental perturbations, clonal populations of the three genotypes were synchronized in common garden conditions – controlled climate chambers with a fixed long day photoperiod (16h light/8h dark) at 20°C- for at least two generations to reduce interference from maternal effect. ADaM medium (Aachener Daphnien Medium: Klüttgen *et al.* 1994) was used as growth medium and for the environmental perturbations. The animals were fed daily with 150,000 cells *Scenedesmus obliquus*/ml. The first generation was cultured at a density of 10 individuals/L, and increased to 50 individuals/L in large aquaria in the second generation to enable the harvesting of enough animals for the environmental perturbation exposures. The second clutch of the second generation was used for exposures to environmental perturbations. Five-day old female juveniles randomly chosen from the offspring of the second generation of the synchronized animals at a density of 100 juveniles/L were exposed to the different environmental treatments. The animal density for the exposures was determined following prior literature studies on *Daphnia* exposures (e.g. Jansen *et al.* 2011).

Up to three investigators performed the environmental exposures and randomly distributed the animals from the aquaria to the experimental vials. Exposures to environmental perturbations for the two natural strains were conducted at the University of Leuven, Belgium. The sequencing for this experiment was performed at the Finnish Institute of Molecular Medicine (FIMM, Technology Centre, Sequencing unit) at the University of

Helsinki. Exposures of the recombinant line were completed at the University of Notre Dame, IN, USA. The sequencing data from this experiment were obtained at the JP Sulzberger Columbia Genome Center (https://systemsbiology.columbia.edu/genome-center). RNA-Seq data were obtained for each treatment and control in triplicates. The quality assessment of the RNA-Seq data in terms of reproducibility across the biological replicates identified the sample I_BN_r3 (toxic *Microcystis* treatment for strain I) as an outlier (Orsini *et al.* 2016). I_BN_r3 was excluded from downstream analyses as it obscured any signal from both the genotype and the treatment. Once this treatment was excluded replicates per treatment clustered as expected (Orsini *et al.* 2016).

RNA-Seq

Library construction was performed on three biological replicates following Nextera workflow (Illumina) with minor modifications. TruSeq PE Cluster Kit v3 (Illumina, San Diego, CA, USA) was used for paired-end sequencing with 101 bp read length and sequenced on an Illumina HiSeq2000 platform (TruSeq SBS Kit v3 reagent kit).

Read sequences were subjected to adapter trimming and quality filtering using Trimmomatic ver.0.33 (Bolger *et al.* 2014). RNA-Seq reads were checked for foreign RNA contamination. Human and mouse contaminant sequences were screened and removed by mapping *D. magna* reads onto ncbigno2014-human.rna and ncbigno2014-mouse.rna using bowtie2 ver. 2.1.0 (Langmead & Salzberg 2012). Finally, 80% of the reads for the inbred genotypes and 99% of the reads for the recombinant genotype were retained (Q>20). The cleaned reads were mapped onto the reference transcriptome of *D. magna* obtained from *de novo* assembly of RNA-Seq data (Orsini *et al.* 2016). These data consisted mostly of the Xinb3 inbred genotype data, but also included a subset of data from the Iinb1 genotype and RNA-Seq available in public databases for *D. magna* at the time of the analysis (mostly, Labbe *et al.* 2009). Details on the generation of reference transcriptome and gene models are provided in (Orsini *et al.* 2016).

Differential expression analysis

Reads mapping uniquely to primary gene transcripts were used to measure differential expression of individual genes. This approach is suitable also for genes with no location or poor match to the *D. magna* draft chromosome assembly (NCBI accession number: LRGB00000000). Differential gene expression was calculated on normalized read counts per treatment as compared to a control (no stress) using DEseq2.0 by use of negative binomial generalized linear models (P_{adj}=0.01; Love *et al.* 2014).

We assessed the conservation beyond crustaceans of genes differentially expressed in response to environmental perturbation by quantifying the correlation between fold change - no differential expression, 2-fold, 4-fold and 16 - fold expression- and the percentage of orthologous genes found in other model species for each category. By partitioning genes in categories based on their magnitude of expression after environmental perturbations, we separated genes that showed basal expression - no differential expression after any perturbation - from genes showing different magnitude of expression under at least one environmental perturbation. For each category we identified orthologs in six other model

species -*Daphnia pulex* (dpul), *Danio rerio* (drer), *Caenorhabditis elegans* (cele), *Drosophila melanogaster* (dmel), *Mus musculus* (mmus), and *Homo sapiens* (hsap). This was done using an *ad hoc* developed orthologue detection pipeline (Appendix 1). This pipeline uses six orthologues detection engines to produce a list of annotated orthologous genes. The combined use of these engines provided robust results as compared to the use of single search engines as orthologous genes function is assigned bases on different criteria, including sequence similarity, domain architecture, and phylogenetic relationship among species. Our pipeline combines the following orthologous genes assignment tools: DODO (Chen *et al.* 2010), OMA (Altenhoff *et al.* 2015), InParanoid8 (Sonnhammer & Ostlund 2015), TreeFam (Ruan *et al.* 2008), Proteinortho (Lechner *et al.* 2011), and OrthoMCL (Li *et al.* 2003).

Weighted gene co-expression network analysis

To gain insights into the regulatory patterns of uncharacterized genes we performed a weighted gene co-expression network analysis with MODA (Li *et al.* 2016) using the normalized expression data for all genes. MODA performs differential expression analysis of gene clusters. The clusters are identified using the WGCNA Topological Overlap Matrices (TOM; Langfelder & Horvath 2008). However, differently than WGCNA, MODA uses a cut-off threshold based on optimal average density to identify clusters of genes (also called modules; Li *et al.* 2016). The cut-off threshold for the clusters identified by the hierarchical clustering is determined based on the optimal average density of modules defined as:

$$Density(A) = \frac{\sum_{i \in A} \sum_{j \in A, j \neq i} a_{ij}}{n_A(n_A - 1)}$$

where a_{ij} is the similarity of gene i and gene j in module A. After the clusters or modules are identified, differential expression is performed using the Jaccard similarity index. The Jaccard index measures similarity between finite sample sets, and is defined as the size of the intersection divided by the size of the union of the sample sets (Jaccard 1902). When two modules appear in networks N_1 and N_2 (each network is represented by several gene clusters or modules), a similarity matrix B is calculated, in which each entry B_{ij} is the similarity between the ith module in network N_1 (denoted by $N_1(A_i)$) and jth module in network N_2 (denoted by $N_2(A_j)$). The similarity index B_{ij} is estimated as:

$$B_{ij} = \frac{N_1(A_i) \cap N_2(A_j)}{N_1(A_i) \cup N_2(A_j)}$$

In our analysis N_1 is the background gene network including genes expressed in all samples and biological replicates whereas N_2 is the network obtained after subtracting gene clusters specific to a treatment or a genotype. We used MODA to identify gene clusters shared among genotypes as well as gene clusters shared among either the biotic or the abiotic treatments. The frequency with which each gene cluster was identified in the treatments and the genotypes is visualized in a bar plot. This visualization enables the identification of

condition-specific and shared modules among genotypes and/or environmental perturbations.

Modules identified by MODA in the treatment and genotype analysis were used for gene enrichment analysis. The genes found in each module were analysed using DAVID (Huang *et al.* 2009) and enriched GO terms and enriched functional-related gene groups were identified based on similarity to the *Drosophila* Uniprot database.

Results and Discussion

Ecoresponsive gene clusters are not conserved beyond crustaceans

A genome wide differential expression analysis revealed that most differentially expressed genes (DEseq $p\text{-}adj < 0.01$) were modulated in a genotype-specific manner, with individual treatment effects obscured by genotype effects (Fig. 1). This early transcriptional response is often mediated by functionally uncharacterized genes (Table S1).

To better contextualize genes of unknown function, we conducted gene modules differential analysis on weighted gene co-expression networks using MODA (https://bioconductor.org/packages/release/bioc/html/MODA.html; Li *et al.* 2016) which enables the association of individual genes in co-responsive modules (gene clusters). Using this approach we identified numerous unannotated genes tightly linked to our differentially expressed genes and gained insights into the pathways and the regulatory mechanisms in which novel genes participate. This association points to the power of exposure biology for the annotation of unknown genes in an ecological context.

Across all genotypes we discovered 62 modules (Table S2). Of these, 5 (6.45%) are shared among the three genotypes whereas the remainder is found uniquely in one of the genotypes (Fig. 2). The shared modules include overall 11,698 genes, approximately a third of the total genes in *D. magna* (Orsini *et al.* 2016), with shared Module #58 (Table S2) including 11,537 genes. This module is enriched for morphogenesis, cell signalling, development and metabolic genes as well as by defence response genes, including oxidative response (Table S3). There are in total 47 genotype-specific modules; of these 28 are unique to XI, 11 to I and 8 to X (Fig. 2). The genotype-specific modules are generally small (< 100 genes) and include many unannotated genes (Table S4). Finally, 11 modules (17.7%) are shared between pairs of genotypes as follows: Modules #144 and #157 are shared between X and I; Modules #1, #79, #106 and #158 are shared between X and XI; Modules #8, #45, #62, #81 and #119 are shared between I and XI (Fig. 2). These findings suggest that there may be a hierarchical activation of stress response with general mechanisms of response at metabolic and cellular level activated as first defence regardless of the genetic background, followed by more specific responses that is genotype or treatment specific. We discuss below about the treatment-specific responses. This implies that whereas a proportion of the transcriptome is shared among genotypes, there is an important component of the transcriptome displaying a genotype-by environment (GxE) interaction. Previous studies on *Daphnia* spp identified a large number of responsive genes to environmental perturbations with a significant GxE interaction (Colbourne *et al.* 2011; Yampolsky *et al.* 2014). Remarkably, more than 60% of these ecoresponsive genes were lineage specific and over-represented in the transcriptome of

the congeneric species *D. pulex* (Colbourne *et al.* 2011). With the rapid growth of sequencing technology overrepresentation of lineage specific genes has been reported in other species. For example, it is found in *Caenorhabditis elegans* in response to extreme environments (Zhou *et al.* 2015). In general, lineage specific genes have been suggested to be one of the principal means of adaptation and one of the most important sources of organizational and regulatory diversity in eukaryotes (Lespinet *et al.* 2002). Intriguingly, we find that responsive genes to ecologically relevant environmental perturbations are systematically less conserved than genes in general. Around half the genes with strong early transcriptional responses to one or more stressors are specific to crustaceans (Fig. 3), and are as-yet functionally uncharacterized (Table S4). Of the 1,396 differentially expressed genes identified in our study, only 612 (44%) were conserved outside of the *Daphnia* lineage and possibly crustaceans (Fig. 3). This is a small number compared to other metazoans where up to 67% of genes have known orthologs in other species (Brown *et al.* 2014). Hence, our findings represent an overall 17 standard deviations from expectation based on other metazoan (normal approximation to binomial p-value < 1e-100). We cannot definitively conclude that these genes are newly evolved or niche specific as it is likely that any error in the transcriptome assembly enriches for the appearance of unconserved genes. However, if we condition on genes that are detectably conserved between *D. pulex* and *D. magna* using information available from the assembled *D. magna* transcriptome (Orsini *et al.* 2016) to mitigate this source of statistical confounding, we see a striking pattern: the degree of induction or suppression of transcript abundance in response to environmental perturbations is inversely correlated with the likelihood of conservation beyond crustaceans (Fig. 3). That is, more strongly induced or suppressed genes are systematically less conserved. Remarkably, 72% of genes that respond 16 fold to at least one assayed perturbation have no orthologues outside crustaceans – a dramatic difference compared to the 25% of genes overall having known orthologues in other species (p-value < $1e^{-100}$; Fig. 3). In summary, our analysis of transcriptional response in multiple genotypes of the same species provides insights into stress response in different genetic backgrounds, critical to our understanding of tolerance and performance in nature (Hofmann & Todgham 2010; Latta *et al.* 2012; Ramu *et al.* 2016). Our results also confirm previous findings about the overrepresentation of lineage specific genes in crustaceans as a potential mechanisms of adaptive response to changing environments (Colbourne *et al.* 2011).

Predictable networks of stress-response genes

Our first approach to identify stress-specific early transcriptional response was an analysis of differential gene regulation. This analysis revealed that only a few individual genes were differentially expressed, many of which under abiotic perturbations (Table S1). In the following, we first discuss the results of this analysis and then present the results obtained leveraging the correlative structure of networks that allowed us to identify co-responsive gene clusters shared among and unique to environmental perturbations.

In total, 1,396 genes were differentially expressed across all treatments and genotypes (Table S1). Generally, very few of these genes were shared among environmental perturbations (Table S5). More specifically, 75 genes (5.4%) were differentially expressed in more than one abiotic treatment (Table S5), whereas only 10 genes (0.7%) were differentially

expressed in at least one biotic and one abiotic treatment (Table S5). Genes that were differentially expressed in multiple treatments did not necessarily change in the same direction across abiotic treatments but consistently changed in the same direction in biotic treatments (Table S5).

Under sublethal heavy metal exposure (Pb and Cd) and UV treatments, genes involved in nucleotide excision and transcriptionally coupled DNA repair pathways were strongly down-regulated, and germ cell proliferation was pervasively shut down (Table S1). The most strongly induced genes under heavy metal exposure and low pH include *glutathione S-transferases* and *synthases*. Interestingly, the *glutathione S-transferases* are upregulated under heavy metals but appear to be both up and down-regulated under osmotic stress (*Dapma7bEVm002921t1, Dapma7bEVm010893t1*; Table S1). In addition to the well-known chitin remodelling response to heavy metals (Bekesiova *et al.* 2008; Lanfranco *et al.* 2004; Poynton *et al.* 2007; Wang *et al.* 2015), mediated by strong up-regulation of genes encoding chitinases, cuticle proteins, and cuticle binding proteins (Table S1), we see the activation and repression of numerous *trypsins* and *trypsin* inhibitors under heavy metals and UV treatments. These differentially expressed genes indicate regulation of digestive enzymes and associated processes, similarly to reported response in *Drosophila melanogaster* under heavy metal exposure (Brown *et al.* 2014).

Under metal stress we also observe the down-regulation of early developmental factors: several transcription factors and RNA binding proteins. These include the *Daphnia* ortholog of *paired* (*prd*) (*Dapma7bEVm010730t1, Dapma7bEVm023055t1*, Table S1, XI genotype), also down-regulated under UV treatment, and the nuclear receptor coactivator, homologous to the splicing factor *Neosin* (*Neos*) in *Drosophila* (*Dapma7bEVm029191t1*, Table S1), indicating that both transcriptional and post-transcriptional regulation play a role in adaptation to heavy metal exposure. Further, the gene homologous to steroid dehydrogenase (*Dapma7bEVm002436t1*) linked to the production of *20-Hydroxyecdysone* (*20E or ecdysone*) in arthropods (Masuoka & Maekawa 2016) also responds, possibly preceding cuticle remodelling, and hence playing a potentially important role in moulting and growth. The activation of larval cuticle proteins in insects, including the model organism *D. melanogaster*, has been reported in response to environmental perturbations (Brown *et al.* 2014). In *Daphnia* spp, body remodelling is a known response to predator cues, in the presence of which they become bulkier or produce neck teeth (Laforsch & Tollrian 2004; Stoks *et al.* 2016). The pervasive activation of cuticle protein precursors in arthropods in response to environmental perturbations prompted us to further examine similarities between genes responsive under environmental perturbations in *D. magna* (present study) and those in *D. melanogaster* (Brown *et al.* 2014; Stoiber *et al.* 2016). The fly studies involved chronic exposures (48 hours) generally at much higher (highly toxic) doses; hence a large overlap was not expected. Consistently, we find a small, but intriguing overlap between the fly studies and the present study, including *Amylase proximal* (*Amy-p*) and *PAR-domain protein 1* (*Pdp1*), which were repressed in both species under heavy metal treatments (Table S6). The steroid dehydrogenase, repressed in *Drosophila*, was either repressed under microcystin-producing cyanobacteria or induced under heavy metals and UV treatment in *Daphnia* (Table S6, *CG32369*). The overlap in gene expression between *Drosophila* and *Daphnia* is suggestive of similarity in mechanisms of response to stress. However, functional

studies are needed to validate these findings and confirm similarities between the two species.

We found strong down-regulation of *SOD1-vtg* fusion in response to osmotic (NaCl) and oxidative stress (UV-B light), whereas we observed up-regulation of *SOD-vtg* under Cd, carbaryl and both microcystin treatments (Table S1). *SOD-vtg* is generally expressed in diapausing insects (Bi *et al.* 2014) and crustaceans (Acton 2012)); hence, the regulation of this gene under environmental perturbation indicates a potential alteration of the diapause process by environmental perturbations (e.g. Slusarczyk & Rybicka 2011). In summary, early response to abiotic perturbations (UV-B, acidification and osmotic stress) was characterized by strong down-regulation of germ cell proliferation and activation of genes regulating either cuticle formation involved in moulting and growth or egg formation. These responses indicate that, despite the sub-lethal concentrations used reflecting current or realistic contamination of human-impacted inland waters, the environmental perturbations are severe enough to potentially affect growth, moulting and reproduction. The observation that *chitinase* and cuticle protein synthesis is upregulated under heavy metals suggest that short term exposures to metals affect animal fitness via their ability to moult. Further, the silencing of *SOD1-vtg* under osmotic and oxidative stress might indicate that the production of resting eggs is impaired or that maturation of the animals is severely delayed, as the production of eggs in *Daphnia* is tightly linked to moulting (Raborn *et al.* 2016). Further experimental work is needed to validate these hypotheses.

As opposed to the abiotic perturbations that provided a number of differentially expressed genes with homology in other species, the biotic responses were challenging to interpret, as the vast majority of responsive genes to biotic stress lack known conserved functional domains (Table S1, X genotype, I genotype). Among the biotic treatments, an interesting response was the one to microcystin treatment. Under this treatment, the suppression of germ cell proliferation and activation of heat shock factors and antioxidant transporters, expected in presence of cell toxicity, was not observed (Table S1). In our environmental exposures to microcystin - producing and microcystin - free cyanobacteria, we observe a typical stress response caused by poor food quality rather than by the presence of toxins; we observe the activation of digestive enzymes (Table S1). The absence of a toxic response is corroborated by the absence of signatures of germ cell suppression and stress proteins in the presence of cyanobacteria coupled with the activation of metabolic stress response genes (e.g. glucose metabolism, *trehalose* transporter and cellular response to starvation). Recent studies have shown genotype-based resistance of *Daphnia* to microcystin (Jiang *et al.* 2013; Lemaire *et al.* 2012). In line with previous studies (Lemaire *et al.* 2012), our results point to different response to mycrocystin dependent on genetic background (genotype) and/or prior environmental exposures, as the transcriptional response to cyanobacteria treatments was more severe in the X than in the I genotype. Indeed, the X genotype was sampled from a temporary rock pool system in which cyanobacteria blooms were never reported. Conversely, the I genotype was sampled from a typical eutrophic lake in central Europe that has likely experienced cyanobacteria blooms (O'Neil *et al.* 2012). The accumulation of cyanotoxins in freshwater environments due to harmful algal blooms is an increasing concern for environmental and governmental agencies because of the potent adverse effect they can have on human health and livestock (Backer *et al.* 2015; Hudnell 2008). The use of

Daphnia to remediate watersheds and drinking water supplies affected by toxic algal blooms is an intriguing possibility for freshwater restoration (see Peretyatko *et al.* 2012; Sarnelle 2007 for applications) and may be envisioned considering the observed response to the toxic cyanobacteria treatment we observed.

The differential expression analysis enabled us to identify key genes regulated in specific environmental perturbations, in particular in abiotic ones. However, given the limited number of differentially regulated genes, we were unable to identify shared pathways among treatments leading to the identification of predictive pathways activated by the environmental stressors studied here. To gain a better understanding of the genetic and functional foundation of tolerance to multiple environmental stressors we leveraged the correlative nature of networks using MODA (Li *et al.* 2016) and identifying modules shared among environmental perturbations. The discovery of gene networks predictive of stress-response in the keystone species *Daphnia* could be used as a monitoring tool of freshwater ecosystems health and eventually lead to an anticipation strategy of response to stress.

We discovered 33 co-expression modules across biotic and 25 across abiotic treatments (Fig. 4 and Table S7 for all genes co-expressed in each module). Intriguingly, only two modules are shared across all conditions – biotic and abiotic (Table S7, bio_abio). One contains essentially all housekeeping genes (ca. 9,000 genes, Module #54). The other (Module #154) includes 531 genes (Table S7, bio_abio), 280 of which have no conserved domain, no known orthologues outside crustaceans, and no known functions. This module is strongly enriched for a variety of *P450 cytochromes* (40 genes, GO terms: 0004497, 0020037, 0009055, 0005506, max Fisher's exact test FDR-corrected *p*-value = 8e-24, Table 8), cell membrane and structural components (84 genes, GO terms: 0042598, 0005792, 0005624, 0005626, 0000267, 0019898, 0016020, 0005783, max FDR-corrected *p*-value = 1e-9, Table S8), and genes involved in cuticle formation, which is critical for growth and moulting (17 genes, GO terms: 0005214, 0042302, max FDR-corrected *p*-value = 5e-9, Table S8). Collectively (141 genes), these enrichments indicate that detoxification and cell proliferation are common features of *Daphnia*'s early response to stress. Whether cytochrome expression or proliferating cells are localized to a single physiological compartment, e.g. the haemolymph (inferred from the respiration signal) or epithelia (from the cuticle signal), or more broadly distributed across multiple organs will require additional transcriptomics analysis in dissected tissues or isolated cells. The association of this large collection of genes of unknown function with genes involved in respiration and cell proliferation may help to focus targeted studies to uncover their functions. For now, all we know about these 280 genes is that they are co-responsive with the genes of known function (see Table S7 for all genes in each module).

Co-expression Module #70 is shared among abiotic conditions (Fig. 4). This module includes ca. 6,800 genes (Table S7) and is enriched for membrane and transporter activity, cell signalling, morphogenesis and redox activity genes; it also contains developmental and growth genes (Table S9). Module #107 shared among UV, pH and osmotic stress contains 45 genes (Table S7) enriched for ion transport activity and metabolic processes as it can be predicted under these stress conditions, suggesting that our unbiased approach reveals biological relevant mechanisms of response to environmental stress.

Module #25 is shared across all biotic conditions. It contains 37 genes (Table S7), 18 of which have no known function, and 10 encode *vitellogenin* lipoproteins (FDR-corrected *p*-value < 1e-9). Notably, these *vitellogenins* are fused to a superoxide dismutase domain (*SOD1-vtg*). The remaining seven genes of known function are likely lipid or lipoprotein transporters, including *Dapma7bEVm010067t1* and *Dapma7bEVm019273t1*, two *CRAL/TRIO-domain* containing proteins and yeast *Sec14p* orthologs; two probable haemolymph coagulation co-factors, one encoding a von Willebrand factor (*vWF*, *Dapma7bEVm022355t1*) and a *BTB/POZ domain* containing gene (*Dapma7bEVm022613t1*); and one uncharacterized, but conserved peptide (*Dapma7bEVm020225t1*) which encodes the conserved domain of unknown function *pfam09172*, *DUF1943*. While changes in v*itellogenins* are not statistically significant in each treatment, the pervasive presence of these genes across treatments indicates that their modulation is linked to numerous environmental perturbations.

Four modules were shared between the vertebrate and invertebrate predator treatments (Modules #25, #102, #123, and #148) and enriched for canonical signatures of body remodelling (chitinase activation) and activation of cuticle proteins, including larval cuticle proteins (Table S10). Discussing each of the modules in turn is beyond the scope of this manuscript, and hence all modules (Table S7), as well as their GO term analyses (Tables S8, S9, and S10), are provided in Supplementary material as a community resource.

Conclusions

We discovered that approximately one third of the *Daphnia* genes, enriched for metabolism, cell signalling and general stress response, is responsible for early transcriptional response to environmental stress and it is shared among genotypes. We also discovered that a large proportion of the transcriptome responds to environmental perturbations with a strong GxE component. This suggests a sequential activation of transcriptional response to environmental stress, which first involves a general stress response, and then activates a genotype and/or condition-specific transcriptional response. The latter is driven by smaller modules of genes enriched for more specific functions - e.g. body remodelling to predator cues or ion transporter for oxidative and osmotic stress. Intriguingly, genotype and condition- specific transcriptional response is found in genes not conserved beyond crustaceans. These genes may be associated with niche-specific adaptation. Finally, our study associated hundreds of uncharacterized genes within co-responsive modules to genes of known function and to specific environmental perturbations. This link represents a fundamental basis for our understanding of the genetic and functional foundation of tolerance to multiple environmental stressors. Establishing these associations is generally challenging, even in the best studied model species [e.g. yeast, (Pena-Castillo & Hughes 2007) and fly, (Brown & Celniker 2015)].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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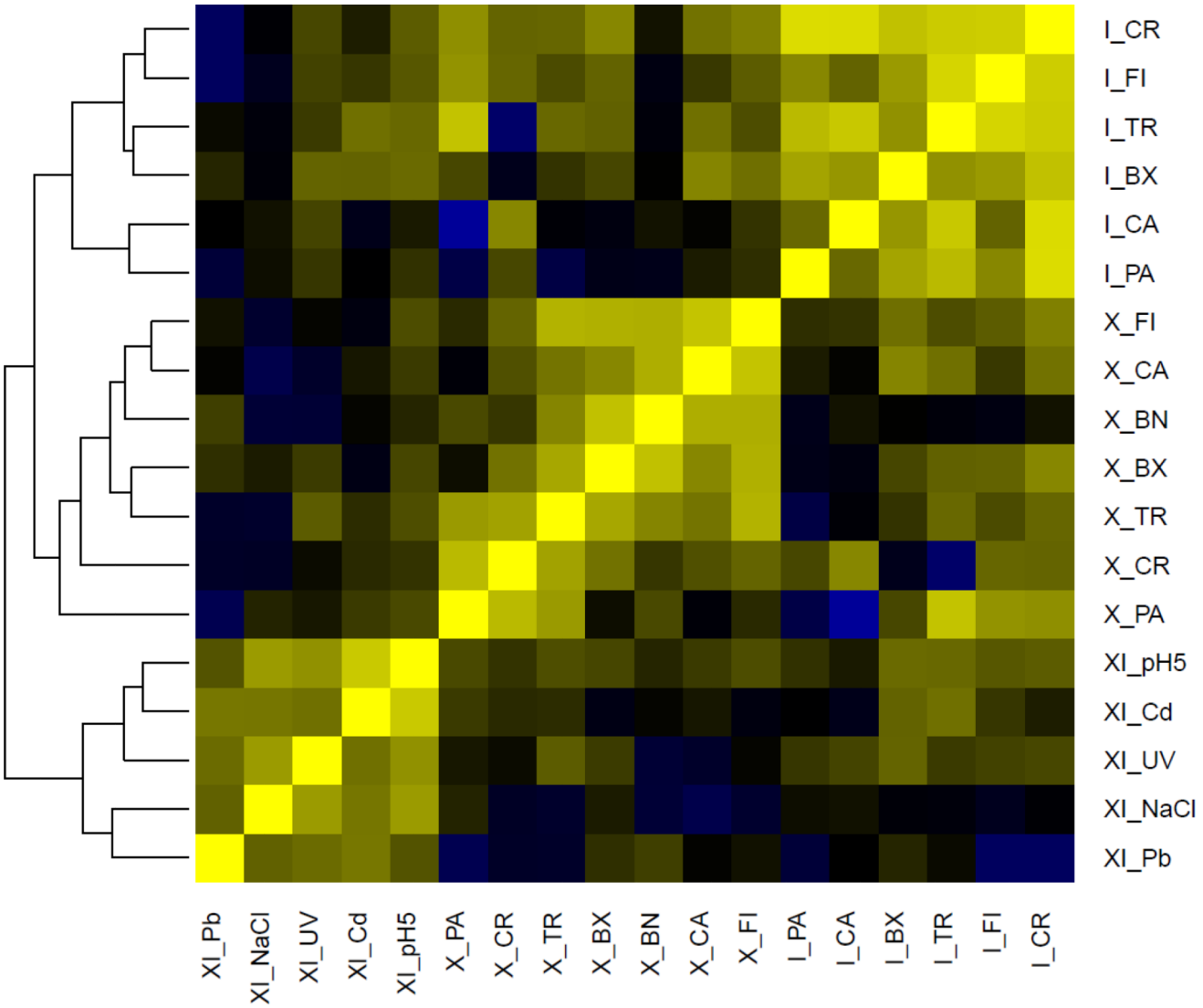

Figure 1. Genome-wide differential expression analysis

Heat map of the most genome-wide scale differentially expressed genes (DEseq *p-adj* < 0.01) in the three genotypes: X, I and XI. The data are obtained from three biological replicates per genotype. Yellow indicates high similarity whereas blue indicates low similarity. The abbreviations for the environmental perturbations are as follows: **FI**: Vertebrate predation; **TR**: Invertebrate predation; **PA**: exposure to the parasite *Pasteuria ramosa*; **CR**: crowding; **BX:** microcystin-producing cyanobacteria; **BN:** microcystin-free cyanobacteria; **CA**: insecticide Carbaryl; **CO**: control. The environmental perturbations imposed on the recombinant genotype are as follows: **CD:** Cadmium; **PB**: Lead; **pH 5.5:** low pH; **UV:** UV-B light; **NaCl:** osmotic stress. The treatment BN for the I strain was not included in this analysis as a PCA plot inclusive of all data identified this treatment as an outlier (Orsini *et al.* 2016).

Figure 2. Co-expressed gene networks identified in the genotype analysis

Significantly co-expressed gene networks (numbered on y-axis) shared among genotypes: X, I and XI. From top to bottom the networks are ordered starting from shared among all genotypes, shared between two genotypes and genotype-specific. The genotypes are color-coded as follows: X - black; I - grey; XI - red. Numbers on the y-axis refer to the module number listed in Supplementary Table S2

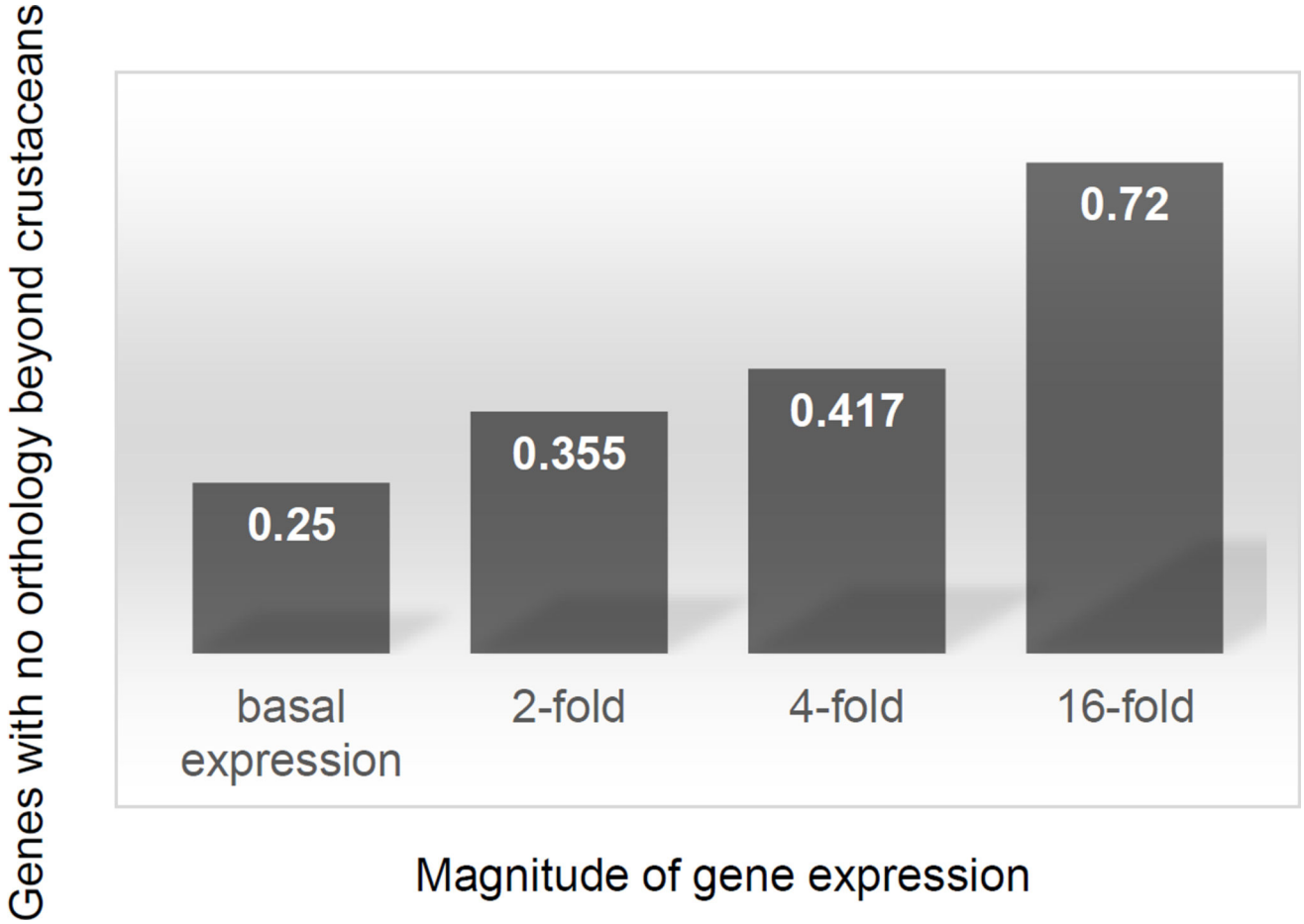

Figure 3. Conservation of genes responsive to environmental perturbations

Genes responsive to environmental perturbations (N = 1,396 genes) are partitioned based on their magnitude of differential expression, ranging from basal expression (no differential expression under any perturbation) to 16-fold differential expression under at least one assayed environmental perturbation. For each category of differentially expressed genes the percentage of non-orthologous genes to other model species outside crustaceans is shown. The following model species were used for orthology searches: *Danio rerio*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Mus musculus*, and *Homo sapiens*. The two crustacean species *Daphnia magna* and *Daphnia pulex* were used in orthology searches to identify genes conserved within crustaceans. The percentage of genes with no orthology beyond crustaceans is indicated as percentage for each category of differentially expresses genes.

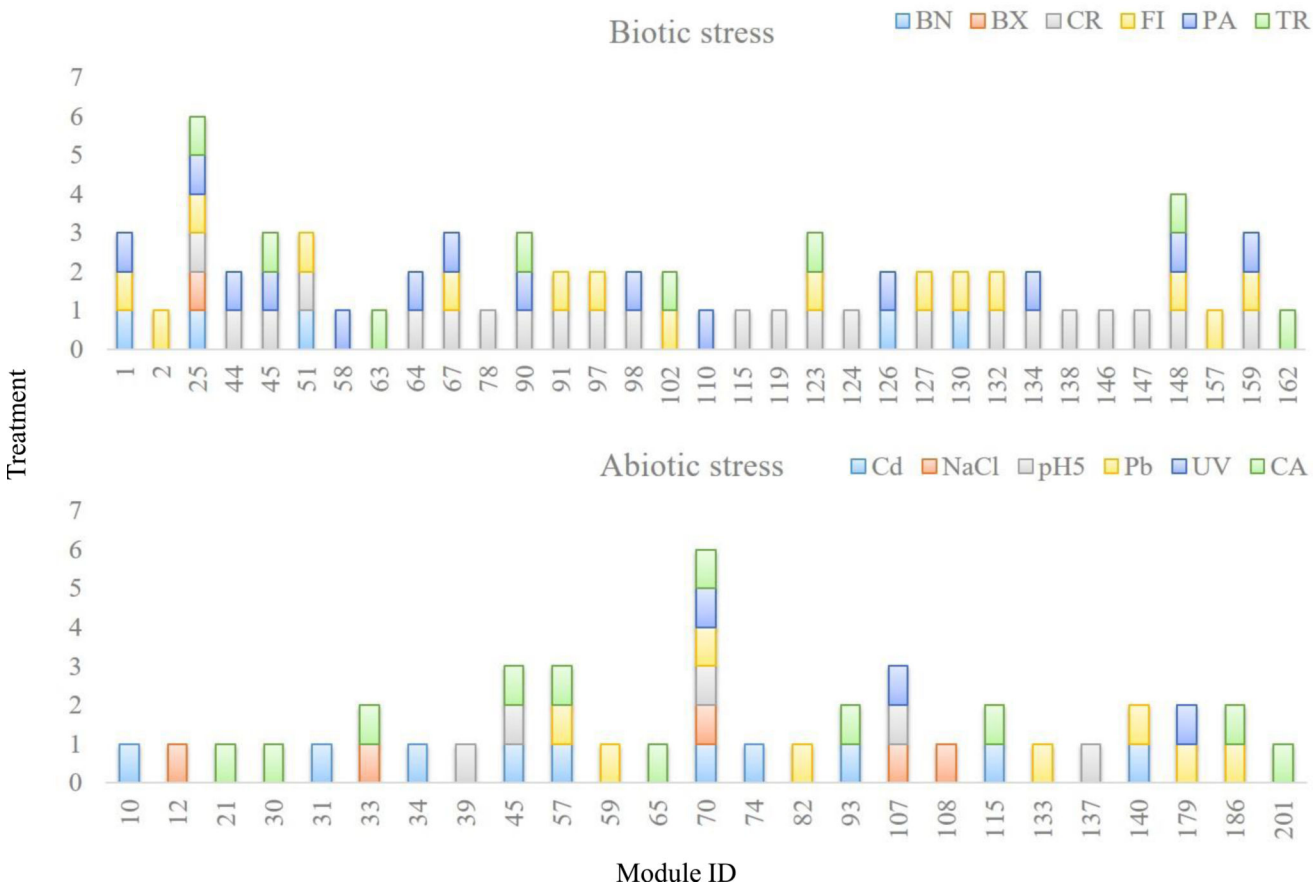

Figure 4. Co-expression networks under environmental perturbations

Significantly co-expressed gene networks (numbered on x-axis) shared among the biotic (N = 6 treatments) and abiotic treatments (N = 6 treatments). Abbreviations for environmental perturbations are as in Figure 1. Colours and cluster numbers in the bar plots are randomly generated, hence identical colours and numbers in the two plots do not indicate the same network. Numbers on the x-axis refer to the module number listed in Supplementary Table S7.