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Conserved transcriptional responses to cyanobacterial stressors are mediated by alternate regulation of paralogous genes in *Daphnia*

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Abstract

Despite a significant increase in genomic data, our knowledge of gene functions and their transcriptional responses to environmental stimuli remains limited. Here, we use the model keystone species *Daphnia pulex* to study environmental responses of genes in the context of their gene family history to better understand the relationship between genome structure and gene function in response to environmental stimuli. *Daphnia* were exposed to five different treatments, each consisting of a diet supplemented with one of five cyanobacterial species, and a control treatment consisting of a diet of only green algae. Differential gene expression profiles of *Daphnia* exposed to each of these five cyanobacterial species showed that genes with known functions are more likely to be shared by different expression profiles, whereas genes specific to the lineage of *Daphnia* are more likely to be unique to a given expression profile. Furthermore, while only a small number of nonlineage-specific genes were conserved across treatment type, there was a high degree of overlap in expression profiles at the functional level. The conservation of functional responses across the different cyanobacterial treatments can be attributed to the treatment-specific expression of different paralogous genes within the same gene family. Comparison with available gene expression data in the literature suggests differences in nutritional composition in diets with cyanobacterial species compared to diets of green algae as a primary driver for cyanobacterial effects on *Daphnia*. We conclude that conserved functional responses in *Daphnia* across different cyanobacterial treatments are mediated through alternate regulation of paralogous gene families.

Keywords: crustaceans, gene structure and function, molecular evolution, transcriptomics

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Introduction

Over the last decade, an increasing number of sequenced genomes, expression profiles and data sets have been published. Yet, our knowledge about these genomes, and in particular, the specific function of their genes remains limited. In general, at least a third of all genes in any genome, even well-studied organisms such as *Escherichia coli*, lack functional annotation (Adams 2013; Anton *et al.* 2014). In addition, an increasing number of new genomes being sequenced contain high numbers of lineage-specific genes lacking homology with known genes (Colbourne *et al.* 2011; Anton *et al.*

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2014). Furthermore, while we know the biochemical function or annotation of some genes, we know little about their environmental or ecological responses and their contribution to phenotypic variation and higher population level effects (Anton *et al.* 2014). Genes with known annotations are often identified in the expression profiles generated from distinct environmental conditions. For example, Swindell *et al.* (2007) studied the transcriptional responses of heat-shock proteins and transcription factors in *Arabidopsis thaliana* to ten different biotic and abiotic stressors. They concluded that heat-shock proteins interact with multiple stress response pathways and therefore to understand the function of these genes their responses need to be studied under a wide variety of environmental conditions. Girardot *et al.* (2004) observed transcriptional responses in *Drosophila melanogaster* specific to each of three different stressors as well as transcriptional responses shared by all three stressors. The latter is often referred to as a general stress response. Yet, we know very little about how and why some genes respond to many environmental conditions while the transcription of others is condition specific. This lack of knowledge hampers our understanding of the organism's biology and their interactions with the environment and suggests that we should study transcriptional responses to environmental conditions in species with tractable and well-characterized ecologies (Colbourne *et al.* 2011). Focussing on species in an appropriate ecological context is crucial as many genes with unknown functions are primarily responsive to ecological conditions commonly experienced by these species. Laboratory model species are often less suitable for these types of studies as these model species are often difficult to study in their natural environment (Pěna-Castukki & Hughes 2007; Colbourne *et al.* 2011).

The freshwater keystone species *Daphnia pulex* is particularly suitable for studying gene responses under environmental conditions due to its well-known ecology (Miner *et al.* 2012) and unique genomic structure (Colbourne *et al.* 2011). As a result, this freshwater crustacean is now emerging as a true model organism in ecological and environmental genomics (Ebert 2011). Unlike most other known genomic models, *Daphnia* has a large number of duplicated genes due to the expansion of many gene families within the genome (Colbourne *et al.* 2011; Simon *et al.* 2011). Gene duplicates are of particular interest as they are highly susceptible to functional diversification (Maere *et al.* 2005). In addition, more than a third of the *Daphnia* genome is specific to its lineage (Colbourne *et al.* 2011). Expression studies highlighted that these lineage-specific genes are more responsive to ecological conditions and gene duplicates demonstrate divergent expression patterns under divergent ecological conditions (Colbourne *et al.* 2011). This interaction between the genome and the environment makes *Daphnia* particularly suitable to study such interactions and infer functional responses from gene expression patterns.

In this study, we characterized transcriptional responses of *Daphnia pulex* challenged with five species of cyanobacteria. Cyanobacteria or blue-green algae are unicellular organisms that can have a significant impact on freshwater environments and some are known to adversely affect many organisms, from zooplankton to fish, livestock and humans (Codd 1995; Duy *et al.* 2000). In particular, for zooplankton species, the cause of these adverse effects remains unclear. Frequently reported deleterious effects on *Daphnia* are reduced reproduction, decreased growth and lower survival rate (Lampert 1987; Kirk & Gilbert 1992; Panosso & Lürling 2010). Others have reported lower feeding rates of *Daphnia* when fed with diets containing cyanobacteria (Chow-Fraser & Sprules (1986), Gilbert & Durand (1990)). These effects have been in part attributed to toxins they produce, yet other factors such as low food quality for zooplankton and feeding impairment cannot be excluded (Rohrlack *et al.* 1999; Von Elert *et al.* 2003; Schwarzenberger *et al.* 2010).

Studies by Wilson *et al.* (2006), Tillmanns *et al.* (2008) and Asselman *et al.* (2014) do not support the long-standing hypothesis of cyanobacterial toxins as a primary driver for cyanobacterial toxicity. Instead these studies suggest that nutritional deficiency may be the primary driver of adverse effects. Cyanobacteria are known to vary in their fatty acid composition across species (Caudales & Wells 1992; Řezanka *et al.* 2003). In addition, studies on the effects of cyanobacteria at the gene level are limited and primarily focused on studying the effects of secondary metabolites produced by cyanobacteria. For example, Schwarzenberger *et al.* (2010) observed significant upregulation of specific digestive proteases at the RNA level in *Daphnia* when exposed to cyanobacterial protease inhibitors. They observed that different digestive proteases responded differently to different cyanobacterial protease inhibitors. Asselman *et al.* (2012) observed differential regulation of the ribosomal proteins, the oxidative phosphorylation pathway and the trypsin gene family when *Daphnia* were exposed to *Microcystis aeruginosa*. Little is known about the effects of other cyanobacterial species on *Daphnia* at the gene level and to what extent these effects are similar across different cyanobacterial species.

This study aimed to improve our understanding of how genes respond to environmental stimuli and how they vary in these responses, as well as to provide a deeper understanding of the mechanisms of cyanobacterial

toxicity, an emerging threat in freshwater ecosystems (Paerl & Huisman 2008). Therefore, this study addressed two major hypotheses by exposing *Daphnia* to five species of cyanobacteria resulting in five expression profiles. As described above, studies such as Swindell *et al.* (2007) and Girardot *et al.* (2004) have highlighted the responsiveness of genes with known functional annotations to many different environments. In contrast, Colbourne *et al.* (2011) have demonstrated that lineage-specific genes are responding to specific ecological conditions. Based on these observations, we put forward as our first hypothesis that general stress responses, that is responses shared by all five expression profiles of *Daphnia* exposed to five different cyanobacteria, are enriched for functionally annotated genes while condition-specific response are enriched for lineage-specific genes.

Second, Colbourne *et al.* (2011) highlighted the evolutionary diversification within paralogous gene families within *Daphnia pulex* in response to different environments. In addition, Schwarzenberger *et al.* (2010) observed differential regulation of different digestive proteases in *Daphnia* in response to different cyanobacterial protease inhibitors. Therefore, we put forward the hypothesis that stress responses to different cyanobacterial species can be mediated through different genes belonging to the same paralogous gene families.

Our results showed that *Daphnia* genes regulated by all cyanobacterial treatments were enriched for genes with a known gene function, which supports the first hypothesis. Furthermore, while only a small number of homologous genes were conserved across treatment type, there was a high degree of overlap in expression profiles at the functional level. The conservation of functional responses across the different cyanobacterial stressors can be attributed to the alternate regulation of different paralogous genes within the same gene family, which supports the second hypothesis. Comparison with available gene expression data in the literature suggests differences in nutritional composition compared to green algae as a primary driver for cyanobacterial effects on *Daphnia*.

Material and methods

Exposure experiments

All exposures were conducted inside a climate-controlled room at a constant temperature (20 ± 1 °C) and photoperiod (16:8 light–dark). Juvenile individuals were obtained from isoclonal brood cultures synchronized in terms of age and reproduction and cultured in 1.5 l no N, no P COMBO medium (Shaw *et al.* 2007). These isoclonal cultures were obtained from the Shaw laboratory and sampled from a pond in Oregon, USA (Paland *et al.* 2005). The obtained juveniles were grown under identical conditions as the control exposure. On the fourth day, these animals were randomly assigned to a control or a cyanobacteria treatment. Each treatment consisted of ten replicate beakers. Each borosilicate beaker contained 30 juvenile *Daphnia* (4 days old) in 1.5 l no N, no P COMBO medium (Shaw *et al.* 2007). To ensure sufficient RNA for all hybridizations, a control treatment was set up for each cyanobacterial treatment resulting in a total of 100 beakers (10 replicates * 5 cyanobacteria * 2 treatments, i.e. control and cyanobacteria treatment). The entire experiment was conducted twice. The full experimental design is illustrated in Fig. S9 (Supporting information).

Animals were fed daily with a mixture of *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* in a 3:1 cell number ratio at a rate of 3 mg dry weight/l in control conditions. In cyanobacterial treatments, this diet was modified by the addition of 50% of a respective cyanobacterial species, on dry weight basis. The medium was renewed every 2 days and reproduction and survival monitored. When the animals reproduced, neonates were counted and removed from the beaker. After 10 days, RNA was extracted from adult-exposed animals for gene expression analysis. Although 10 replicates per treatment were available, only four were selected for gene expression analysis. The six additional replicates served as back-up replicates in case of loss of sample during the experiment or shipment to the array facility, failed RNA extractions, failed RNA amplifications, failed hybridizations or even insufficient amount RNA from a single replicate. For each treatment, each of the ten replicates was assigned a random number and a random number generator picked out four replicates per treatment for gene expression analysis for each replicated experiment.

Culturing of cyanobacteria and green algae

All cyanobacterial strains used in this study originated from certified culture institutions (Table S1, Supporting information) and were cultured in modified referenced culture medium to allow optimal growth (Allen 1968; Kotai 1972) (Tables S8-S10, Supporting information). All strains were cultured under standardized conditions in a clean environment. Cultures were incubated in 6-l volumetric flasks at 20 ± 1 °C under constant light conditions with gentle aeration and allowed to grow until mid-late log phase. Afterwards, cultures were concentrated by centrifugation and cleaned by resuspension and centrifugation using reconstituted water (modified COMBO medium) three times before use. Density of the cultures was determined with a counting chamber.

Dry weight was determined by drying 2 ml of algal suspension in a preweighed aluminium cup at 40 °C for at least 24 h.

Gene expression analysis

Gene expression patterns were analysed using the 12-plex two-colour oligonucleotide *D. pulex* microarray developed by Roche NimbleGen and Colbourne *et al.* (2011) (GPL11278). Each individual subarray contains 137 000 probes covering the full transcriptome of *Daphnia pulex*. Sample preparation followed methods as described in Asselman *et al.* (2012) and Lopez & Colbourne (2011). RNA was extracted using the RNeasy kit and Qiashredder (Qiagen, Venlo, the Netherlands) following manufacturer's protocol. DNA contamination was removed by a DNAse treatment (Qiagen). One microgram of RNA was subsequently amplified with the MessageAmp II aRNA Amplification kit (Ambion, Applied Biosystems, Carlsbad, CA, USA) following manufacturer's protocol. Next, 10 μg of cRNA was used to synthesize cDNA with the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) following clean up (alkaline hydrolysis and Qiaquick columns; Qiagen). Labelling, dual-colour hybridization, washing and scanning were carried out according to manufacturer's protocol (dual-colour DNA labelling kit, Hybridization Kit, Hybridization Wash Buffers, NIMBLESCAN 2.6 Software; Roche Nimblegen, Madison, WI, USA). Four competitive hybridizations (control samples vs. cyanobacterium exposed samples) were conducted with dye swaps for each cyanobacteria treatment. These hybridizations included four replicates per treatment from each of the repeated experiments, resulting in a total of eight replicates per treatment (four from repetition 1 and four from repetition 2). Data images were analysed with the statistical software package R (Ihaka & Gentleman 1996) and Bioconductor (Gentleman *et al.* 2004). We used an in-house pipeline based on the LIMMA (Smyth 2004) package with additions and modifications according to Colbourne *et al.* (2011). First, we determined the correlation between replicates within each experiment as well as the correlation between replicates across the replicated experiments. In particular, the correlation coefficient between biological replicates across and within experiments for each treatment was determined to evaluate the quality of the data. Within the same replicated experiments, biological replicates were correlated with any other replicate of the same treatment. Only replicates with a correlation coefficient of at least 0.9 with all other replicates were retained for further analysis, other replicates were excluded from the analysis. We then compared the correlation coefficients between replicated experiments. Biological replicates were correlated with all other replicates of the same treatment in pairwise manner. Only replicates with a correlation coefficient of at least 0.85 with all other replicates were retained for further analysis, and other replicates were excluded from the analysis. Second, all signal distributions from the remaining replicates were then quantile normalized across chips, samples, and replicates. Third, differential expression of a gene was determined based on the median M-value of probes that represent the gene in question using the function *lmFit* to fit a linear model to the data. The linear model consisted of six coefficients, one for each cyanobacterial species and an additional coefficient to account for the variation between the two replicated experiments. Significant differential expression for each cyanobacterial species separately was determined using the function *contrasts.fit* followed by applying empirical Bayes statistics to the resulting fit using EBayes. The M-value for a gene was defined as the $\log_2$ ratio of the expression in the exposed animals and the expression of the animals in the control treatment. Finally, the Benjamini–Hochberg method (Benjamini & Hochberg 1995) was implemented to determine significance of expression and adjust for multiple testing. Raw data are deposited in GEO under GEO62763.

Analysis of gene lists

Gene lists were analysed in R, where the gene lists were combined with annotation information on each gene available through wfleabase.org (Colbourne *et al.* 2005). For each expression profile, significant genes with their full annotation information were extracted for further analysis based on Asselman *et al.* (2012). In addition, we used the supplementary information from Colbourne *et al.* (2005), available at http://wfleabase.org/release1/current_release/supplement/SOM_IV.4/Tandy/, to identify tandem duplicated genes. Venn diagrams were then constructed with the R package venn diagram to determine the number of shared genes, shared annotation definitions based on KOG (eukaryotic orthology groups) classifications and GO terms within the list of genes that were significantly regulated in at least one expression profile, that is one cyanobacterial exposure. Fisher's exact test was used to determine significant differences between proportions of different groups (Fisher 1922). Conserved and divergent expression was determined by bootstrapping all significant gene lists containing only genes with functional annotations and randomly assigning genes to each annotation definitions. For each bootstrap, the standard deviation in differential gene expression across all genes in the functional annotation across all transcriptomic profiles was calculated (e.g. standard deviation of a functional

annotation with 10 significantly expressed genes in treatment 1 and 15 significantly expressed genes in treatment 2 was then calculated by first bootstrapping 10 genes out of the gene list for treatment 1, 15 genes for the gene list of treatment 2 and then calculating the standard deviation). This was repeated 1000 times. Afterwards, the actual standard deviation of the annotation definition was compared with the results of the bootstrap analysis. If the standard deviation was smaller than the 95% interval of the bootstrap data, expression was determined conserved. If the standard deviation was larger than the 95% interval of the bootstrap data, expression was defined as divergent.

Results and discussion

Functional characterization of transcriptomic responses

We generated transcriptomic profiles of *Daphnia* after exposure for 10 days to five different diets each supplemented with a single cyanobacterial species. Five cyanobacterial species were selected to represent five major taxa of toxic cyanobacteria (van Apeldoorn *et al.* 2007), some of which are known to produce different toxins (Table S1, Supporting information). To increase statistical power and reproducibility of the data, experiments consisted of four biological replicates per treatment and were executed twice, resulting in eight biological replicates per treatment and allowing a full validation of the results by comparing the first and second independent replication. In all five transcriptomic profiles, significant responses compared to control conditions were observed at the gene level (Fig. 1) while observing a decrease in total reproduction at the life history level (Fig. S1, Supporting information). We observed both species-specific responses, that is genes only responding to a single cyanobacterial species, as well as general stress responses (Figs 1 and S2, Supporting information). Furthermore, the majority of the genes in all categories had a positive M-value, that is, expression was significantly higher in the treatment than in control conditions (Fig. S3, Supporting information). We also observed large differences between the number of differentially expressed genes across the different treatments. Exposure to *Anabaena*, *Cylindrospermopsis* or *Nodularia* resulted in about five to ten times more differentially expressed genes compared with the number of differentially expressed genes in response to *Aphanizomenon* or *Oscillatoria*. This difference does not correlate with our observations of *Daphnia* reproduction as the largest decline in reproduction was observed in response to *Nodularia* and *Oscillatoria*. At present, it remains unclear why some cyanobacteria affect more genes in *Daphnia* than others.

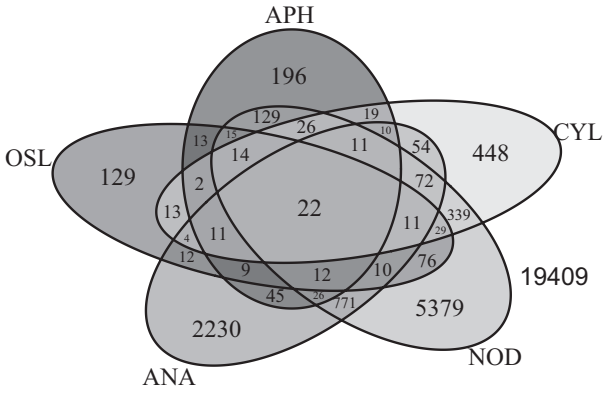

Fig. 1 Venn diagram of genes significantly regulated (*q*-value < 0.05) for each transcriptomic profile. Intersections denote the number of shared significant genes between two or more profiles. 19409 genes were not significantly regulated in any of the transcriptomic profiles. (*Aphanizomenon*: APH, *Anabaena*: ANA, *Cylindrospermopsis*: CYL, *Nodularia*: NOD, *Oscillatoria*: OSL). In total, for each cyanobacteria 29545 was tested for differential expression.

In support of the first hypothesis, all genes responding to all cyanobacterial species have homologues with known functional annotations in other species while not a single lineage-specific gene was significantly regulated by all cyanobacterial exposures. In particular, the 22 genes demonstrating a general cyanobacterial stress response had diverse annotations ranging from cytochrome P450 to trypsin and neurexin (Table S2, Supporting information). Out of the 22 genes, 12 were shared with the transcriptomic profile in response to *Microcystis aeruginosa*, another common cyanobacterial species (Asselman *et al.* 2012, Table S2, Supporting information).

Overall, differentially expressed lineage-specific genes (i.e. with no homology and no functional annotation) were more likely to be unique to a single expression profile than to be shared by all profiles (P-value < 0.05, Table S3, Fig. S4, Supporting information). In contrast, differentially expressed genes with homology were more likely to be shared by all profiles than to be unique to a single expression profile (P-value < 0.05, Table S3, Fig. S5, Supporting information). Furthermore, for all cyanobacterial exposures excluding *Aphanizomenon* (P-value > 0.05, Table S3, Supporting information), differentially expressed lineage-specific genes were more likely to be unique to a single expression profile than to be shared with at least one other expression profile (P-value < 0.05, Table S3, Supporting information). Taken together, these results suggest that lineage-specific genes are primarily involved in responding to specific environmental stressors, whereas genes with homology are more likely to be involved in a conserved

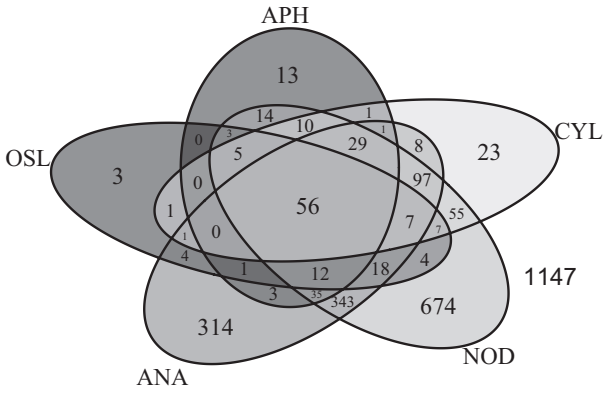

Fig. 2 Number of shared annotation definitions corresponding to the significant genes (q-value < 0.05) for each transcriptomic profile. Intersections denote the number of shared annotation definition between two or more transcriptomic profiles. 1147 annotation definitions that correspond to genes in the genome, did not correspond to any of the significant genes in any profile (*Aphanizomenon*: APH, *Anabaena*: ANA, *Cylindrospermopsis*: CYL, *Nodularia*: NOD, *Oscillatoria*: OSL). A total of 2892 functional annotations were tested.

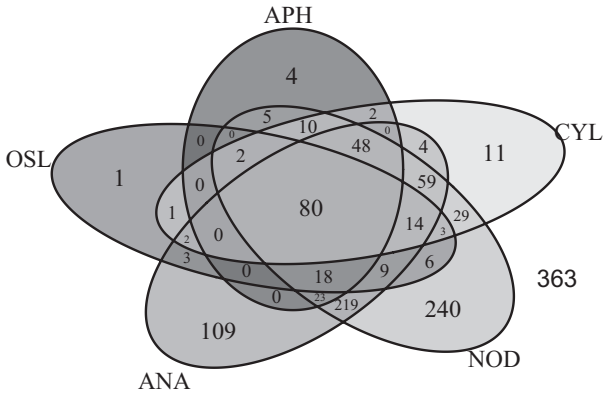

Fig. 3 Number of shared Gene Ontology (GO) terms corresponding to the significant genes (q-value < 0.05) for each transcriptomic profile. Intersections denote the number of shared GO terms between two or more transcriptomic profiles. 363 GO terms that correspond to genes within the genome, did not correspond to any of the significant genes in any profile. (*Aphanizomenon*: APH, *Anabaena*: ANA, *Cylindrospermopsis*: CYL, *Nodularia*: NOD, *Oscillatoria*: OSL). A total of 1185 GO terms were tested.

response, comparable across a set of environmental stressors. These results also highlight one reason that lineage-specific genes may be difficult to annotate as these genes most likely respond to a limited and specific set of environmental stressors for a particular organism. In addition, it also suggests that lineage-specific genes are less evolutionary conserved because they respond to particular set of specific environments while those genes that do have functional annotations are often more evolutionary conserved because their responses are much broader.

Despite the small number of shared genes among treatments (Fig. 1), a high number of functional annotations, based on KOG classifications, were shared among the different transcriptomic profiles (Figs 2 and 3). Analysis of the number of functional annotations and gene ontology (GO) terms in the significant gene lists revealed 56 functional annotations and 80 GO terms shared among all significantly expressed genes across the five expression profiles (Figs 2 and 3). The shared functional annotations comprised a variety of functions, such as chitinases, collagen, cytochrome P450 families and glutathione-S-transferase and these functional annotations corresponded well with the shared GO terms, for example trypsin activity, chitin metabolism, glutathione transferase activity (Tables S4 and S5, Supporting information). The number of differentially expressed genes in these 56 functional annotations differed across the different treatments (Fig. S6, Supporting information). Enrichment analysis for each of these functional annotations individually did not show significant enrichments within each of the treatments. However, for all treatments, the proportion of differentially expressed genes within all 56 functional annotations was significantly higher than expected based on a Fisher's exact test ($p_{anabaena}$ = 1.7 e-8, $p_{aphanizomenon}$ < 2.2 e-16, $p_{cylindrospermopsis}$ = 1.6 e-16, $p_{nodularia}$ = 7.1 e-8, $p_{oscillatoria}$ < 2.2 e-16). In addition, expression profiles for *Anabaena* and *Nodularia* also contained a large number of annotation definitions specific to these profiles, which is not surprising as these annotation definitions comprise a large number of genes, that is 2230 and 5379, respectively (Figs 1–2).

Evolutionary diversification of duplicated genes

The discrepancy between the observations of overlapping transcriptional response across treatments at the gene level and at the functional level of shared functional annotations and GO terms (i.e. 22 shared genes vs. 56 and 80 shared functional annotations and GO terms) could in part be attributed to the high number of duplicated genes within the *Daphnia* genome [3]. Genomes often have multiple paralogs with the same functional annotation. In particular in genomes with an increased gene copy number, the paralogous genes may be assigned to the same functional annotation and GO term due to their close sequence similarity. Here, the 56 shared functional annotations cover between 171 and 908 differentially expressed genes in any particular transcriptional profile (Fig. S6, Supporting information), and we observed that 83.6–99.6% of these genes within the 56 shared functional annotations were duplicated genes, whereas only 0–47.9% of the genes within

functional annotations unique for each expression profile were duplicated genes (Tables 1 and S6, Supporting information). Fisher's exact test confirmed that shared functional annotations were enriched for differentially expressed paralogous genes (Tables 1 and S6, Fig. S7, Supporting information). In contrast, functional annotations unique to just one cyanobacterial exposure were enriched for differentially expressed nonduplicated genes (Tables 1 and S6, Fig. S7, Supporting information). This pattern was observed for all cyanobacterial exposures excluding *Oscillatoria* for which *P*-values were not significant (Table S6, Supporting information). These results suggest that different cyanobacterial species trigger the same mechanisms or functional annotations in *Daphnia,* but the transcriptional responses are mediated through different paralogs, most likely gene duplicates. We also tested whether this pattern could be observed for lineage-specific genes but found no significant differences between the proportion of paralogous lineage-specific genes in shared and unique expression profiles (Table S6, Supporting information).

Gene duplication may occur through many different mechanisms; two examples are tandem duplication or whole genome duplication (Maere *et al.* 2005). In plants, it has been observed that genes generated through different duplication mechanisms may vary significantly in their gene function and functional responses (Maere *et al.* 2005). In particular, Hanada *et al.* (2008) observed that genes generated through tandem duplication are involved in biotic stress response and responses to environmental stimuli in *Arabidopsis thaliana*. We therefore tested whether condition-specific responses were enriched for differentially expressed tandem duplicated genes while shared responses would be enriched for differentially expressed nontandem duplicates. However, no difference was observed between duplicates and tandem duplicates for any of the cyanobacterial exposures (Table S5, Fig. S8, Supporting information).

To better understand how these duplicated genes are responding to the different cyanobacterial species, we investigated the expression patterns within each specific functional annotation. We specifically tested if two expression patterns existed. The first, the conserved expression pattern, describes expression of the genes within a specific functional annotation group, which vary less than expected by chance between the genes within that functional annotation group and between exposures. The second, the divergent expression pattern describes the pattern in which the expression of genes within a functional annotation group varies more than expected by chance between the genes of that group and between the exposures. Here, we observed significantly conserved expression across all transcriptomic profiles for seven annotation definitions (i.e. alpha-amylase, lipid exporter ABCA1, neurexin IV, predicted transporter, triglyceride lipase-cholesterol esterase, von Willebrand factor and zinc carboxypeptidase). For four annotation definitions: acyl-CoA synthetase, fasciclin and related adhesion glycoproteins, serine proteinase inhibitor family, and type I phosphodiesterase-nucleotide pyrophosphatase, gene expression was divergent. The conserved expression of these eight annotation definitions indicates a strong conservation of these functions across the different cyanobacterial exposures and tight regulation of this transcriptional response. Indeed, even though the differentially expressed genes within this functional annotation differ between the cyanobacterial exposures, they are regulated in the same manner. Figure 4 (left panel) clearly highlights these expression patterns for the alpha-amylase functional annotation. Five genes were excluded, as they were not significantly expressed in any treatment. The remaining ten genes can be divided into two paralogous clusters and one single copy gene. In contrast, Fig. 4 (right panel) highlights the divergent expression pattern for the functional annotation serine proteinase inhibitor. This functional annotation contains five single copy genes and 25 duplicated genes divided across seven paralogous gene clusters. Fifteen genes were excluded, as they were not significantly expressed in any treatment. Figure 4 (right panel) highlights the divergent regulation of genes across treatments within a single functional annotation. In particular, the variation across genes across treatment is much smaller within the alpha-amylase functional annotation compared to variation within the serine protease inhibitor functional annotation (Fig. 4, denoted by black arrows). When taking into account the genetic phylogeny of the genes within these

Table 1 Proportions of duplicated genes within the different groups of all significant genes (q-value < 0.05) with an annotation definition for each treatment. (*Aphanizomenon*: APH, *Anabaena*: ANA, *Cylindrospermopsis*: CYL, *Nodularia*: NOD, *Oscillatoria*: OSL), Figure S3 (Supporting information)

	APH	OSL	ANA	NOD	CYL
% of Duplicated genes within all annotation definitions	65.9	67.7	72.1	81.0	70.2
% of Duplicated genes within 56 shared annotation definitions	88.4	83.6	97.7	99.6	93.7
% of Duplicated genes within unique annotation definitions	22.2	0	26.1	47.9	16.0

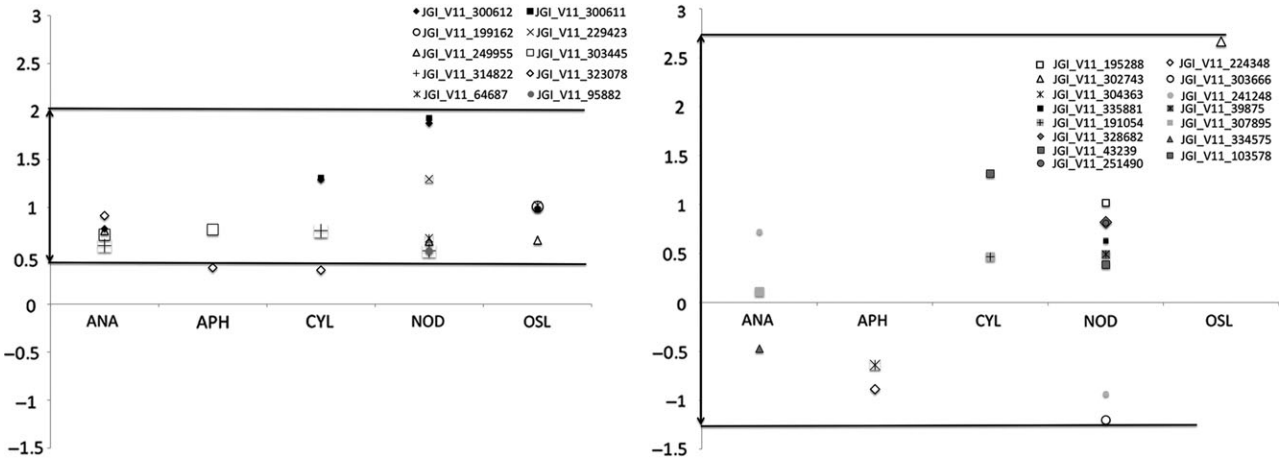

Fig. 4 Expression patterns of genes within the alpha-amylase functional annotation (left panel) and serine proteinase inhibitor functional annotation (right panel) that are significantly regulated in at least one transcriptomic profile. (*Aphanizomenon*: APH, *Anabaena*: ANA, *Cylindrospermopsis*: CYL, *Nodularia*: NOD, *Oscillatoria*: OSL). Arrows denote the spread of M-values within the functional annotations across genes and treatments.

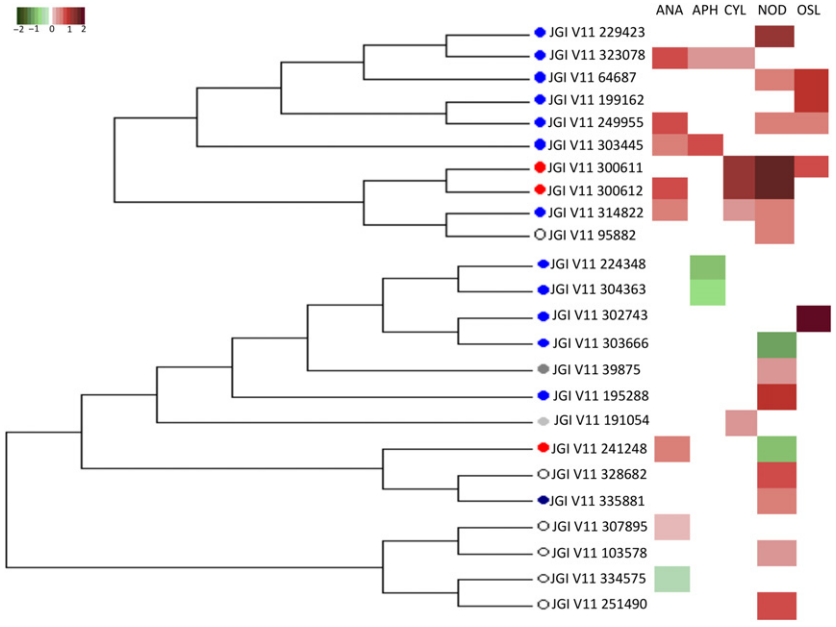

Fig. 5 Heatmap of the expression values of ten genes within the alpha-amylase functional annotation significantly regulated in at least one transcriptomic profile (top panel) and for the fourteen genes within the serine protease inhibitor functional annotation significantly regulated in at least one transcriptomic profile (bottom panel). Phylogenetic tree is based on the protein sequences of these genes. Circles denote genes belonging to the same paralogous gene cluster, black unfilled circles denote single copy genes. The M-value of expression is represented using a colour scale bar, that is the darker the red the higher the M-value and the darker the green the lower the M-value. The gene with id JGI_V11_43239 was excluded from the analysis as some pairwise distances could not be calculated.

two functional annotations (Fig. 5), we observed that genes showing similar expression patterns (Fig. 4, left panel) are not necessarily closely related in terms of their phylogeny (Fig. 5, top panel). In addition, genes showing different expression patterns (Fig. 4, right panel) are not necessarily distantly related in terms of their phylogeny (Fig. 5, bottom panel).

The divergent expression of four functional annotations including acyl-CoA synthetase indicates that although all cyanobacterial species affect these functions, the effects are divergent for the different cyanobacterial species. In particular, the paralogous genes within these functional annotations are individually regulated in a different manner. Differences in the expression of acyl-coA synthetases, the rate-limiting enzyme in the fatty acid synthesis (Zinke *et al*. 2002), suggest a differential impact on fatty acid metabolism caused by various cyanobacterial stressors, potentially mediated through the differences in total fatty acid content or different fatty acid composition of the cyanobacteria. Significant differences in fatty acid composition and total fatty acid content between different genera of cyanobacteria have been document by Řezanka *et al*. (2003) and by Caudales & Wells (1992). The majority of

shared annotation definitions lacked clear conserved or divergent expression patterns suggesting a complex pattern of conserved mechanistic targets under cyanobacterial stress response and species-specific effects on these targets.

Functional multistress responses and specific cyanobacterial stress responses

We observed 56 functional annotations responding significantly to all cyanobacterial exposures yet the question remains whether these functional annotations demonstrate a conserved general stress response or rather a conserved cyanobacterial stress response. We therefore compared these functional annotations with the functional annotations reported to be significantly regulated in other data sets in response to various stressors (cadmium, salinity, altered phosphorous content in green algae and one other cyanobacterial species *Microcystis aeruginosa*,) (Jeyasingh *et al.* 2011; Asselman *et al.* 2012; Latta *et al.* 2012; De Coninck *et al.* 2014). If the functional annotations are specific for cyanobacterial stress response, we expect the majority of the annotations to be also significantly regulated in the data set of *Microcystis* exposure but to be absent in the other three data sets. If functional annotations are demonstrating a general stress response, we expect to find the majority of the functional annotations significantly regulated in all data sets.

Fifty-five of the fifty-six functional annotations shared by all transcriptomic profiles of cyanobacterial stress in the present study were also identified in the transcriptomic profile of *Microcystis aeruginosa* stress (Asselman *et al.* 2012). In contrast, only a limited number of these functional annotations were identified in transcriptomic profiles under cadmium (nine functional annotations) and salinity stress (14 functional annotations) (Table S3, Supporting information) (Latta *et al.* 2012; De Coninck *et al.* 2014). Only five functional annotations were shared between the transcriptomic profiles in response to cyanobacteria, salinity and cadmium (Table S3, Supporting information). This observation suggests that 55 of the 56 functional annotations common to all transcriptomic profiles in response to cyanobacterial stress are specific to cyanobacterial exposure, regardless of the cyanobacterial species, rather than an overall general stress response. Furthermore, although the five cyanobacteria tested here are filamentous species, *Microcystis* is a coccal species suggesting that the response of the 55 functional annotations in *Daphnia* is conserved across cyanobacterial species regardless of the latter's morphologies. The few functional annotations shared with salinity and cadmium profiles primarily pertain to functions that have been referred to in other studies as related to general stress response: chitinases, trypsins and von Willebrand factor (Poynton *et al.* 2007; Heckmann *et al.* 2008). However, we also identified 48 functional annotations common in response to diets grown on medium with an altered phosphorous content and diets containing one of six cyanobacterial stressors. Jeyasingh *et al.* (2011) pointed out that the functional responses in *Daphnia* to altered phosphorous in the diet are not necessarily directly involved in phosphorous metabolism, but potentially in various other physiological processes. Jeyasingh *et al.* (2011) fed *Daphnia* with two diets of green algae, which are generally considered as a standard food source in *Daphnia* experiments. The first diet consisted of green algae that were grown in standard medium and the second diet consisted of green algae grown in the same medium, containing only a tenth of the phosphorous concentration of the standard medium. They concluded that the differences in gene expression in *Daphnia* in response to these two diets most likely reflected a difference in biochemical (and thus nutritional) composition of the two diets that resulted from the physiological response of the green algae to low vs. high phosphorus in the medium. These observations in combination with our results suggest that cyanobacteria have a shared nutritional feature across species to which *Daphnia* responds via genes within these 48 shared functional annotations. Furthermore, this shared nutritional feature elicits a response similar to the differential response of *Daphnia* when exposed to diets of green algae with different nutritional properties due to differential phosphorous concentration in the growth medium of these algae. Previous studies have put forward the hypothesis of nutritional deficiency in diets supplemented with cyanobacteria to explain observed adverse effects during cyanobacterial exposure (Von Elert *et al.* 2003; Brett *et al.* 2006; Asselman *et al.* 2014). Fatty acids and sterols have been indicated as the primary nutrients lacking in cyanobacteria compared to other green algae (Von Elert *et al.* 2003; Tillmanns *et al.* 2008). Pathway analysis of the data resulted in significant enrichment of pathways related to different nutritional features, but the specific results differed among the different cyanobacterial exposures (Table S7, Supporting information).

Based on these results, we suggest that a general difference in nutritional properties across all cyanobacterial species compared to control food invokes environmental responses of particular set of functional annotations related to nutrition. However, given the morphological and genetic differences between the cyanobacterial species, the exact nutritional composition may well be species specific and as a result trigger different genes with an identical functional annotation.

Concluding statements

Gene expression profiles describing the environmental response of *Daphnia pulex* to different cyanobacterial species have answered two major research questions. First, our results supported the hypothesis that conserved stress responses are enriched for genes with known homology, whereas treatment-specific responses are enriched for lineage-specific genes. Second, we observed an environmental diversification of the functional annotations through paralogous genes involved in cyanobacterial stress response. Indeed, a high proportion of functional annotations was shared by all cyanobacterial stress expression profiles, and these functional annotations contained a significant proportion of duplicated genes which supports our second hypothesis. All cyanobacterial species affect the same functional annotations, but the specific environmental responses are mediated through paralogous genes that demonstrate a cyanobacteria-specific response.

Furthermore, a comparison of these functional annotations with functional annotations in other data sets has improved our knowledge on the mechanisms of cyanobacterial stress response. Few functional annotations were shared with expression data describing cadmium and salinity responses while many were shared with expression data describing responses to green algae with different nutritional properties as these green algae were cultured in media with a different phosphorous content. These results implicate differences in nutritional properties in cyanobacteria compared to control food as a general *Daphnia* response mechanism common to all cyanobacterial stressors. However, species-specific differences in cyanobacterial nutritional quantity or quality will lead to environmental responses mediated through different paralogous genes. Finally, our results highlight the need to study environmental responses of genes with regard to their gene family history to better understand their environmental response.

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J.A. and K.D.S. designed the study. J.A., D.D.C. and J.L. performed the experiments. J.A. analysed the results with comments and suggestions from K.D.S., M.P., J.S. and D.D.C. J.A. has written the manuscript with comments and suggestions from K.D.S., M.P., J.S., D.D.C. and C.J.

Data accessibility

All microarray data are deposited in GEO under GEO62763, and the R script used for data analysis is available as Supplemental information.

Supporting information

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

Fig. S1 Reproduction in exposure treatments (*Cylindrospermopsis*: CYL, *Nodularia*: NOD, *Anabaena*: ANA, *Oscillatoria*: OSL, *Aphanizomenon*: APH) relative to corresponding control treatments. Error bars represent standard deviation per replicated experiment.

Fig. S2 Significant genes (q-value < 0.05) shared among all transcriptomic profiles.

Fig. S3 Venn diagram of genes significantly upregulated (q-value < 0.05 and M-value > 0) for each transcriptomic profile.

Fig. S4 Venn diagram of genes significantly regulated (q-value < 0.05) with no functional annotation for each transcriptomic profile.

Fig. S5 Venn diagram of genes significantly regulated (q-value < 0.05) with functional annotation for each transcriptomic profile.

Fig. S6 Number of significant genes (q-value < 0.05) for each transcriptomic profile.

Fig. S7 Venn diagrams for significantly regulated(q-value) duplicated genes and nonduplicated genes, the number of annotation definitions within the significantly regulated duplicated and non-duplicated genes and the number of gene ontology (GO) terms within the significantly regulated duplicated and non-duplicated genes for all transcriptomic profiles.

Fig. S8 Venn diagrams for significantly regulated (q-value) duplicated genes and tandemduplicated genes, the number of annotation definitions within the significantly regulated duplicated and tandem-duplicated genes and the number of gene ontology (GO) terms within the significantly regulated duplicated and tandem-duplicated genes for all transcriptomic profiles.

Fig. S9 Schematic illustration of the experimental design.

Table S1 List of cyanobacterial species obtained from different culture institutions with their respective identification number (ID), culture medium and geographic origin.

Table S2 Genes with JGI id and functional annotations that are shared among all transcriptomic profiles.

Table S3 P-values, corrected for multiple testing, of fisher exact tests comparing the proportion of lineage and non-lineage specific genes in (1) unique expression profiles vs. shared all profiles, (2) unique expression profiles vs. shared by at least two profiles (*Aphanizomenon*: APH, *Anabaena*: ANA, *Cylindrospermopsis*: CYL, *Nodularia*: NOD, *Oscillatoria*: OSL).

Table S4 Shared functional annotations across all transcriptomic profiles and their presence (+) or absence (−) in transcriptomic profiles exposed to *Microcystis aeruginosa* (MC, Asselman *et al*. 2012), salinity (NaCl, Latta *et al*. 2012), cadmium (Cd, De Coninck *et al*. 2014;.) and changes in phosphorous content of the diet (LoC-HiP, Jeyasingh *et al*. 2011).

Table S5 List of Gene Ontology terms shared by all transcriptomic profiles

Table S6 P-values, corrected for multiple testing, of fisher exact tests comparing the proportion of functional annotations shared by all expression profiles relative to functional annotations unique to a single profile for duplicated genes vs. non-duplicated genes (1) and duplicated vs. tandem duplicated genes (2).

Table S7 Pathways statistically enriched (P-value < 0.05) with significantly regulated genes (q-value < 0.05), based on a fisher exact test. Only pathways that were statistically enriched in at least three cyanobacterial exposure profiles are represented.

Table S8 Medium composition of BG110.

Table S9 Medium composition of BG11.

Table S10 Medium Composition of Z8.