Transcriptional consequences of acute copper exposure in *Daphnia pulex*

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Preface

Contribution of Authors

I am the main author of this thesis and all of the manuscripts within it.

I conducted toxicity tests along with Piumi Abeynayaka. I performed gene expression experiments and molecular work. I analysed RNA-sequencing data with the help of Dr. Frederic Chain.

My advisors, Melania Cristescu and Teresa Crease contributed to all phases of the development of this research, including thesis proposal, experimental design, experimental guidance, data analysis, and provided comments on earlier drafts of this thesis. Dr. Frederic Chain also provided experimental guidance and provided comments for writing of the thesis.

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Abstract

Copper (Cu) pollution in aquatic ecosystems is a worldwide problem, and continues to grow due to contamination of waste from many forms of industrial processes, from mining to agriculture. It is particularly harmful to aquatic invertebrates such as *Daphnia pulex,* which are very sensitive to environmental changes. My objectives was to investigate the toxic mode of action and mechanisms of tolerance to Cu stress in three genetically and phenotypically divergent *Daphnia pulex* lineages with high (Clone K) and low (Clones D and S) tolerance to Cu. I used RNA-sequencing to compare transcriptomes of these lineages exposed to sublethal Cu for 24 hours to non-exposed controls. Analysis across all lineages showed differential expression (DE) of 207 genes, of which 166 were upregulated and 41 downregulated under Cu exposure. Enriched Gene Ontology (GO) categories of upregulated genes include genes involved in digestion, molting and growth, suggesting these pathways may be involved in mitigating the adverse effects of Cu. Enriched GO categories of downregulated genes are involved in the metal-regulatory system, immune response and epigenetic modifications, suggesting that excess Cu interferes with these processes. The *metallothionein*s (*mt*s) are a gene family important for Cu tolerance; *mt1* was the only DE gene of the five *D. pulex mt*s, in contrast to previous work on metal-specific expression patterns. I also looked for DE gene in each clone separately. The gene encoding the anti-oxidant important for Cu tolerance *glutathione-S-transferase* (*gst*) was DE except in the sensitive clone S, suggesting that lower relative expression of *gst* may be involved in lower Cu tolerance in this clone. The sensitive clone S was the only lineage to show significantly lower expression in the Cu treatment of genes involved in digestion and molting suggesting that these processes were impaired to some degree in this clone. Moreover, the other sensitive clone, D was the only lineage to show significantly lower expression of a hemoglobin complex gene as well as an

ATPase gene involved in transporting Cu out of the cell. This suggests that oxygen transport and removal of excess Cu is more severely affected in this clone. All the unique DE genes in the most tolerant clone, K, have no known function. My results indicate that the *D. pulex* lineages used in this study, with different genetic backgrounds and tolerance, respond to acute Cu stress using the same major pathways. However, there are differences between lineages in the expression of genes that are known to be important for coping with Cu stress which may be important for tolerance to Cu.

Resumé

La pollution de cuivre (Cu) dans les écosystèmes aquatiques est un problème mondial, et ça continue à grandir à cause de la contamination des déchets de nombreuses formes de procédés industriels, de l'exploitation minière à l'agriculture. Il est particulièrement nocif pour les invertébrés aquatiques tels que *Daphnia pulex*, qui est très sensible aux changements environnementaux. Pour comprendre comment Cu affecte négativement les invertébrés aquatiques, on peut examiner des changements dans les modes d'expression des gènes en réponse au stress de Cu. Outils de recherche génomique telle que le séquençage de l'ARN permettent l'identification des gènes avec une expression altérée en réponse à cette toxine environnementale envahissant. Mes objectifs étaient de 1) enquêter sur le mode d'action toxique et les mécanismes de tolérance à Cu et 2) examiner les différences entre les trois lignées de *Daphnia pulex* avec élevée (Clone K) et faible (Clones D et S) tolérance à Cu. Pour ce faire, j'ai généré la première étude transcriptome basée sur ARN-séquençage en *D. pulex* utilisant trois clones génétiquement et phénotypiquement divergents exposés au stress Cu aiguë. Une analyse sur toutes les lignées a montré 207 (DE) gènes exprimés de manière différentielle (MD), 166 surexprimés et 41 réprimés sous exposition de Cu. Les catégories d'Oncologie Génétique Enrichi (OG) des gènes surexprimés comprennent les gènes qui sont impliqués dans la digestion, la mue et la croissance, suggérant le rôle de ces voies dans l'atténuation des effets néfastes de Cu. Les catégories de GO Enrichi de gènes réprimés sont impliquées dans le système de réglementation des métaux, la réponse immunitaire et les modifications épigénétiques, ce qui suggère que l'excès de Cu interfère avec ces processus. Les *métallothionéines (mt*s*)* constituent une famille des gènes importante à la tolérance Cu; MT1 est le seul gène MD de cinq *mt*s de *D. pulex*, contrairement aux précèdent travaux sur les motifs d'expression spécifique au métal. Je regardais aux gènes qui

étaient seulement MD, comparant chaque clone à son contrôle. Expression de l'antioxydant important pour Cu tolérance *glutathion-S-transférase (gst)* était MD sauf dans le sensible clone S, suggérant que l'expression relative plus faible de la GST peut être impliquée dans la tolérance inférieure à Cu dans ce clone. Le clone sensible S était la seule lignée d'avoir l'expression significativement plus faible dans le traitement Cu des gènes impliqués dans la digestion et mue, suggérant que ces processus ont été altérés dans une certaine mesure dans ce clone. En outre, l'autre clone sensible, clone D, est la seule lignée de montrer l'expression significativement plus faible d'un gène de l'hémoglobine, ainsi qu'un gène de l'ATPase impliqué dans le transport de Cu hors de la cellule. Ceci suggère que le transport de l'oxygène et l'élimination de l'excès de Cu est plus sévèrement affecté dans ce clone. Tous les gènes MD uniques dans clone le plus tolérant, clone K, n'avait pas de fonction connue. Mes résultats indiquent que les lignées *D. pulex*. utilisées dans cette étude avec différents fonds génétiques et tolérance répondent aux contraintes Cu aiguës en utilisant les mêmes voies principales. Cependant, j'ai aussi découvris des différences entre les lignées dans l'expression des gènes qui sont connus pour être importants pour faire face aux contraintes de Cu ainsi que la régulation négative de certaines catégories de gènes seulement dans certains clones. Pris ensemble mes résultats suggèrent qu'il existe des nuances dans l'expression des gènes à l'exposition Cu entre lignées de *D. pulex* avec différentes origines génétiques qui peuvent être importants pour la tolérance au Cu.

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General Introduction

1. Copper pollution and aquatic invertebrates

Environmental stress caused or exacerbated by anthropogenic actions continues to be a growing concern for the health of our ecosystems worldwide (Su et al., 2014). This is particularly true for aquatic ecosystems because they are a major target for human pollution including but not limited to acidification, eutrophication, calcium decline and contamination of metals (Altshuler et al., 2011). Copper (Cu) belongs to a group of trace metals that are essential micronutrients that function as cofactors for many enzymes (Zhu et al., 2014). However, in excess it can be a particularly toxic heavy metal, and is considered a dangerous environmental toxin because of its widespread use in industry and agriculture which leads to elevated environmental concentrations (Fernandes and Henriques, 1991).

Pollution of Cu and other heavy metals occurs through many avenues such as dispersion into the air from combustions or other industrial processing, car emissions, waste from tanneries, electroplating, mining, batteries and agricultural pesticide run-off (Jarup 2003; Zhu et al., 2014). This can lead to detrimental effects on an ecosystem, which have been studied for many years (Tyler, 1989; Heugens et al., 2001). Heavy metals pose a serious threat to aquatic ecosystems due to their synergistic effects with other biotic or abiotic stressors (Heugens et al., 2001), and bioaccumulative properties (Zhou et al., 2008). They are also a serious threat to some of aquatic ecosystems' most vulnerable yet important class of organisms: zooplankton. Zooplankton are an essential part of the food web, and while many environmental regulations are set by standards to other groups of organisms such as fish, zooplankton are known to be much more sensitive to environmental contaminants (Schindler, 1987). Understanding the adverse effects of Cu pollution on aquatic invertebrates must be done on many levels, from molecular processes to populations

(Soetaert et al., 2007). The ecotoxicogenomic approach seeks to integrate genetic data from an organism to understand molecular pathways that are affected by metal toxicity. This forms the basis of knowledge that can be used to help explain and predict changes in high levels of biological organization to metal toxicity.

2. Ecotoxicogenomics: a bottom-up framework

Ecotoxicogenomics aims to integrate ecotoxicology, the understanding of how pollutants and toxins affect the ecosystem, with genomic information such as gene expression. The ecotoxicogenomics framework incorporates multiple levels of biological organization, from the molecular and cellular processes, to the whole organismal level, and ultimately the ecosystem (Snape et al., 2004; Schirmer et al., 2010). The molecular basis includes patterns of gene expression, and protein and metabolic activity that are used to understand underlying molecular mechanisms of toxicity of a toxin. When this is put in the framework of ecotoxicogenomics and linked to other organismal metrics such as growth and development, population dynamics such as colonization and reproduction, one can extrapolate how the ecosystem as a whole is altered (Kim et al., 2015). It has been stated that it is imperative to understand the ecotoxicogenomics framework on the most basic molecular level to fully harness the ability to predict and mitigate future environmental changes (Eggen et al., 2004).

2.1 Ecotoxicogenomics in action

Transcriptomic data reveals altered gene expression in biological pathways to stress and thus can be linked to specific phenotypic outcomes that are most relevant for regulatory endpoints (Biales et al., 2015). Gene expression signatures using a suite of toxin-specific biomarkers are thought to serve as early warning indicators of environmental effects and as reliable and distinct endpoints for toxicity tests (Aardema and MacGregor, 2002; Fedorenkova et

al., 2010). Furthermore, characterization and grouping of mode of toxicity can be used to understand the growing list of new environmental pollutants that are poorly studied (Versteeg and Naciff, 2015). Ecotoxicogenomic approaches have been used in many taxa exposed to metals to investigate toxic modes of action, detect biomarkers of exposure, and integrate this information into biomonitoring programs (Jamers et al., 2013; Kim et al., 2013; Mussali-galante et al., 2013; Koedrith et al., 2013). Mode of action assays have been paired with traditional toxicology tests to provide both genomic and phenotypic evidence for risk assessment reviews conducted by the Environmental Protection Agency (Wilson et al., 2013; reviewed by Waters and Fostel, 2004).

New genomic tools such as transcriptomic profiling offer great advantages that can be complementary to traditional toxicology endpoints, such as survivorship and reproduction. These genomic methods provide faster, reproducible responses and allow inferences about the specific biological pathways involved in the mechanism of toxicity that were not otherwise possible (Robbens et al., 2007).

2.2 Genetic tools for ecotoxicogenomics

Genomic tools open up the possibility of asking new scientific questions and looking at traditional questions in a new light; we can now better understand how organisms react to environmental change at the molecular level by looking at whole genome gene expression patterns (transcriptomics). For example, we can understand how one part of the genome may have trans-acting effects on another. Previously, genetic mechanisms of toxicity and tolerance had been linked to a few genes (Van Straalen, Janssens, Roelofs, 2011), but now we have the ability to use the entire gene expression profile to see that molecular mechanisms are far more complicated and interconnected (Van Straalen et al., 2011). For example, classical case studies

looking at adaptation of the housefly to DDT showed a single gene was responsible for the resistance (Kettlewell, 1955), but more recent studies using whole transcriptome sequencing paired with qPCR found evidence of a more complex mechanism of tolerance through trans-acting factors (Roelofs et al., 2009; Straalen et al., 2011).

RNA sequencing is becoming the tool of choice for ecotoxicogenomics studies, especially with the decreasing price. RNA sequencing also offers some major advantages over microarrays (Zyprych-Walczak et al., 2015) including a larger dynamic range for quantifying gene expression levels (Wang et al., 2009), less background noise (such as cross hybridization of probes) (Zhao et al., 2014), the possibility of detecting alternative splicing isoforms (Wang et al., 2008; Pan et al., 2008), and the power to detect novel genes, gene promoters, isoforms, and allele-specific expression (Landau and Li, 2013).

3. Study species: Daphnia

3.1 Daphnia in Ecotoxicogenomics

There are many compelling reasons why the freshwater micro-crustacean *Daphnia* is considered an ideal study species to address ecotoxicogenomic questions. These include *Daphnia'*s ecological significance, decades of studies covering many fields, a reference genome, and clonal reproduction. *Daphnia*, found in lakes and ponds across the world, is an integral part of freshwater ecosystems, being an important link in aquatic food webs. It is considered a sentinel for environmental disruptions due to its sensitivity to toxins (Schindler, 1987). *Daphnia* species are widely studied in ecology (Hebert, 1978; Lampert, 2006 and 2011; Seda and Petrusek, 2011), and ecotoxicology (Sarma and Nandini, 2006; Altshuler et al., 2011), and are used to develop environmental regulations (Gunatilaka and Puzicha, 2000; Le et al., 2016). This

provides a rich knowledge base to put ecotoxicogenomic studies into an ecological context.

Ecotoxicogenomic studies in *Daphnia pulex* are made possible by the availability of a genome sequence; it was the first crustacean genome to be sequenced. *D. pulex* is considered to have an ecologically responsive genome; about one third of *D. pulex* genes that are most likely to be responsive to environmental changes are considered to have no homology in closely related species (Colbourne et al., 2011). Furthermore, a large portion of *Daphnia* genes underwent duplications, which reveal insights into the diversification of the gene pairs related to function and regulatory mechanisms. This allows for discovery of functions in responsive genes that have no known homolog in a closely related species or possibly have taken on a novel function (Alzarez et al., 2015), such as the *metallothionein* genes in *D. pulex* (Shaw et al., 2007).

The fact that genes of unknown function are likely to be responsive to environmental changes shines light on the bias towards investigating candidate gene pathways (Miner et al., 2012). This suggests that studies should take a non-targeted approach to discover new genes that may be involved beyond already known candidates. In fact, studies on non-model organisms without the complement of a well understood genome limit research by focusing on only previously discovered pathways (Neave et al., 2012). Non-targeted approaches like the use of microarrays and RNA-sequencing should be used to expand the genetic knowledge base for ecological species such as *D. pulex*, and allow exploration of less-understood molecular pathways (Collins et al., 2008; Vera et al., 2008).

Another reason *Daphnia* are often used in ecotoxicology studies is the ease with which they are cultured in a lab, and their clonal mode of reproduction, which effectively minimizes variation among individuals and thus generates more reproducible results compared to other species (Haap and Kohler, 2009). However, given the wide range in responses between clones

(Barata et al., 2002), it is essential to take into account genetic variation when carrying out ecotoxicogenomic studies.

3.2 Implications of Interclonal variation in Daphnia for Ecotoxicogenomics

One reason *Daphnia* are chosen for toxicity experiments is their clonal mode of reproduction, which yields more consistent results between individuals. However there has been much variation in toxicity test results between laboratories that used different clonal lineages (Barata et al., 2000). It is important to use an ecotoxicogenomic approach and look at changes in gene expression to understand these phenotypic differences between clones. Given *Daphnia*'s widespread use in ecotoxicology, and the broad applications of these studies, it is important to determine the link between genetic variation and gene expression response (Baird and Barata, 1998). Phenotypic studies on variation in other arthropods have suggested differences in the underlying mechanisms of tolerance (Neumann and Galvez, 2002). However, the role of gene expression in metal tolerance is not well understood in *Daphnia*; previous targeted gene expression studies in *D. magna* have focussed on cadmium (Cd) stress (Haap et al., 2016). However there are few studies on *D. pulex* in response to Cu stress. As ecotoxicogenomics takes a bottom-up approach, addressing the previously characterized variation in toxicity between lineages, the role of gene expression studies cannot be understated as they provide mechanistic information about the toxin.

3.3 Gene expression studies in Daphnia: Mechanistic information

The overall goal of ecotoxicogenomics is to make predictions about the toxic effects a toxin will have on each level of biological organization and thus use this knowledge to reduce negative effects on the environment (Schirmer and Fischer, 2010). To this end, information about

toxic mode of action gained through gene expression experiments is essential. Microarray gene expression studies have shown that heavy metals produce distinct gene expression profiles by metal., dosage, duration of exposure (Poynton et al., 2007 and 2008; Shaw et al., 2007), and life stage (Muyssen and Janssen, 2007). However there is much work to be done to understand the mechanism of toxicity in *Daphnia* (Soetaert et al., 2007), although toxic effects have been predicted including digestion suppression, oxidative stress, immune suppression and disruption of molting (Poynton et al., 2007).

On the other hand, organisms are able to combat stressors to some extent. The current accepted mechanism of tolerance to metal stress in a range of species, including *Daphnia*, is the metallothionein proteins that bind to extraneous metal ions (Asselman et al., 2012). While the response of these genes to metal stress has been characterized, we must recognize that the evolution of tolerance may be more complex than a single gene adaptation (Van Straalen, Janssen and Roelofs, 2011). Thus analysis of gene expression of thousands of genes, instead of select candidates, is required to identify responsive gene networks and bring us closer to understanding the mechanisms of Cu toxicity and tolerance of *D.pulex*.

4. Regulation and Risk Assessment

Water chemical regulations are set using a wide variety of ecotoxicology studies utilizing a number of traditional toxicological endpoints such as immobilization, death and reproduction, as well as molecular endpoints such as transcriptomics, proteomics and other metabolites. *Daphnia* are routinely utilized to determine the quality of inland waters (Gunatilaka et al., 2000), which is then referenced by environmental protection agencies worldwide to evaluate the risks the toxins pose to the environment (Weber, 1991). In North America, these agencies include the United

States Environmental Protection Agency, Environment Canada, and the Organization for Economic Co-operation and Development.

In the past, single gene biomarkers were widely used to identify the toxin damaging the ecosystem; however they have their disadvantages (Forbes, Palmqvist and Bach, 2006) including complex dosage interactions and their utility in environments dealing with multiple stressors. As a result, transcriptomics offers a promising alternative with thousands of toxicological endpoints which can then be linked to protein levels and other metabolites (Poynton et al., 2008). Many studies have now shifted to considering a suite of genes rather than a single biomarker as a more robust means to identify toxin-specific expression profiles.

To combine ecotoxicogenomics with ecological monitoring, a rigorous set of regulatory benchmarks must be developed. Some methods utilize differential expression analysis paired with benchmark dose analysis (another proposed statistical model to draw ecologically applicable information from toxicology data) of genes belonging to certain Gene Ontology (GO) groups (Thomas et al., 2007). However, this is limited to species with well annotated genomes, otherwise assigning GO terms can be very difficult (Poynton et al., 2008).

Other methods, such as No Observed Transcriptional Effect (NOTEL) seek to find a dosage at which no genes have differential expression (Lobenhofer et al., 2003; Ankley et al., 2006). This method also has limitations; there have been no studies confirming that NOTEL occurs in organisms exposed to toxins (Poynton et al., 2008). Even more complex methods have been developed such as the Transcriptional Effect Level Index (TELI) method which takes into account number of genes with altered expression, the magnitude of change, as well as the temporal pattern of gene expression (Gou et al., 2011).

Ecotoxicogenomic tools have great potential to gain understanding of the mechanism of

toxicity and tolerance to a toxin. The ultimate goal is to apply this information in a risk assessment context. This involves integrating gene expression information with other ecotoxicological endpoints to set robust suites of biomarkers. We must continue to improve and integrate ecotoxicological techniques to put regulations in place that prevent and temper calamitous effects on our natural environment.

5. Main objectives of this study

In study we look at gene expression patterns in response to Cu stress of *D. pulex* clones with different genetic backgrounds and with different Cu tolerance. We identify pathways that are affected by Cu stress and either serve as coping mechanisms to deal with the stress or that are negatively affected by excess Cu. We also look at differences in gene expression between clonal lineages to see which biological pathways are differentially affected. This helped us gain an understanding of gene expression changes in response to Cu stress differs between genetic lineages. This included genes known to be responsive as well as through biological pathways proposed to be involved in toxic effects.

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Investigating gene expression responses to acute copper stress in distinct *Daphnia pulex* lineages

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Abstract

Copper pollution is pervasive in aquatic habitats and is particularly harmful to invertebrates. Mechanisms of toxicity and tolerance to copper are not well understood. We used RNA-sequencing to investigate these mechanisms in three genetically distinct *Daphnia pulex* lineages with different copper tolerance. Enriched Gene Ontology (GO) categories correspond to upregulated genes after copper exposure were involved in digestion, molting and growth, suggesting the role of these pathways in mitigating adverse effects of copper. Enriched GO categories correspond to downregulated genes after copper exposure were involved in the metal-regulatory system, immune response and epigenetic modifications, suggesting excess copper interferes with these processes. We detect lower relative expression of the genes known to be important for copper tolerance, *metallothionein* and *glutathione-S-transferase*, in more sensitive than in resistant clones. The results indicate that the *D. pulex* lineages in this study respond to copper stress using the same major pathways; however sensitive clones may fail to regulate key genes as well as tolerant clones, suggesting there are important nuances in gene expression between clones in affected pathways which may be important for copper tolerance.

Keywords
Daphnia, copper, toxicity, RNA-seq, transcriptomics, intraspecific variation, ecotoxicogenomics

INTRODUCTION

Environmental stressors are of great concern, especially to the health of aquatic ecosystems, which are a large target for toxin deposition. Cu is a common water contaminant, which is especially toxic to aquatic invertebrates in high doses and is known to be detrimental to ecosystems (Heugens, 2001). Over the last decades, ecotoxicological approaches have been used to assess the effects of excess Cu on a variety of aquatic species. Traditional practices have investigated survivorship and reproduction, however the use of these endpoints is too slow for routine laboratory use (Robbens et al., 2007) and does not provide mechanistic information (Fedorenkova et al., 2010). However, the integration of genomic tools such as microarrays and RNA-sequencing has proven to be very valuable for ecotoxicology, as these tools provide information about the mechanism of toxicity on a molecular level that was missing previously (Robbens et al., 2007). Despite the growing interest in patterns of gene expression in response to Cu stress, the mechanisms underlying the toxic mode of action as well as the mechanism of defence against Cu toxicity is still not understood (Soetaert et al., 2007). Moreover, we do not understand how genetic background and history of exposure to Cu will influence the response of an organism. It is essential to take this into account in laboratory research to ultimately understand how Cu contamination will affect natural populations. With the development of genomic tools including RNA-sequencing, we are now able to answer mechanistic questions with exceptional depth.

Copper pollution

Cu pollution in aquatic ecosystems is a growing concern worldwide because of the ubiquitous use of Cu in industrial manufacturing, agriculture and Cu mining practices (Jarup 2003; Zhu et al., 2014). This heavy metal is known to be highly toxic, can have synergistic

effects with other stressors (Heugens et al., 2001), can bioaccumulate (Zhou et al., 2008), and its effects cannot be easily mitigated (Davis et al., 2001). Despite its toxic effects in high concentrations, Cu is essential for life and has an important function as a cofactor for many enzymes (Zhu et al., 2014), particularly those involved in hemoglobin synthesis (Lee et al., 1968). However, even at sublethal levels, Cu can have negative impacts on growth, reproduction and immune function of aquatic invertebrates (De Schamphelaere et al., 2007; Poynton et al., 2007). These adverse effects illustrate the need for robust monitoring of aquatic ecosystems. In the genomic age, biomonitoring can take a bottom up approach, such as changes in gene expression, the impact of which can be extrapolated to higher levels of biological organization, such as population dynamics (Snape et al., 2003). Changes in gene expression provide a genetic signature to indicate adverse physiological endpoints and are used as risk assessment tools (Eggen et al., 2003). An important aquatic invertebrate for risk assessment is the freshwater micro-crustacean, *Daphnia*. This bottom-up approach to biomonitoring can be implemented using *Daphnia* as an ecotoxicogenomics model species to investigate gene expression patterns in response to acute Cu stress.

Daphnia are found in lakes and ponds across the world and are an integral part of freshwater ecosystems. As such, they are considered to be a sentinel organism for environmental issues (Schindler, 1987). *Daphnia* species are widely studied in ecology (Hebert, 1978; Lampert, 2006 and 2011; Seda and Petrusek, 2011), ecotoxicology (Sarma and Nandini, 2006; Altshuler et al., 2011), and have been used to develop environmental regulations (Gunatilaka and Puzicha, 2000; Le et al., 2016). This provides a rich knowledge base to put ecotoxicogenomic studies into a broader ecological context. Ecotoxicogenomic studies on *Daphnia pulex* are made possible by the availability of a genome sequence; *D. pulex* was the first crustacean to have its genome

sequenced. *D. pulex* is considered to have an ecologically responsive genome; about one third of all *D. pulex* genes are likely to be sensitive to environmental changes and are generally considered to have no homologs in closely related species (Colbourne et al., 2011). This allows for discovery of functions in responsive genes such as the newly annotated *metallothionein* genes (Shaw et al., 2007). Subsequent follow-up functional assays can also be used to discover novel functions. Another reason *Daphnia* are often used in ecotoxicology studies is their clonal mode of reproduction, effectively minimizing variation among individuals and enabling generation of more reproducible results compared to other species (Haap and Kohler, 2009). However, given the wide range in responses between *Daphnia* clones in the same species (Barata et al., 2002), it is essential to take into account genetic variation when carrying out ecotoxicogenomic studies.

Implications of Interclonal variation in Daphnia for Ecotoxicogenomics

Understanding the link between genetic variation and gene expression response to a stressor is a question that has been minimally explored. While differences in stressor response between phenotypes have been shown in arthropods, such as differences in insecticide tolerance (McKenzie and Yen, 1995), and suggest different genetic mechanisms of tolerance (Neumann and Galvez, 2002), there has been little investigation using gene expression analysis. In *Daphnia*, the use of genetically different clones locally adapted to different lakes or ponds leads to much variation in toxicity test results between laboratories (Barata et al., 2000) as well as field studies (Haap and Kohler, 2009). Given the widespread use of *Daphnia* in ecotoxicology, and the broad applications of these studies in risk assessment and environmental regulation, it is important to address the effect of genetic variation and gene expression response (Baird and Barata, 1998). Moreover, understanding the interaction between genetic variation and gene expression has applications beyond ecotoxicogenomics and can advance areas such as ecosystem and population

health monitoring (Zayed, 2009), laboratory use of microbiota strains (Kvitek et al., 2008), and human health (Aardema and MacGregor, 2002).

The degree to which gene expression patterns under metal stress vary across *Daphnia* lineages (inter-clonal variation) remains little understood. Genetic variation between clones has been studied mainly in terms of fitness and biokinetic parameters (Muyssen et al., 2010; De Coninck et al., 2013). These studies document differences in physiological responses (growth, ingestion rates, energy reserves and electron transport activity) between clones and thus provide a basis to investigate underlying gene expression. Previous gene expression studies incorporating different clonal *Daphnia magna* lineages have focused on interclonal differences in expression of a few genes before and after acquired tolerance (multi-generational) to Cd metal stress (Haap and Kohler, 2009; Haap et al., 2016). However there is a lack of studies on the response of *D. pulex* to Cu stress. Similar to the work on metal homeostasis and tolerance conducted on hyper-tolerant plants (reviewed in Clemens, 2001), there is a need to integrate genetic variation among *Daphnia* clones with gene expression studies in order to identify genes or gene networks that can help disentangle complex mechanisms of toxicity and tolerance to Cu stress.

Ecotoxicogenomics approach

Molecular mechanisms of Cu toxicity can be studied using ecotoxicogenomic approaches that integrate toxicology with transcriptomics or other genomic tools. Transcriptomic data reveals altered gene pathways to cope with stress and thus can be linked to specific phenotypic outcomes that are most relevant for regulatory endpoints (Biales et al., 2015). Furthermore, characterization and classification of mode of toxicity can be used to make predictions of mode of action for toxins on the growing list of new environmental pollutants that are poorly studied (Verteeg et al., 2015). Ecotoxicogenomic approaches have been used in many taxa undergoing

metal exposure to investigate toxic modes of action, detect biomarkers of exposure, and integrate this information into biomonitoring programs (Jamers et al., 2013; Kim et al., 2013; Mussali-galante et al., 2013; Koedrith et al., 2013). Mode of action assays have been paired with traditional toxicology tests to provide both genomic and phenotypic evidence for risk assessment reviews conducted by the Environmental Protection Agency (Wilson et al., 2013; reviewed by Waters and Fostel, 2004). New genomic tools such as transcriptomic profiling offer great advantages that can be complementary to traditional toxicology endpoints such as survivorship and reproduction. These genomic methods provide faster, reproducible responses and allow inferences about the specific pathways involved in the mechanism of toxicity as well as pathways involved in coping mechanisms that were not otherwise possible (Robbens et al., 2007).

Gene expression studies: Mechanistic information

There has been growing interest in using gene expression profiling to gain mechanistic information on Cu toxicity particularly using *Daphnia* as a model organism. Microarray gene expression studies have shown that heavy metals produce distinct gene expression profiles by metal, dosage, duration of exposure (Poynton et al., 2007 and 2008a and b; Shaw et al., 2007), and life stage (Muyssen and Janssen, 2007). Gene expression profiles can give us information in two regards: the mode of action of toxicity and the mode of tolerance of the organism. Despite all the interest, the exact mechanism of toxicity has yet to be fully understood in *Daphnia* (Soetaert et al., 2007), although effects of Cu toxicity have been predicted using gene expression experiments including digestion suppression, oxidative stress, immune suppression and disruption of vital exoskeleton processes (Poynton et al., 2007). It is also essential to understand how an organism combats Cu stress. The best-known mechanism of tolerance is the production

of metallothionein proteins that bind to extraneous metal ions; however, the mechanism of tolerance has been hypothesized to involve more than just a single crucial gene (Van Straalen et al., 2011; Janssens and Roelofs, 2011). It is essential to use expression information on thousands of genes instead of select candidates to broaden our understanding of gene pathways involved in Cu tolerance.

The objective of this study is to use RNA-sequencing to investigate differences in the whole transcriptome of phenotypically and genotypically different *D. pulex* clones exposed to Cu. To explore how differences in response among clones may be important for Cu tolerance, we first look at biological pathways responsive to Cu stress which we identify in our differential expression analysis including all clones. In particular, we discuss how our data compares with previously proposed effects of toxicity and candidate genes that are involved in metal tolerance. We further extend the analysis to consider differences in Cu induced gene expression among lineages to draw implications related to relative Cu tolerance. We identify genes that can subsequently be investigated in targeted mode of action studies.

METHODS

Daphnia Clones

Daphnia pulex clones were isolated from lakes and ponds in Illinois and Michigan, USA and from a lake in Sudbury Ontario, Canada. The sampled lakes and ponds in the USA are not known to be contaminated with heavy metals, while Cu contamination has been documented in Kelly Lake near Sudbury according to the Ontario Waters Resource Commission (1965), and recently by the Cooperative Freshwater Ecology Unit (2004). *Daphnia* clones were established from single-wild caught individuals and cultured in the lab for about one year prior to use in toxicity testing.

Toxicity test

Preliminary toxicity assays were conducted to determine the relative tolerance of *Daphnia* clones and to determine the concentration to use in the subsequent Cu exposures for gene expression analysis. We performed 48 hour toxicity tests on unfed neonates. Initially 21 *Daphnia* lineages (9 lake and 12 pond lineages) were tested of which 15 lineages were chosen for further testing. Selection of the 15 lineages was based on retaining genetic diversity from different populations from both ponds and lakes (8 lakes and 7 ponds). Groups of 5 neonates of each lineage were randomly selected for exposure to a gradient of Cu concentrations, and this was repeated to create a total of 3 replicates for each lineage. Neonates were taken from the 3rd to 8th clutch from 4-5 mothers of each lineage and randomly distributed across replicates and concentrations within a lineage. Neonates were chosen to establish LC50 tolerance since neonates are considered to be the most sensitive life stage and are commonly used in standard toxicity tests (Hanazato, 2001). The established LC50 tolerance based on neonates was used to determine the treatment concentrations for the subsequent acute and chronic experiments.

Toxicity tests were performed using Cu concentrations from 150 to 178 µg/L in 5 µg/L increments as well as a control containing only baseline Cu concentration in FLAMES media of 0.2842 µg/L (Celis-Salgado et al., 2008). Source *Daphnia* were reared in control conditions for at least 3 generations to control for maternal effects. The exposure of all groups of five neonates less than 48 hours old involved the transfer to an intermediate vial with 30 mL of media containing the target concentration of Cu, and then transferred from the intermediate to an experimental vial also containing 30 mL of media at the target Cu concentration. The neonates were not fed and their survivorship was observed after 48 hours. Neonates were considered dead if immobilized or if no movement after light agitation with a pipette was observed. Lethal Concentration at 50% mortality (LC50) was calculated with a Probit analysis (Finney, 1952) using the "MASS" package (Venables and Ripley, 2002) for R with the *dose.p* function. Clones for the gene expression analysis experiment were chosen based on LC50 values ranging from sensitive to tolerant. The function *comped* from the "drc" package (Ritz et al., 2015) for R was used to perform a ratio test to determine if LC50 values between populations were significantly different (Wheeler et al., 2006).

Copper exposures for gene expression analysis

Six clones were chosen for acute Cu exposure for gene expression analysis. The exposure involved adult primiparous *D. pulex* exposed for 24 hours to 90 µg/L of Cu. Three tanks contained FLAMES media with Cu and one tank without Cu served as control. FLAMES media with Cu was made from a Cu stock of 100,000 µg/mL which was diluted to a final concentration of 90 µg/L. The pH of each tank was monitored during the duration of the experiment. The six clonal lineages were each replicated 5 times in each of the four tanks. Each of the 8 L polypropylene tanks contained a total of 30 falcon tubes (50 ml each) suspended from a

plexiglass scaffold. Tubes had an opening at the bottom covered with 300 micron nylon mesh to allow for free flow of media. Tanks were placed on stir plates and constantly stirred at a rate of 120 rpm. Tanks were also covered with a plexiglass lid to reduce evaporation and cross contamination. Three species of algae, *Ankistrodesmus*, *Pseudokirchneriella*, *Scenedesmus*, were grown in Bold's Basal Medium (Stein, 1979), centrifuged, and the pellet was resuspended in FLAMES for feeding. The algae concentration used for feeding was kept at 20,000 cells/mL. The algae for the Cu treatment tanks were suspended in FLAMES containing Cu. Tanks were kept in a controlled temperature chamber at 18°C with a 16:8 light-dark cycle. The tissue collection was done by transferring *Daphnia* individuals and a small amount of media into a 1.5 mL microcentrifuge tube with a wide-bore pipette. Media was then removed and 300 µl of RNAlater® (Qiagen) was added to the tube. Samples were placed at 4°C for 24 hours and then transferred to -80°C for storage.

RNA Extraction

Three out of the six clonal lineages were chosen for sequencing in order to evaluate interclonal variation while optimizing sequencing depth per sample (Figure 1). Whole *Daphnia* samples were stored in RNAlater® (up to 4 adult primiparous individuals per sample in 300 µl) at -80°C prior to extraction. To extract RNA, samples were thawed on ice and the animals were transferred from the RNAlater® to the column extraction buffer. Total RNA was then purified using the RNeasy Plus Universal Mini Kit (Qiagen) as per the protocol. Modifications include homogenization of *Daphnia* with a sterile pestle and motor mixer for 2 minutes, or until no particles remained visible. RNA was aliquoted into 3 separate tubes for short term and long term storage in the freezer. An ethanol precipitation was performed to clean each RNA sample of unwanted salt contaminants leftover from extraction buffers. The following were added to a

sample of 10 µl total RNA: 1 µl of the carrier glycogen at 20 mg/ml, 1.1 µl 3.0 M sodium acetate, and 28 µl 100% ethanol. This was mixed gently by pipette and stored overnight at -80°C. Samples were then centrifuged at 4°C for 30 minutes at 12,000 x g. The supernatant was carefully discarded to avoid disturbing the pellet, then 500 µl of freshly made 75% ethanol was added and the pellet was washed by inverting the tube once. Samples were spun for 15 minutes at 4°C at 12,000 x g and then allowed to stand at room temperature for 15 minutes to dissolve co-precipitated salts. Samples were centrifuged for 5 minutes and the supernatant was discarded. This washing was repeated twice. Pellets were air-dried for 5 minutes and then resuspended in 30 µl RNase-free water for 15 minutes to ensure complete solubilization. The concentration of RNA in each sample was determined using 1.5 µl of each sample and a NanoDrop Spectrophotometer.

RNA Sequencing Library Preparation

RNA Sequencing libraries were prepared using the NEXTflex™ Rapid Directional mRNA-Seq Kit (Bioo Scientific), which is compatible with the Illumina sequencing system. For the first lane of sequencing, 100 ng of total RNA per sample was used as starting material. mRNA was selected from each sample using NEXTflex™ poly(A) Beads and libraries were prepared as per the manufacturer's protocol. For the second lane of sequencing, 200 ng of total RNA per sample was used to obtain a higher concentration in the final libraries. Libraries were quantified at Genome Quebec using an Agilent Bioanalyzer 2100.

Sequencing

Two lanes of sequencing were performed on the Illumina HiSeq 2000 at Genome Quebec using Paired-End (PE) 100 base-pair reads. Lane one included 15 libraries that were differentiated by the addition of unique nucleotide sequences (barcodes) at each end (NEXTflex™ RNA-Seq Barcodes). Lane two included 15 libraries, 6 of which were included in

this data set for a total of 21 libraries; the others from lane 2 were part of a chronic exposure experiment that is currently being analysed.

Bioinformatic pipeline

The FASTQ format of each sequencing file was analyzed using FastQC (Andrews, 2010; http://www.bioinformatics.babraham.ac.uk/projects/fastqc) as a quality control check. Programs in the MUGCIQ RNA-Seq Pipeline on the Calcul Quebec High-computing cluster (https://bitbucket.org/mugqic/mugqic_pipelines/src/master/pipelines/rnaseq) were used to perform subsequent analyses. These included Trimmomatic (Bolger, Lohse and Usadel, 2014) to trim low quality sequences and adaptors, STAR (Dobin et al., 2012) to map sequences to the *Daphnia* reference genome (Colbourne et al., 2011), Picard (http://broadinstitute.github.io/picard) to mark read duplicates, RNA-SeQC (Deluca et al., 2012) to evaluate alignment metrics, HTSeq-count (Anders, Pyl and Huber, 2014) to determine raw read counts per gene, the cufflinks package (Trapnell et al., 2010) to assemble the transcriptome, and the software packages EdgeR (Anders and Huber, 2010) and DESeq (Robinson, McCarthy and Smyth, 2010) to perform differential expression analysis. A gene was determined to be differentially expressed if the adjusted p-value after Benjamini-Hochberg correction (Hochberg and Benjamini, 1990) was less than 0.05 in both DESeq and EdgeR analyses. Gene ontology (GO) enrichment analysis was performed with topGO (Alexa and Rahnenfuhrer, 2006) using GO terms acquired from Ensembl (v30) (Aken et al., 2016). GO terms were identified as significantly enriched using a False Discovery Rate (FDR)-corrected weighted p-value < 0.05 in topGO.

Candidate genes involved in Cu toxicity were investigated based on previous studies on *Daphnia* exposed to Cu and other metals (Table S4). We also explored co-expression of genes to

generate modules of highly correlated genes (using Pearson correlation) by applying a weighted gene co-expression network analysis using the WGCNA package for R (Langfelder and Horvath, 2008). We used differentially expressed genes with adjusted p-values below 0.1 according to at least one of DESeq or EdgeR in analyses of all clones together, or each clone separately. One of the six control samples (Control for Solomon Pond clone- A2 7S) was excluded from the network analysis according to recommendations in the manual following hierarchical clustering implemented in WGCNA. However, in general, this sample followed the same trend as the other control samples.

RESULTS

Toxicity Test Results

Three of the six clones were chosen for sequencing; two clones from non-contaminated environments called Dump Pond 4 (D clone) and Solomon Pond 7 (S clone), and one clone from the contaminated Kelly Lake 12 (K clone). Based on the toxicity tests that evaluate LC50 (Table S1), the K clone had an LC50 of 181 µg/L Cu whereas the D and S clones had an LC50 of 154 µg/L and 170 µg/L, respectively, suggesting that K is more tolerant while the S and D clones are more sensitive. The exposure concentration was ~49%, 53% and 59% of the LC50 for clones K, S and D, respectively. LC50 ratio pairwise comparisons between populations rejected the hypothesis that the populations were the same and are therefore considered significantly different in LC50 values.

Sequencing and Mapping results

A total of 21 RNA libraries were sequenced which included 6 control samples (two from each clonal lineage, from one tank) and 15 Cu treated samples (5 from each clone line, spread between 3 tanks, Figure 1). A total of 245,671,301 raw paired-end reads were obtained from 21 sequenced libraries from the three clones that were chosen for sequencing. The average percentage of reads that mapped to the reference genome was 89% (ranging from 84 to 92%). The mean number of reads per gene was 493 (ranging from 319 to 643). The average depth of coverage for each sample was 16x (ranging from 12 to 21x), or 16,230,359 read pairs (ranging from 12,746,931 to 21,680,702). This depth is considered acceptable for finding differentially expressed genes that are not relatively highly expressed (Lei et al., 2015). The average number of expressed genes in a sample was 17,128 (ranging from 15,134 to 17,869).

Overall Patterns of Gene Expression

Comparing all Cu exposed samples (n = 15) with all of the control samples (n = 6) resulted in 207 significantly differentially expressed (DE) genes; 41 were downregulated and 166 were upregulated under Cu exposure. Six genes were differentially expressed at $p<0.01$ and had a fold change four times that of the control group, highlighting them as strongly responsive to acute Cu exposure (Table 1). The majority (135) of the 207 DE genes could be annotated with gene ontology (GO) categories, however 36% of the DE genes were unannotated. The major GO categories represented among DE genes include metabolism, which accounted for about a quarter of the genes, cell membrane-related proteins, ion transport and peptidase activity, as well as proteins related to metal binding, transport, homeostasis and metalloenzymatic activity (Figure 2a). The representation of DE genes in all categories is higher than expected compared to the number of expressed genes in each category, except for DNA binding and cellular activity (Figure 2b). The expected proportion of genes for each category was calculated by dividing the number of expressed genes for each category by the total expressed genes in the data set. This established a baseline proportion for each category. The actual proportion of genes for each category was calculated by dividing the number of DE genes for each category by the total DE genes. Genes upregulated under Cu exposure were significantly enriched with the functional categories proteolysis, serine-type endopeptidase activity, metallocarboxypeptidase activity, chitin binding and metabolism. Downregulated genes were enriched with the GO terms carbonate dehydratase activity, extracellular matrix structural constituent, structural constituent of cuticle, carbohydrate binding, and one-carbon metabolic process.

A co-expression analysis was performed using a more liberal differential expression threshold (p-value < 0.1; see Methods) to cluster genes into modules with similar expression

profiles across samples. This analysis involved 600 protein-coding genes grouped into 5 modules, three of which (M1, M2 and M3) consisted of genes generally upregulated in Cu-exposed samples compared to control samples, and two oh which (M4 and M5) included genes that were mainly downregulated in Cu-exposed samples (Figure 3a). The co-expression analysis concurred with the overall patterns of gene expression; module M1 corresponded to the significantly upregulated DE genes ($p<0.05$), module M4 corresponded to the significantly downregulated genes, whereas most of the genes with higher adjusted p-values (>0.05) were found in module M5 (Figure 3c).

Lineage specific patterns of gene expression

Given the established difference in LC50 values among the clones used for sequencing, it is possible to investigate whether clonal lines with different tolerance to Cu respond differently to Cu exposure, in contrast to our DE analysis consisting of all clones grouped together. To this end, differential expression analysis using DESeq and EdgeR was performed for each clone individually (Cu treatment n = 5, control n = 2 per clone), resulting in 2 upregulated genes uniquely DE in the K clone (out of 37 DE genes total for the K clone), 4 (three downregulated, one upregulated) in the D clone (out of 33 DE genes total for the D clone), and 14 (eight downregulated, six upregulated) in the S clone (out of 43 DE genes total for the S clone) (Figure 4). The function of many unique DE genes is not known, including both unique DE genes in the K clone (Table 2a). The 14 unique DE genes in the S clone included downregulation of six genes encoding exoskeleton proteins and one gene encoding endopeptidase activity involved in digestion, while a gene for another digestive enzyme, serine protease was upregulated. The functions of another six (one downregulated, five upregulated) unique DE genes in the S clone are not known (Table 2b). The four unique DE genes in the D clone correspond to

downregulation of one gene related to hemoglobin activity, one gene encoding an ATPase activity involved in ion transport, and one gene of unknown function as well as upregulation of a *metallothionein* gene (Table 2c). Nine genes were differentially expressed (six downregulated, three upregulated) in each individual clone as well as in all clones grouped together (Table 3).

Candidate Genes for copper tolerance

Metallothionein genes

Metallothionein genes function to mediate metal homeostasis and detoxification (Janssens, Roelofs and Van Straalen, 2009). Of the five annotated *metallothionein* genes in *D. pulex* (Shaw et al., 2007; Asselman et al., 2012), only *mt*1 was significantly upregulated when grouping all clones (Figure 5a and 5b). *Metallothionein* 1a (*mt*1a) was significantly upregulated only when all clones were grouped together ($pval_{DESeq}$ = 0.00056, $pval_{edgeR}$ = 1.80E-06), but not in clone-specific DE analyses. *Metallothionein 1b* (*mt*1b) was significantly differentially expressed in the D clone ($pval_{DESeq}$ = 0.0013, $pval_{edgeR}$ = 0.00033), and in the K clone in the EdgeR analysis ($pval_{edgeR}$ = 0.027; $pval_{DESeq}$ = 0.13), but not in the S clone (Figure 5b). The genes encoding metallothioneins that are known to respond to Cu exposure, *mt*2 and *mt*4 were not differentially expressed in any DE analysis (Figure 5c).

Proteins that have been documented to interact with metallothioneins are the antioxidant, glutathione transferase and the metalloprotein, metallocarboxypeptidase (Yadav, 2010). The *glutathione transferase* gene was significantly upregulated when all clones were grouped together ($pval_{DESeq}$ = 8.20E-16, $pval_{edgeR}$ = 1.20E-14), in the D clone ($pval_{DESeq}$ = 8.90E-05, $pval_{edgeR}$ = 0.0027) and the K clone ($pval_{DESeq}$ = 4.00E-10, $pval_{edgeR}$ = 2.60E-08), but not in the S clone ($pval_{DESeq}$ = 0.73, $pval_{edgeR}$ = 0.18) (Figure 5d). Metallocarboxypeptidase is encoded by two *Daphnia* genes (*DAPPUDRAFT_117945* and *DAPPUDRAFT_195011*). Both genes were

significantly upregulated when all cones were grouped together (117945: $pval_{DESeq} = 0.027$, $pval_{edgeR} = 0.0019$, 195011: $pval_{DESeq} = 0.00053$, $pval_{edgeR} = 0.00028$), but not in any of the individual clones, although the latter was just beyond our significance threshold in the D clone using DESeq ($pval_{DESeq} = 0.07$, $pval_{edgeR} = 0.24$) (Figure 5e and f).

DISCUSSION

OVERALL PATTERNS OF GENE EXPRESSION

We investigated patterns of gene expression common to all lineages in the context of Cu tolerance and toxicity. This entails understanding the results of our DE analysis including GO categories of upregulated genes involved in proteolysis, serine-type endopeptidase activity, metallocarboxypeptidase activity, chitin binding and metabolism could be involved in reducing the toxic effects of Cu. We also investigate how GO categories of downregulated genes involved in carbonate dehydratase activity, extracellular matrix structural constituent, structural constituent of cuticle, carbohydrate binding, and one-carbon metabolic process may result in toxic effects of Cu on the organism.

We find that most major cellular pathways are affected in all *Daphnia* clones indicated by similar gene expression patterns. Based on previously proposed toxic effects of Cu we confirm that we see evidence of oxidative stress and immune suppression. Contrary to previously proposed toxic effects we see evidence that suggests molting and digestive processes are in fact enhanced, perhaps in an effort to combat toxic effects. Differences in gene expression between lineages were nuanced within these biological pathways and differences varied by clone. One sensitive clone did not express as highly genes known to binding excess metals and to reduce oxidative stress. The other sensitive clone showed downregulation of genes involved in oxygen transport and pumping metal ions out of the cell.

GENE EXPRESSION PATTERNS BY CELLULAR PROCESS CATEGORY

Immune suppression

Our study supports the hypothesis that some recognition genes of the *Daphnia* immune system are downregulated and thus suppressed in response to Cu exposure, which is an apparent

adverse response. In this study, downregulated genes include six carbohydrate binding genes with C-type lectin domains, and one gene that is involved in a defense response to viruses. However certain immune recognition genes are upregulated in this study including genes involved in scavenger receptor activity, response to a viral capsid and lysozyme activity. A potential negative physiological effect that could be attributed to this gene upregulation is depletion of energy reserves over a long period of time, or at high concentrations. Depletion of energy reserves as nickel (Ni) concentration increased has been observed in *D. magna* (Vandenbrouck et al., 2009). If an organism cannot sustain a heightened state of immune response for a long time, increasing certain immune functions may be a last resort effort to control the acute stressor.

Suppression of the immune system has been a proposed toxic effect specific to Cu (and not other metals) based on reduction in blood cell count and activity, (Parry and Pipe, 2004) and increased disease susceptibility (Yeh et al., 2004) in other organisms. In *Daphnia*, this is supported by down regulation of immune genes encoding proteins involved in recognition of infection, even at low doses of Cu, and consistently from short to long exposures (Poynton et al., 2007 and 2008a). Although there is much to be understood about the molecular mechanisms of the *Daphnia* immune system (Rozenberg et al., 2015), a recent in silico identification of the immune-related genes, whose functions were inferred by comparing homologues in other arthropods, has advanced our understanding (McTaggart et al., 2009; reviewed by Auld, 2014). For example, we know the *Daphnia* immune system is comprised only of an innate immune system; the three main stages are recognition, regulation and attack. In this study, genes that encode C-type lectins responsible for recognition of pathogens were downregulated. However genes encoding other types of recognition proteins such as scavenger receptors and

peptidoglycan catabolism were upregulated. The genes encoding recognition proteins belong to many gene families (McTaggart et al., 2009), and this may suggest that the different types of recognition proteins play different roles in the immune system. Further functional information about different types of recognition proteins may help us to understand which specific part of the immune system is affected by excess Cu ions.

It is also interesting to note that the attack stage of an innate immune response includes chitinase activity, and chitinase gene expression was upregulated in all three clones, suggesting that chitinase is not only important for molting but also for the immune response. There is certainly more work to be done to understand how Cu stress is affecting the expression of genes involved in the *Daphnia* immune system, and here we show that expression of some immune genes is suppressed, while other genes are in fact upregulated.

Oxidative stress

As seen in other studies in *Daphnia* (Poynton et al., 2007, 2008a, Shaw et al., 2007), we found that nine genes related to oxidation reduction processes, response to oxidative stress, and peroxidase activity were upregulated including *glutathione–S-transferase (gst)*. The transcription of *gst* has been shown to be regulated by oxidative stress (Casalino et al., 2004). Cu is known to cause reactive oxygen species when present in excess amounts (Kawakami et al., 2008). Oxidative stress is a common effect of other heavy metals and toxins (Melegari et al., 2013); Cu among other metals produces mutagenic and carcinogenic by-products through redox reactions (Valko et al., 2005). Other known biomarkers of oxidative stress, including superoxide dismutase and catalase (Ruas et al., 2008) were not differentially expressed in our study. One gene (DAPPUDRAFT_234836) related to oxygen binding and transport was downregulated. The

upregulation of genes that are anticipated to respond as part of the antioxidant pathway indicate that oxidative stress is a mode of action of Cu toxicity. The role of antioxidants to protect the organism will be discussed below.

Digestion

In this study we see upregulation of 27 genes involved in peptidase activity, including serine type endopeptidase activity, which is a digestive enzyme involved in lipid metabolism, carbohydrate metabolism, and hydrolase activity (von Elert et al., 2004). We also see upregulation of 28 genes involved in hydrolase activity. This suggests that digestive processes may have been increased by Cu exposure, although we also see downregulation of genes involved in lipid transport. This is in contrast to a proposed toxic effect of Cu involving disruption of digestive processes based on evidence in *Daphnia* and other crustaceans. Supporting evidence includes downregulation of genes encoding digestive enzymes (Poynton et al., 2007, 2008a), reduction in feeding behaviour (DeCoen and Janssen, 1998), and physiological evidence of necrosis of the hepatopancreas in other crustaceans (Li, Zhao and Yang, 2007), an organ with functions similar to the liver (Griffiths, 1980).

One possible reason for the upregulation of genes encoding digestive enzymes, as opposed to their expected down regulation, could have to do with the mode of Cu exposure: waterborne compared to dietborne Cu exposure elicits different gene expression profiles in fish (Minghetti et al., 2008). In our study, algae species were suspended in Cu media and adsorption to the outside of the algae cell would occur, thus *Daphnia* would be exposed to both waterborne and dietborne Cu. Dietborne exposure to Cu could mean Cu is more bioavailable to the organisms, although the link between bioavailability and toxicity is not clear (Clearwater et al., 2002). Our data may align more with studies such as De Schampelaere and Janssens (2004a) in

which chronic dietary Cu exposure resulted in a significant increase in growth and reproduction in *D. magna* that was not seen in waterborne exposures. This concept is supported by studies in invertebrates (Chen et al., 2002) and mammals (Kirchgessner et al., 1976). Digestion is linked to growth and reproduction by providing metabolism of essential macromolecules. On the other hand, even in the few studies assessing dietary metal exposure, other chronic studies show the opposite trend in reproduction to previously discussed studies (Chang and Sibley, 1993, De Schamphelaere et al., 2007).

Comparing the studies involving chronic Cu exposure mentioned above to our study involving acute exposure provides a complementary picture and helps to integrate our knowledge of the energy allocation theory. Dynamic energy budget models (Nisbet et al., 2000) and laboratory experiments on *D. magna* (Nogueira et al., 2004) suggest that increased energy consumption to mitigate stressors draws energy away from growth and reproduction. These increased metabolic demands to defend against Cu damage will lead to a reduction in energy reserves, which will reduce further over time, as shown by a gradually widening gap in brood production and size in *D. magna* compared to non-exposed individuals (De Schamphelaere et al., 2007). An initial increase in metabolic and digestive processes after exposure to Cu may serve to provide energy to mitigate the immediate toxic effects of Cu if the stressor is severe enough. If the stressor is sublethal, these processes may be downregulated to prioritize other pathways. The resulting increase or decrease in growth and brood production may be dictated by the amount of energy that is ultimately used to deal with the stressor, and depends on factors such as concentration and duration of exposure (Bossuyt, Escobar and Janssen, 2005). Therefore we suggest that for the duration and concentration, and mode of Cu exposure in this study, disruption of digestion does not appear to be a toxic effect of Cu. In this case, upregulation of

genes involved in digestion could actually be protecting the organism by providing energy for use in other pathways that defend against Cu toxicity.

Exoskeletal proteins

In our study, all 11 significantly differentially expressed genes related to chitin binding, chitinase activity and metabolic processes were upregulated. In contrast to this, Cu toxicity has been proposed to repress development and reproduction as suggested by downregulation of exoskeletal genes in *D.magna* exposed to Cu (Poynton et al., 2008a). Exoskeletal proteins play an important role in molting, development and reproduction of *Daphnia* as degradation and rebuilding of the exoskeleton is essential for these processes. Molting is highly controlled by hormone expression. Toxicants such as PCBs and other heavy metals have been shown to disrupt hormone synthesis (Zou and Fingerman, 1997) and thus interfere with the molting process.

There are a few explanations for the upregulation of genes encoding exoskeleton-related proteins that we observed. The first is that we used individuals actively preparing to shed their exoskeleton (primiparous). Genes related to molting activity were upregulated in individuals exposed to Cu compared to the controls, perhaps as a stress response to release the brood as soon as possible; either in an effort to reduce metal exposure to the brood, or as a self-preservation technique as molting can be used to eliminate metals. Another reason for an unexpected upregulation of exoskeleton genes could be the concentration of the stressor, and therefore the severity. In other studies using a lower concentration of Cu (Poynton et al., 2008a), exoskeleton genes were downregulated, perhaps to prioritize more important functions. When *D. pulex* was exposed for 48 hrs to Cd, a more toxic metal, chitin-related genes were upregulated (Shaw et al., 2007). This was also seen in *D. magna* exposed to Cd (Connon et al., 2008). In the present study, the Cu stressor was more severe and potentially elicited a response similar to Cd, suggesting that

molting is an approach to mitigate it quickly and effectively. Other studies show downregulation of chitinase genes in response to Cu exposure (David et al., 2011; Hook et al., 2014), therefore this study presents a unique perspective on the proposed effect of Cu in regards to growth and reproduction. We suggest that for the concentration of Cu and primiparous state of animals in this study, upregulation of exoskeletal genes may be advantageous for *Daphnia* and appears to be a means to eliminate excess Cu.

Hemoglobin production

In our study we observe a decrease in expression of a gene involved in oxygen binding, transport, and hemoglobin complex, as well as heme-binding. However we see upregulation in other heme-binding protein encoding genes, some of which have other corresponding GO terms such as response to oxidative stress. The gene involved in oxygen binding and transport as well as the hemoglobin complex was significantly downregulated when grouping all clones together, and in the sensitive D clone alone, but not in the other clones separately. Downregulation of these oxygen and heme-binding genes would suggest the decrease in production of hemoglobin (Hb) and thus oxygen transport, which results in respiratory impairment of the organism. It is possible Cu interrupts metabolic processes that regulate oxygen concentration (Untersteiner et al., 2003) as Hb production is regulated by oxygen availability. We suggest that the downregulation of *Hb* genes and potential impairment of oxygen binding and transportation is an interesting future target of study in the toxicity of Cu in *Daphnia*, especially in light of the fact that Cu is essential for Hb synthesis.

The effects of Cu on hemoglobin production in *Daphnia* are not well understood. Dave (1984) observed a complex dose-response relationship between Cu concentration and Hb

production in *D. magna* wherein Hb was decreased at low and high concentrations but increased at intermediate concentrations. *D. magna* exposed to other metals such as zinc (Zn) and Cd showed a slight decrease in Hb concentration (Berglind et al., 1986) and *Hb* genes were downregulated in *D. pulex* exposed to Cd (Shaw et al., 2007). However in other cases Zn did not have any effect on Hb production in *D. magna* (Pane et al., 2003). *Daphnia* studies are difficult to compare to studies in other crustaceans because the latter use a Cu based oxygen transporting enzyme, hemocyanin. However, in the present study, downregulation of some genes involved in heme-binding occurred, while others were upregulated. This could suggest a trade-off between heme-binding proteins potentially similar to the metal sensing ferric uptake regulator protein Fur which controls expression of genes involved in iron transport (Lee and Helmann, 2007). Future studies should seek to understand this complex relationship in the context of Cu toxicity.

CANDIDATE GENES FOR CU TOLERANCE

We recognize all clonal lineages used in this study regulate broad biological pathways responsive to Cu toxicity in similar ways. That being said, this may be expected in organisms of the same species, and differences in gene expression overall may be more nuanced with only large differences in expression of major genes. Thus now consider candidate genes known to respond to metal stress.

Patterns of Metallothionein Expression

One major mechanism of Cu tolerance in invertebrates is the induction of *metallothionein* genes (Janssens, Roelofs and Van Straalen, 2009). Metallothioneins (MTs) are a class of proteins that bind specifically to metal ions to regulate intracellular concentrations of essential heavy metals, including Cu, to regulate Zn-regulated proteins, and to mitigate the effects of free

radicals. These proteins are also able to protect the cell from heavy metal toxicity, possibly by binding these extra metal ions and chaperoning them to be incorporated in metalloproteins that require Cu ions to perform essential cellular tasks (Udom and Brady 1980, Cano-Gauci and Sarkar, 1996, Maret et al., 1997). Incorporation into metalloproteins is a reversible process that is assisted by glutathione (Maret, 1994, Jiang et al., 1998). *Mt* genes are found in all eukaryotes and some prokaryotes, often in multiple copies (Palmiter, 1998), which may allow for *mt* specialization for a particular heavy metal. *D. pulex* has 5 total *mt* genes, as *mt*1 is duplicated. *Mt*2 and *mt*4 are known to respond to Cu exposure (Asselman et al., 2013) while *mt*1 and *mt*3 are known to respond to Cd exposure (Shaw et al., 2007).

The patterns of *mt* expression we observed do not align with previous results; *mt2* and *mt4* were either expressed at the same level in the treatment and control or slightly downregulated after Cu exposure. In contrast, *mt*1a and *mt*1*b* were the only *mt* genes to be significantly upregulated (*mt1a* only when grouping all clones together, *mt1b* only in the D clone individually). Since Cd is a more toxic stressor than Cu, it may be possible that Cu becomes as potent a stressor as Cd at a certain concentration, thus inducing expression of *mt1*. Asselman et al. (2013) found that chronic EC50 Cd exposure caused upregulation of *mt1* (day 4) followed by repression of that *mt* while the other Cd-responsive *mt4* was upregulated later in the exposure period (day 8). This suggests different *mt* homologs may not respond only to specific metals, but are more dependent on exposure duration as well as concentration.

There are several viable hypotheses that help explain the dynamic expression patterns of the *D. pulex mt* genes. These include a time-response relationship between exposure and gene expression (as shown in Asselman et al., 2013), a dose-response relationship (Onosaka and Cherian, 1981, reviewed in Amiard et al., 2006), or an acclimation-response, which can occur

over a few hours to generations (Muyssen and Janssen, 2004). Furthermore, the mechanisms of regulation of the different homologs of *mt*s are not well understood. Current proposed mechanisms include 1) differences in promoter regions (Asselman et al., 2013) 2) an inhibition-induction mechanism (Asselman et al., 2012) 3) metal chelation (Amiard et al., 2006) and 4) ecdysis mediated metal elimination (Riddell et al., 2005), which we will discuss briefly below.

The regulation of the different *mt* homologs likely involves a complex interaction between promoter regions, which can differ in number, location and sequence of metal response elements (MREs) (Asselman et al., 2013). According to the inhibition-induction mechanism, the homolog most able to combat the stressor will be expressed while the others will be repressed (Asselman et al., 2013). This is seen in other types of stress responses such as heat shock proteins (Franzellitti and Fabbri, 2005), the response elements of which are also found in the promoter region of the *D. pulex mt*s. Chelation of extraneous metal ions can occur after MT proteins are synthesized, thus continual transcription of the *mt* genes becomes unnecessary over time (Amiard et al., 2006).

Ecdysis or molting has been shown to remove metals from the body in *Daphnia* (Muyssen and Janssen, 2002; Riddell et al., 2005). Cu-specific *mt*s have been linked to molting metabolism and changes in metal localization in other species (Engel and Brouwer, 1987, and 1991) suggesting that life stage plays an important role in metal detoxification. Additionally, ecdysone-responsive elements are found only in the promoter region of *mt1a/b* (Asselman et al., 2013). This is an interesting link between significant upregulation of *mt1* in our data set and the primiparous life stage at which the *Daphnia* were exposed.

A highly conserved pathway from insects to mammals for *mt* activation is through Metal Transcription Factor 1 (MTF-1), which binds to MREs within the promoter region of the *mt*

gene. In this context, we are not able to explain why *mt1a* and *mt1b* were activated as the other *mt*s have more MREs and in similar positions (Asselman et al., 2013). The mechanism of activation may involve other factors such as position in relation to binding sites for other transcription factors (Hockner, Dallinger and Sturzenbaum, 2015).

As Asselman et al. (2013) suggest, cyclical-, time- and dose-dependent expression patterns between homologs highlight the need for temporal comparisons of induction between all the *mt* genes. Understanding the mechanism of activation of the *mt* genes in *D. pulex* can help understand changes in *mt* expression in response to different metal stressors. The question still remains as to why *mt*s are differentially expressed over time and dose; further studies looking into the amount of bound and unbound metal ions found in the organism as well as cellular functions to actively eliminate the metal all together may lead to further insights into the mechanisms underlying detoxification of metals by metallothioneins.

Patterns of Glutathione-S-transferase Expression

The role of glutathione-S-transferase (GST) is to catalyse the conjugation of glutathione (GSH) with toxic substrates caused by oxidative stress such as reactive oxygen species (Lee et al., 2008). GST is used as a biomarker because it plays an important role in the survival of an organism when exposed to toxins (Cunha et al., 2007). GSH also plays a role in cellular distribution of Cu (Steinebach and Wolterbeek, 1994), possibly aiding in the formation of metalloproteins (Jiang et al., 1998). The *gst* gene (DAPPUDRAFT_210571) was found to be significantly upregulated when grouping all clones together and individually in the D and K clone, but not in the S clone, although it was upregulated, but by 1.5-2 fold less. This could suggest that the S clone, a sensitive clone, does not detoxify Cu and mitigate oxidative stress as well as the other clones. Metalloproteins including two genes encoding metallocarboxypeptidase

(DAPPUDRAFT_117945 and DAPPUDRAFT_195011) were significantly upregulated when all clones were grouped together. Metallocarboxypeptidases have a wide range of biological actions and function by catalyzing the cleavage of peptides at the C-terminus (Alonso-del-Rivero et al., 2009). Again, expression of these genes was slightly lower after Cu exposure in the S clone compared to the other clones. Given the role of GST in metalloprotein synthesis, this supports the idea that Cu ions are being incorporated into metalloenzymes at a higher rate, and more so in the D and K clones compared to the S clone. This could suggest that the S clone is not as efficient at sequestering the metal ions, which can free up MTs again, and thus detoxify the cell. Taken together, lower expression of *gst* and genes encoding metallocarboxypeptidases suggests that the ability to mitigate oxidative stress and detoxify the cell is likely a key pathway involved in Cu tolerance. However it must also be recognized that Cu tolerance is likely a complex interaction between many defence mechanisms.

In a study comparing sensitive and tolerant plants exposed to Cu, sensitive plants displayed depletion of GST caused by the synthesis of the metal-binding protein, phytochelatin (De Vos et al., 1992), the production of which has been linked to increased Cu sensitivity (Hartley-Whitaker et al., 2001). Phytochelatins have been found in some invertebrates but not in *Daphnia* as of yet (Janssens et al., 2009). Future work should seek to identify possible phytochelatins in *Daphnia* and to understand their role in Cu sensitivity through oxidative stress.

CO-EXPRESSION ANALYSIS

Co-expression of genes in enriched Gene Ontology categories in the M1 module such as chitin binding and metabolic processess, digestive enzymes and peptidase activity (serine-type endopeptidase activity, metallocarboxypeptidase activity), suggests that responses involving

exoskeleton proteins and digestion are similar. In fact, the peritrophic membrane (PM) in arthropod guts is made of chitin and is an important structural and immunological defence against ingested pathogens (Dinglasan et al., 2009). The thickness of the PM can be regulated by chitinases to create a structural barrier for parasites or other molecules (Filho et al., 2002). Since we know that there are gut-specific chitinases in other arthropods (Kramer et al., 1993; Shen and Jacobs-Lorena 1997; Arakane et al., 2005; Dinglasan et al., 2009), it would be interesting to know if *D. pulex* is expressing a specific chitinase in response to Cu exposure. As noted by Beckerman et al. (2013), it may be developmentally advantageous to develop one physiological pathway in response to many types of stressors. It is also interesting to note that other proteins with chitin binding domains are not able to bind to chitin and thus cannot remodel the PM (Shi and Paskewitz 2004; Badariotti et al. 2007; McTaggart et al., 2009). Their function is yet to be determined but it is thought that they are predominantly active in the gut and are involved in an immune-related response (Siva-Jothy et al., 2005).

Co-expression of downregulated genes enriched in the M4 module such as structural constituent of cuticle, carbonate dehydratase activity, and collagen trimer support the hypothesis suggested by Engel and Brouwer (1987) that metallothioneins, ecdysteroids and metallo-enzymes (including carbonate dehydrogenase, also known as carbonic anhydrase) all interact with each other in the molt cycle of blue crabs (*Callinectes sapidus*). This hypothesis is supported by studies in *Daphnia* (Bodar et al., 1990) and other crustaceans (Abidi et al., 2016). For example, metals such as Cu and Cd are known to disrupt carbonate dehydrogenase enzymatic activity in crustaceans as well as *in vitro* erythrocyte bioassays (Vitale et al., 1999; Lionetto et al., 2005). Studies have also reported that Cd delays molting in fiddler crabs (Weis, 1976) and increases time between molting in *Daphnia* (Bodar et al., 1990). Genes encoding the

structural constituent of the cuticle are downregulated in this co-expression module with a P value between 0.1 and 0.05, so they were not included in our interpretation of differentially expressed genes ($P<0.05$) in which exoskeletal genes were upregulated. This incongruence requires future work to fully understand how dosage and duration factor into the complex relationship between the expression of exoskeletal genes and Cu exposure.

Other downregulated genes that were enriched in the M4 module were associated with GO annotations for the one-carbon metabolic process, which is the process involved in epigenetic regulation of converting methyl groups from dietary sources to methyl donors that cause DNA methylation (Anderson et al., 2012). One carbon metabolism is known to be a major donor for the methyl groups used for methylation, most notably DNA methylation (Kruman and Fowler, 2014). Downregulation of genes involved in methylation activity suggests epigenetic modifications were beginning to take place during Cu exposure. Changes in methylation patterns have been seen in *D. magna* when exposed to Zn, however Cd did not result in any changes (Vandegehuchte et al., 2009a and b). Two DNA methylases have been identified in the *D. pulex* genome (Colbourne et al., 2011), and methylation was recently shown to occur in this species (Strepetkaite et al., 2015). However, there have been no studies of methylation in response to any metals in *D. pulex* despite keen interest. Although epigenetic regulation of gene expression in response to stressors and toxins has been studied widely in mammals, there has been little work done to understand these processes in *Daphnia* (Asselman et al., 2015). Taken together, previous research and the downregulation of genes involved in the one-carbon metabolic process in this study suggest that epigenetic modifications should be taken into account in future ecotoxicogenomics studies (Vandegehcuchte and Jansen, 2011)

INTERCLONAL VARIATION

Comparing gene expression profiles among *Daphnia* clones using whole transcriptome sequencing has not previously been done; other studies looked at candidate genes (Haap and Kohler, 2009; Haap, Schwarz and Kohler, 2016) and at methylation patterns (Asselman et al., 2015). In fact, interclonal differences in toxicology have not been well studied in *Daphnia* despite the implications for risk assessment (Baird and Barata, 1998). The fact that all three *Daphnia* clones in our study, with different sensitivity to Cu, show largely similar patterns of gene expression (upregulation of genes involved in digestion and molting, oxidative stress response and metal binding; downregulation of certain immune and oxygen transport genes) shows that the major stress pathways respond similarly in all clones. Genes with highest log fold change with the smallest P values (Table 1) were also some of the genes that were differentially expressed in all cases (Table 3), including carbohydrate binding genes involved in an immune response (downregulated), and peptidase genes involved in digestion (upregulated). These results suggest that Cu affects the clones in the same way, as might be expected for lineages of the same species. However, differences in expression levels of certain genes may be very important overall if they have central roles in tolerating Cu stress. An example of this is the *gst* gene, discussed previously, that was upregulated in all clones, but by about half as much in the S clone, which shows high sensitivity to Cu. The enzyme encoded by this gene has been shown to play a pivotal role in the ability of the organism to detoxify Cu (Hossain et al., 2012). It is interesting that *gst* is often used as a biomarker for many toxins that are known to cause oxidative stress (Cunha et al., 2007). However, it would not have been detected as differentially expressed in one of our sensitive clones. This raises the need to both further investigate the role of *gst* expression in Cu toxicity in *Daphnia*, as well as understand how it relates to Cu sensitivity

in different *Daphnia* clones.

The genes that were only differentially expressed in one clone could provide insight into how these clones respond differently to acute Cu stress. The only two genes uniquely DE to the most resistant K clone do not have any known function (Table 2a); the closest Blastp hit in *D.magna* also has no known function and no predicted protein signatures were found using InterPro Scan. There were also six uniquely DE genes in clone S that do not have a known function (Table 2b). Other uniquely DE genes in the S clone show downregulation of one gene encoding the digestive enzyme, serine peptidase and upregulation of one gene encoding another digestive enzyme, endopeptidase. The six other unique DE genes in the S clone showed downregulation of structural constituents of the cuticle. This contradicts the trend observed when all clones are grouped together, and in the D and K clone alone. This sharp downregulation in the S clone under Cu exposure could potentially be due to differences in basal gene expression between clones. The D clone was the only comparison to show significant upregulation of the *mt*1a gene after Cu exposure (Table 2c). This could suggest that different clones are prioritizing the expression of one copy of the gene. It could also be due to mapping of the reads to one of the closely related duplicated genes more than the other, as the genes share 99% cDNA similarity in the reference genome. Only a hemoglobin complex gene was significantly downregulated in the D clone, suggesting that oxygen transport was significantly impaired in the D clone and not in the others. An ATPase activity gene was also only significantly downregulated in the D clone, ATPase activity is known to be a mechanism of transporting Cu ions out of cells and organelles in mammals (Dameron and Harrison, 1998). This could suggest that the D clone is not able to pump Cu ions out of the cell as effectively as the other clones.

It should be noted that gene expression is a first measure to estimate final gene product

expression. There are many factors that contribute to the differences between mRNA expression and protein abundance, including the relative stability of mRNA and protein molecules. While some studies find upregulation of gene expression and abundance of proteins to be well correlated, the same trend has not been observed with downregulation of gene expression (Vogel and Marcotte, 2012). In fact protein to mRNA ratios differ by organism and study (de Sousa Abreu et al., 2009), and has proven to be a controversial topic (Conesa et al., 2016). While there is still much to understand, the use of Next Generation Sequencing coupled with more advanced methods of quantifying protein abundance is an important advancement for applications such as novel isoform detection (Conesa et al., 2016) and understanding the relationship between mRNA and protein stability with protein function, particularly environmentally relevant ones (Evans, 2015).

Taken together, these unique significantly DE genes between different clonal lineages suggest that there are differences in the way each clone responds to Cu stress. De Coninck and colleagues (2013) caution against using single clonal lineages to extrapolate findings to an entire species. Different lineage sensitivities to Cu stress (and other metals) can have important population level implications (Venancio et al., 2016). Although there has been research on phenotypic and fitness differences between clonal lineages to metal stress, to our knowledge there have been no large scale gene expression studies in *D. pulex* than investigate differences between clonal lineages. The challenges of interpreting a large transcriptome dataset of over 17,000 expressed genes include identifying specific genes with major roles in Cu toxicity without a priori knowledge. It may be difficult to discern which differences in expression are biologically consequential. There is a need for more work to be done to understand the nuances in gene expression patterns between clonal lineages in *Daphnia*.

CONCLUSIONS

We have identified over 200 genes whose expression responds to acute Cu stress in *D. pulex.* They can now be studied in follow-up assays that would enhance our knowledge of their involvement in toxic Cu response in this species. Although major proposed pathways involved in toxicity were shared by all clonal lineages, our comparison of gene expression between clones shows that genetic background does influence the expression patterns of genes responsive to acute Cu stress. Regulation of key genes, such as *gst*, differed between clones cautioning the use of one lineage to draw conclusions for a whole population or species. This illustrates the need for future studies to incorporate more clonal lineages to further understand the breadth of variation in gene expression.

The results of our study have important implications for ecotoxicogenomics, specifically *Daphnia*'s important role as a widely used ecotoxicogenomic model. We showed that previously proposed toxic effects of Cu exposure, including suppression of the immune system and oxidative stress are important in *D. pulex*. Other proposed toxic effects such as disruption of molting and digestion were not supported by our study. These differences raise interesting questions about the use of different dose, duration and mode of exposure between studies. Each study is only a 'snap shot' of the gene expression for those given conditions. Future studies should incorporate gene expression levels monitored from acute to chronic timeframes over varying dosages.

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Table 1: Eleven differentially expressed genes with large log fold change (log |FC| >4) and significant (p< 0.01) in *Daphnia pulex* clones exposed to copper. Columns indicate EnsemblMetazoa gene ID, log fold change for all clones grouped together, P value for analysis using DESeq and EdgeR, functional annotations based on GO, InterPro Scan (predicted function; integrated signatures) and BLASTp (for those that did not have any GO annotation of result in InterPro scan).

Gene ID	All clones grouped			GO annotation	InterPro scan	NCBI BLASTp
	Log FC	DESeq	EdgeR			
225009	-4.74	6.80E-30	7.20E-31	Carbohydrate binding	C-type lectin	
104167	-5.775	9.40E-27	3.90E-36	Carbohydrate binding	C-type lectin	
319341	-5.918	1.50E-05	3.10E-21	Carbohydrate binding	C-type lectin	
109980	-4.826	0.00094	1.00E-12	Protein binding	Caprin, C1q domain, Tumour necrosis factor-like domain	
101472	6.517	9.60E-13	1.20E-15	Growth factor activity; Positive regulation of cell division; Membrane	Cystine-knot cytokine, PDGF/VEGF domain	
247441	4.04	8.10E-05	0.00011	G-protein coupled receptor signaling pathway; G-protein coupled receptor activity; Integral component of membrane	G protein-coupled receptor (rhodopsin-like), GPCR, rhodopsin-like, 7TM	
222529	6.878	8.00E-06	3.60E-10	NA	Unintegrated signatures (None)	Similar to many *Daphnia pulex* hypothetical proteins, no known function
300382	7.728	0.0066	2.00E-07	NA	Unintegrated signatures (None)	Similar to many *Daphnia pulex* hypothetical protein, no known function
313428	5.001	5.80E-06	1.50E-10	NA	Unintegrated signatures (None)	Chitinase, Putative glycosyl hydrolases, Hypothetical protein

317348	4.731	0.0027	2.20E-08	NA	Unintegrated signatures (None)	Similar to many *Daphnia pulex* hypothetical proteins, no known function
332119	5.33	0.004	0.00017	NA	Retrotransposon gag domain; Unintegrated signatures	Similar to many *Daphnia pulex* hypothetical proteins, no known function

Table 2A: Uniquely differentially expressed genes in each clone of *Daphnia pulex* exposed to copper. The ID is EnsembleMetazoa gene ID, P value is given for both DESeq and EdgeR analyses. If available, the Gene Ontology (GO) annotation and the category of the GO ID are presented. Any predicted functions from InterPro scan are shown. The BLAST results from the Uniprot database as well as BLASTp using the NCBI non-redundant (nr) database are shown. **A:** Unique to clone K, **B:** Unique to clone S, **C:** Unique to clone D.

Gene ID	Log FC	P value DESeq	P value EdgeR	GO ID	Category	InterPro Scan	BLAST-Uniprot	NCBI-BLASTp
Unique K								
242030	1.861	0.021	0.0091	NA	NA	None	Uncharacterized protein, *D. magna*	Uncharacterized protein, *D. magna*
264253	2.289	0.011	0.0014	NA	NA	None	Uncharacterized protein, *D. magna*	Uncharacterized protein, *D. magna*

Table 2B.

Gene ID	Log FC	P value DESeq	P value EdgeR	GO ID	Category	InterPro scan	BLAST-Uniprot	NCBI-BLASTp
Unique S								
224273	-2.93	7.50E-06	3.00E-06	0003824	Catalytic activity, proteolysis, serine-type endopeptidase activity	Peptidase S1	Serine protease, *D. magna*	Hypothetical protein, *D. pulex*/ Uncharacterized protein, *D. magna*
117311	-2.453	0.011	0.0095	NA	NA	None	Uncharacterized protein, *Danio rerio*	Hypothetical protein, *Penicillium digitatum* PHI26
235814	3.121	5.90E-05	0.039	NA	NA	Signature matches: collagen triple helix repeat	Uncharacterized protein, *D. magna*	Uncharacterized protein, *D. magna*
239216	3.658	0.039	0.0065	NA	NA	None	Von Willebrand factor (VWF) type C domain protein, *D. magna*	None
303510	3.528	0.0017	0.00032	NA	NA	None	Uncharacterized protein, *D. magna*	Uncharacterized protein, *D. magna*
307666	4.41	1.80E-09	0.00038	NA	NA	None	Uncharacterized protein, *D. magna*	Uncharacterized protein, *D. magna*
325141	5.745	0.0025	3.60E-07	NA	NA	None	Putative uncharacterized protein,	Hypothetical protein, *D. pulex*

							D. pulex	
43239	4.878	0.05	0.0057	0043086	Regulation and negative regulation of catalytic activity, endopeptidase activity and peptidase activity; Regulation of serine-type endopeptidase inhibitor activity.	Serine-type endopeptidase inhibitor activity	Putative serine protease inhibitor, *D. magna*	Hypothetical protein, *D. pulex*/Putative serine protease inhibitor, *D. magna*
253512	-3.235	6.70E-05	0.00056	0042302	Structural constituent of cuticle	Structural constituent of cuticle	Putative uncharacterized protein, *D. pulex*	Hypothetical protein, *D. pulex*
269506	-3.164	0.0015	0.0038	0042302				
28927	-3.561	0.00018	1.30E-05	0042302				
301390	-3.934	6.70E-06	0.00013	0042302				
307539	-5.119	0.0096	2.60E-05	0042302				
324898	-6.297	7.40E-06	1.00E-11	0042302				

Table 2C.

Gene ID	Log FC	P value DESeq	P value EdgeR	GO ID	Category	InterPro scan	Blast-Uniprot	NCBI-Blastp
Unique D								
302394	-2.71	0.0029	0.0034	0042626	ATPase activity, coupled to transmembrane movement of substances, metabolic process, obsolete ATP catabolic process, transmembrane transport, transport	ATPase activity, coupled to transmembrane movement of substances	Putative uncharacterized protein, *D. pulex*/ V-type proton ATPase subunit D, *D. magna*	Hypothetical protein, *D. pulex*/V-type proton ATPase subunit D, *D. magna*
311662	-2.138	0.00061	0.026	0005576	Extracellular region, heme binding, hemoglobin complex, iron ion binding, oxygen binding, oxygen transport, oxygen transporter activity, transport	Heme binding; Iron ion binding, oxygen binding, oxygen transporter activity	Hemoglobin, *D. pulex*	Hemoglobin, *D. pulex*

290505	4.26	0.0013	0.00033	NA	NA	None	Metallothionein B, *D. magna*/ Metallothionein protein crs5, *D. magna*	Metallothionein B, *D. Magna*/Putative metallothionein 2, *D. pulex*
306152	-2.885	3.60E-05	0.00017	NA	NA	None	Uncharacterized protein, *D. magna*	Salivary gland secretion-like protein, *D. magna*

Table 3: Gene Ontology annotations for genes differentially expressed in all four *Daphnia pulex* clone and group comparisons. Nine differentially expressed ($p<0.05$) genes were found in all comparisons (all clones together and each clone individually). Gene ID indicates EnsembleMetazoa gene ID, GO ID indicates GO annotation ID, GO Description indicates the functional description of the GO ID and logFC D, S and K indicate the log fold change in gene expression for the D, S and K clone, respectively.

Gene ID	GO ID	GO Description	logFC D	logFC S	logFC K
229607	0016998	Cell wall macromolecule catabolic process	3.592	2.89	3.321
	0009253	Peptidoglycan catabolic process			
	0003796	Lysozyme activity			
220200	0004869	Cysteine-type endopeptidase inhibitor activity	4.068	3.942	3.23
	0043086	Negative regulation of catalytic activity			
	0010951	Negative regulation of endopeptidase activity			
	0010466	Negative regulation of peptidase activity			
	0050790	Regulation of catalytic activity			
	0052548	Regulation of endopeptidase activity			
	0052547	Regulation of peptidase activity			
319612	NA	NA	2.817	3.46	4.123
226732	0071918	Urea transmembrane transport	-3.046	-2.491	-3.278
	0015204	Urea transmembrane transporter activity			
	0016021	Integral component of membrane			
104167	0030246	Carbohydrate binding	-5.672	-5.642	-5.976
221339	0030246	Carbohydrate binding	-3.456	-3.482	-3.545
225009	0030246	Carbohydrate binding	-5.378	-4.457	-4.686
319341	0030246	Carbohydrate binding	-5.076	-6.052	-6.153
303054	NA	NA	-3.145	-3.099	-3.053

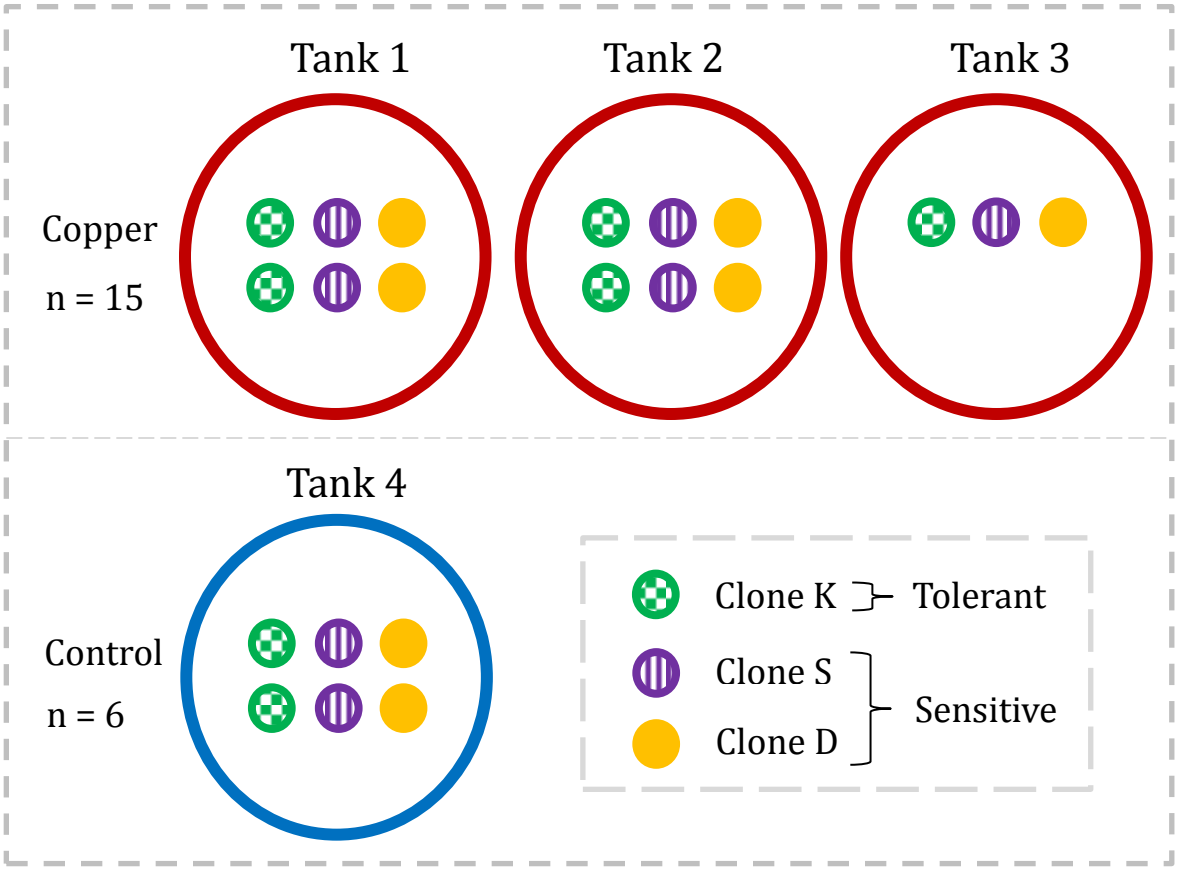
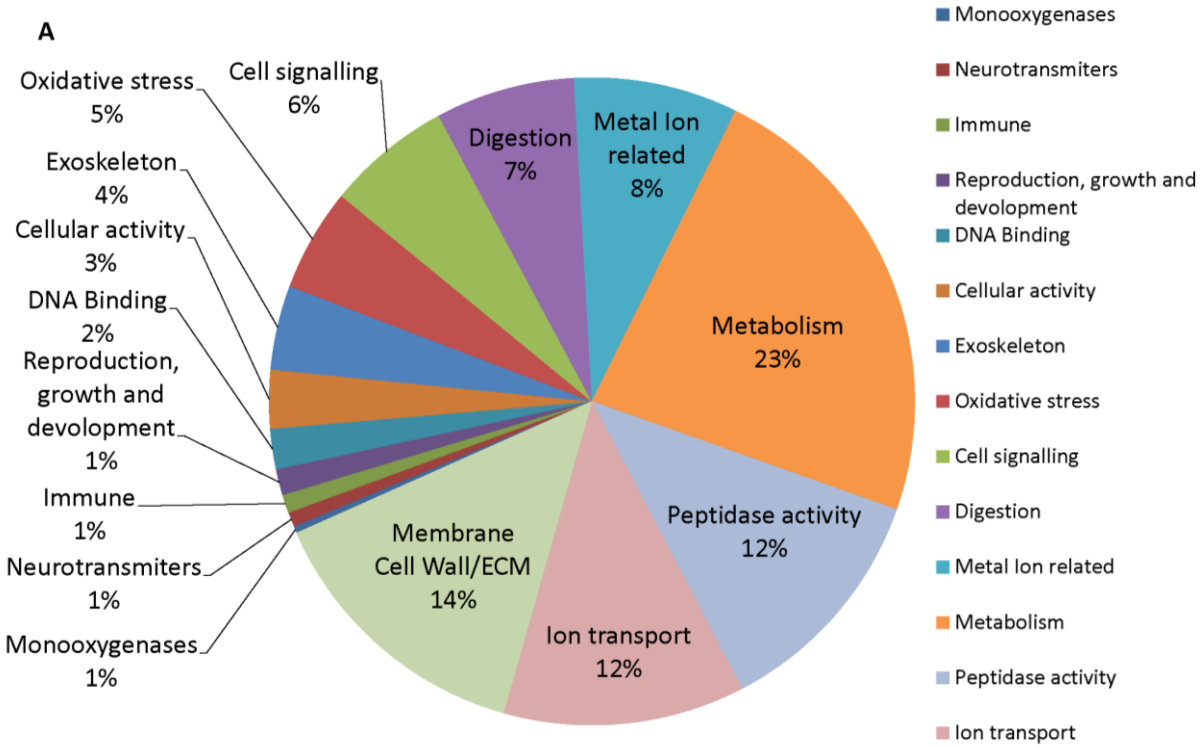


Figure 1: The sequencing sample layout for Illumina 100 bp paired-end RNA sequencing. Differential gene expression analyses in *Daphnia pulex* exposed to copper were done by grouping all copper treatments together compared to all controls together, as well as each clone compared to its respective control separately (four comparisons: all clones, Solomon Pond [clone S], Dump Pond [clone D] and Kelly Pond [clone K]). Differential expression was assessed using both DESeq and EdgeR.


A
Oxidative stress 5%
Cell signalling 6%
Exoskeleton 4%
Cellular activity 3%
DNA Binding 2%
Reproduction, growth and devolopment 1%
Immune 1%
Neurotransmiters 1%
Monooxygenases 1%
Digestion 7%
Metal Ion related 8%
Metabolism 23%
Peptidase activity 12%
Ion transport 12%
Membrane Cell Wall/ECM 14%
Monooxygenases
Neurotransmiters
Immune
Reproduction, growth and devolopment
DNA Binding
Cellular activity
Exoskeleton
Oxidative stress
Cell signalling
Digestion
Metal Ion related
Metabolism
Peptidase activity
Ion transport
Membrane/Cell Wall/ECM

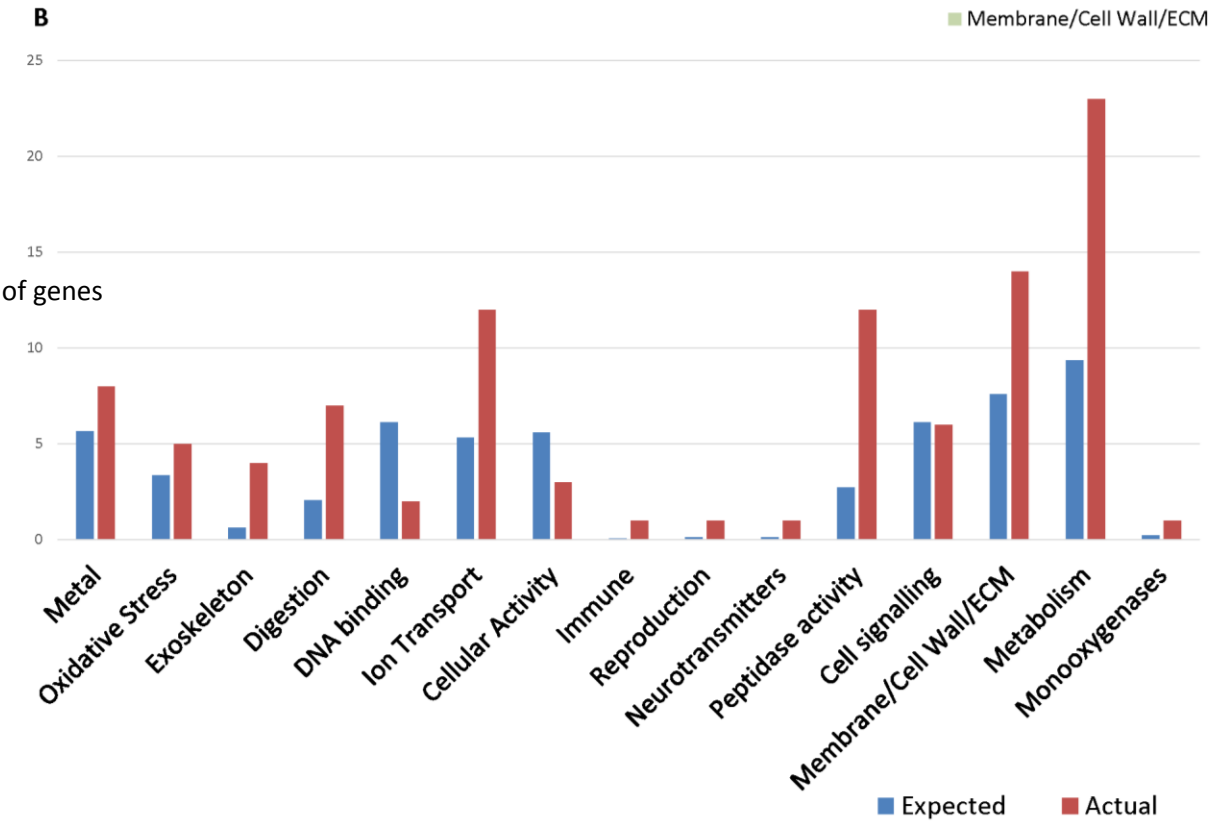

B
Percent of genes
25
20
15
10
5
0
Metal
Oxidative Stress
Exoskeleton
Digestion
DNA binding
Ion Transport
Cellular Activity
Immune
Reproduction
Neurotransmitters
Peptidase activity
Cell signalling
Membrane/Cell Wall/ECM
Metabolism
Monooxygenases
Expected
Actual

Figure 2: A Pie graph of functional annotations for differentially expressed genes (annotated and gene categories).The 207 genes that responded significantly ($p<0.05$) to copper exposure in the "all clones" grouping are included in the graph. Annotations are based on the Gene Ontology annotation in the *Daphnia pulex* genome from wFleabase. **B** The expected proportion of genes was calculated by dividing the number of expressed genes for each category by the total expressed genes. The actual proportion of genes was calculated by dividing the number of differentially expressed (DE) genes in the data set for each category by the total number of DE genes. All gene categories were represented in higher proportions by DE genes than expected based on all the 17,508 expressed genes in the data set, except for DNA binding and cellular activity.

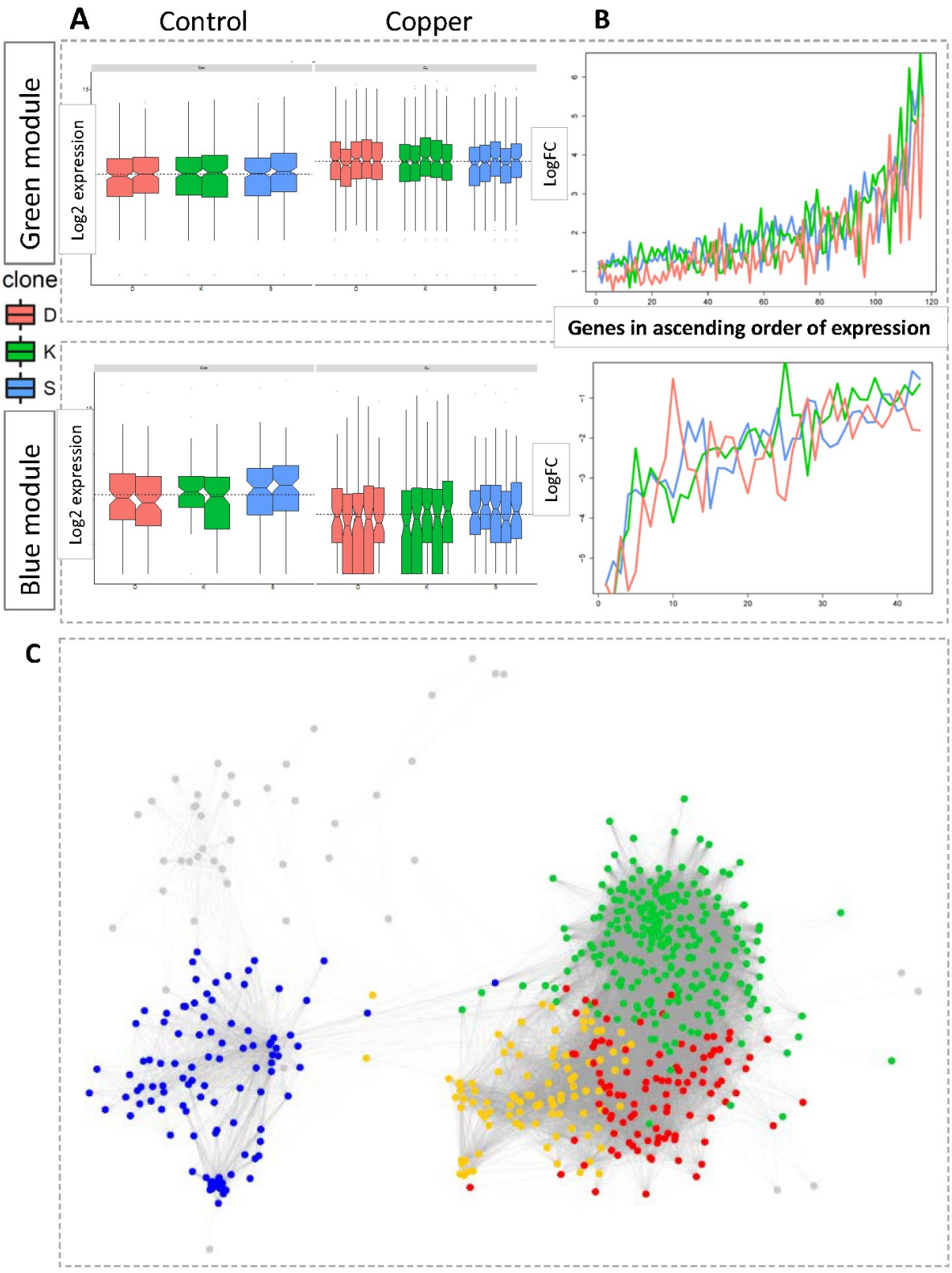

A
Control
Copper
B
Green module
Log2 expression
LogFC
clone
D
K
S
Genes in ascending order of expression
Blue module
Log2 expression
LogFC
C

Figure 3: Patterns of gene expression for each clone grouped by co-expression modules. M1 (green), M2 (yellow) and M3 (red) modules consist of genes generally upregulated in copper-exposed samples compared to control samples. The M4 (blue) and M5 (grey) modules include genes that were downregulated in copper-exposed samples. The M4 (blue) module consists of significantly downregulated genes and the M1 (green) module consists of significantly upregulated genes. **A:** Average expression of genes in the M1 (green) and M4 (blue) modules by replicate and by clone. **B:** Expression in log fold change (logFC) of differentially expressed genes by each clone grouped in the M1 and M4 modules. Genes appear in ascending order of expression. Clones are colour-coded the same as panel A. **C:** Co-expression network diagram made using Weighted Gene Co-expression Network Analysis (WGCNA). The 600 significantly copper-responsive genes in *Daphnia pulex* (P value <0.1) in any comparison in either DESeq or EdgeR are included in the network. Co-expressed genes are grouped into modules 1-5. M1, green; M2, yellow, M3, red, M4, blue and M5, grey.

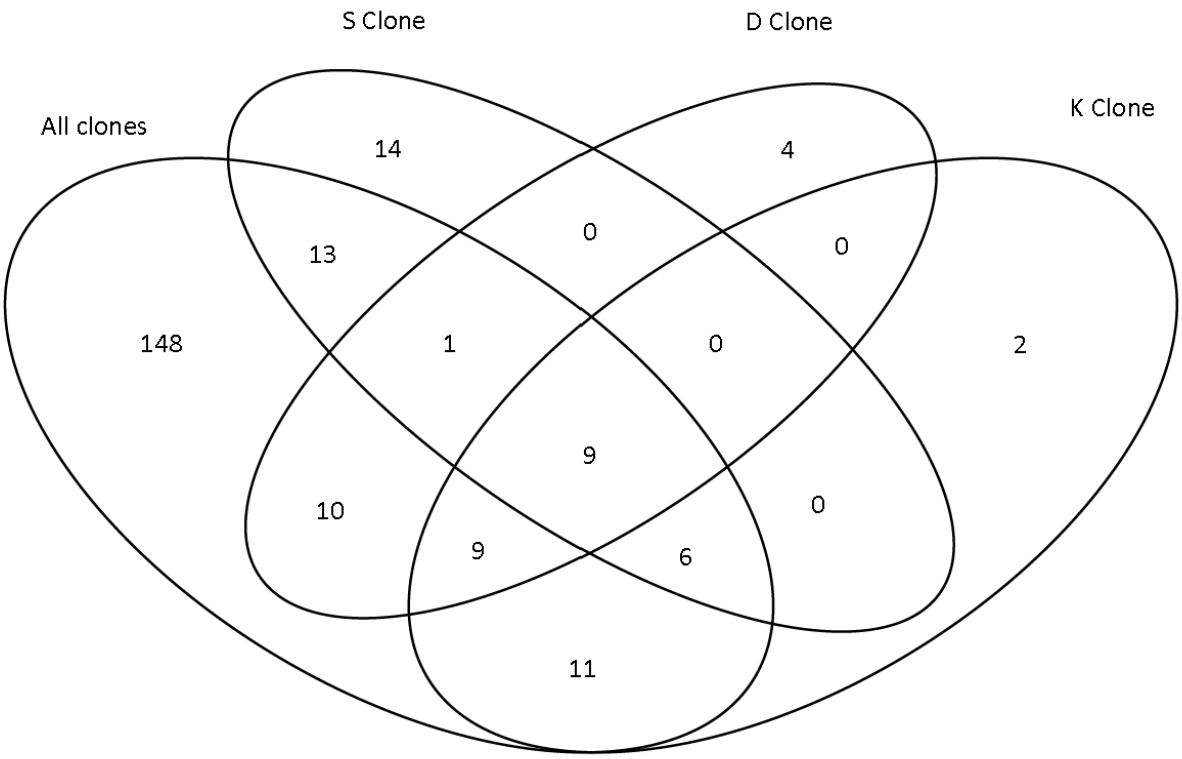


Figure 4: Venn diagram of differentially expressed (DE) genes ($p<0.05$) in *Daphnia pulex* clones exposed to copper. Copper exposed samples were compared to control samples from each lineage individually and then grouping all the clones together for a total of four DE analyses.

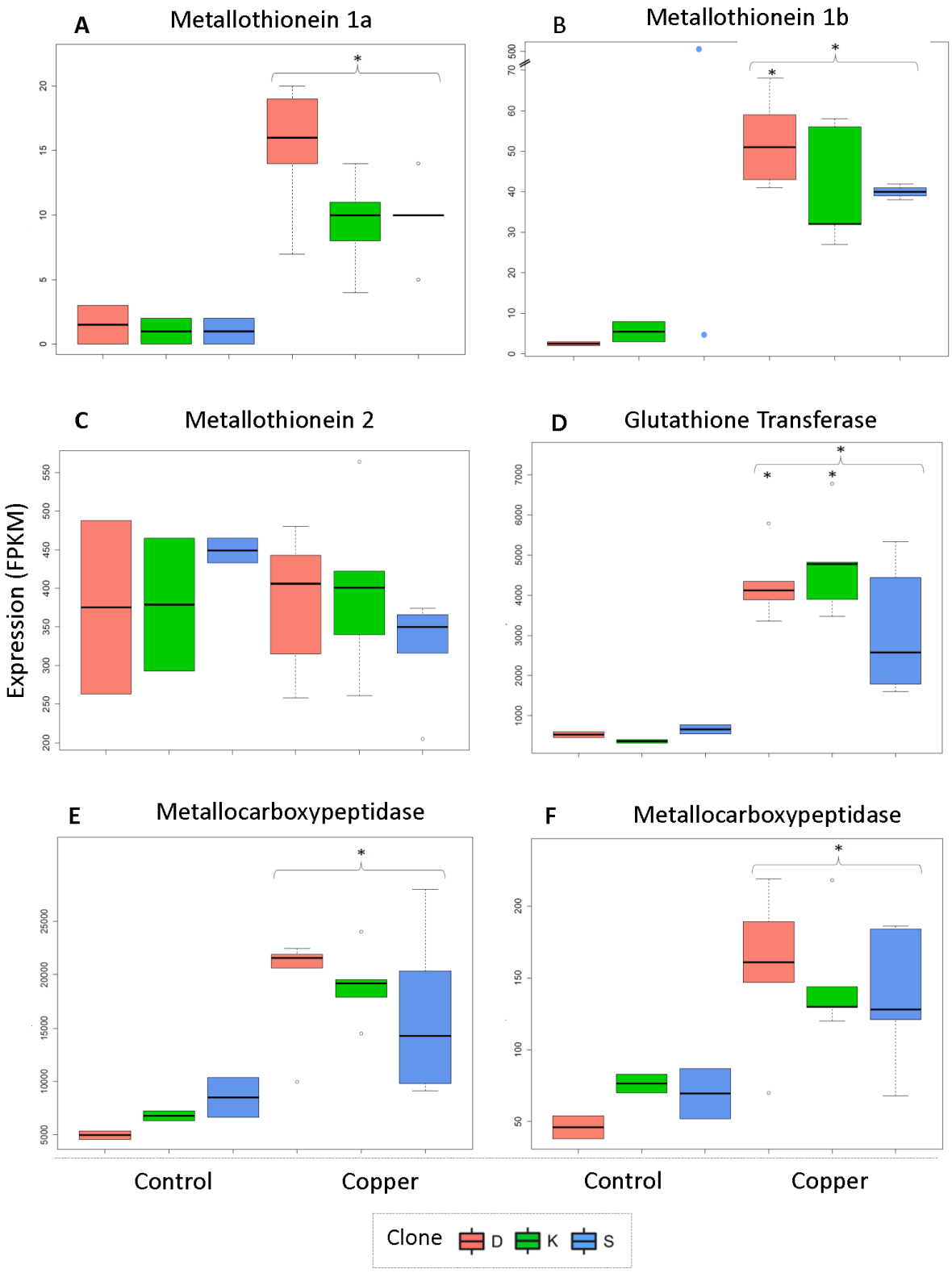

A
Metallothionein 1a
B
Metallothionein 1b
C
Metallothionein 2
D
Glutathione Transferase
E
Metallocarboxypeptidase
F
Metallocarboxypeptidase
Expression (FPKM)
Control
Copper
Control
Copper
Clone
D
K
S

Fig 5: Box plots of expression level for *metallothionein* genes, *glutathione-S-transferase* and *metallocarboxypeptidase*. The y-axis is expression in Fragments Per Kilobase of transcript per million mapped reads (FPKM) for control (n=2) and copper treatment (n=6) samples from each clone. The bold line indicates the median value, box edges are the 25th and 75th percentile, whiskers represent 1.5 x IQR (inter-quartile range). Any points beyond the whiskers are marked as individual points. Significance ($P < 0.05$) is denoted with an asterisk. **A, B:** The two genes encoding metallothionein 1(*mt*1). *Mt*1a is differentially expressed only when all clones are grouped together. *Mt*1b is significant for each clone individually as well as all together. **C:** The copper specific metallothionein (*mt*2) gene does not show differential expression in any case. **D:** The gene encoding glutathione-S-transferase was differentially expressed in the D and K clones and when all clones were grouped together, however it was not differentially expressed in the S clone. **E, F:** The two genes coding for metallocarboxypeptidase (117945 and 195011) were differentially expressed only when grouping all clones together (P.DESeq= 0.027 and 0.00053, respectively).

GENERAL CONCLUSIONS

Modes of action

Proposed toxic effects of Cu, including disruption of digestion, oxidative stress, interruption of exoskeletal functions and immune suppression were investigated and compared to previous studies on species in the genus, *Daphnia*. Digestion was a proposed toxic effect of Cu due to downregulation of digestive enzymes and other behavioural and physiological evidence in *Daphnia* and other crustaceans. However, in our study, many digestive enzymes were upregulated possibly indicating that the mode of exposure (foodborne and waterborne in this study compared to waterborne in previous studies) could help explain differences between studies. Digestion is also linked to growth and reproduction via the energy budget model. Future work should investigate how dose and duration of the stressor affects these energy reserves.

Regulation of exoskeletal proteins is essential for growth and molting in *Daphnia*, and gene expression for this process is known to be downregulated in Cu-exposed *Daphnia*. However, in this study we see upregulation of the genes encoding such proteins, similar to Cd-exposed *Daphnia*. There is much to understand about how the type of metal, life-stage during exposure and severity of stressor affect exoskeletal processes in *Daphnia*.

Immune suppression has been proposed to be a toxic effect of Cu due to immune suppression in other crustaceans and downregulation of immune genes in Cu-exposed *Daphnia*. We saw downregulation of some immune genes while others in the same category were upregulated. The *Daphnia* immune system is not well understood and more work needs to be done to understand why certain immune elements are responsive to Cu stress.

Co-expression analysis

Co-expression analysis identified groups or modules of differentially co-expressed genes.

One module (M1) included upregulated genes involved in exoskeletal protein regulation and digestion. Another module (M4) composed of downregulated genes supported the theory that metalloproteins and molting processes are interconnected. Co-expression analyses could be powerful tools to help understand complex gene networks.

Differences in gene expression between clones

Results from the transcriptome analysis of three distinct genetic lineages of *D. pulex* exposed to acute Cu stress revealed that all clones respond using the same molecular pathways. However, we identified significant differences between lineages in expression of genes encoding proteins that play key roles in the detoxification of Cu.

The metallothionein proteins are known to bind to excess metal ions to aid in the detoxification of the cell. One of the sensitive clones, clone D, was the only lineage to significantly upregulate an *mt* gene, although it was significantly upregulated when all clones were grouped together. The *glutathione-s-transferase* gene, known to provide anti-oxidant relief under heavy metal stress, was also significantly upregulated in this clone. However this lineage was the only one in which the heme-M4binding gene, important for oxygen transport, as well as ATPase activity implicated in pumping Cu ions out of the cell, was significantly downregulated. This could suggest that chelation is prioritized in this clonal lineage to compensate for the reduced ability to remove Cu from the cell.

Cu exposure is known to cause oxidative stress and genes encoding antioxidants are known to be upregulated in *Daphnia* exposed to Cu and other heavy metals. We see the same pattern in this study when all clones were grouped together. However, in the other sensitive lineage, clone S, the antioxidant *glutathione-s-transferase* gene was not significantly upregulated. Another metal binding protein, phytochelatin, and its interaction with glutathione-S-

transferase to confer Cu tolerance or sensitivity has been shown in other species and would be an interesting aspect to investigate in *Daphnia* in the future. The S clone also showed lower expression of the gene encoding the metalloenzyme, metallocarboxypeptidase. These two lines of evidence suggest that the S clone is less efficient at detoxifying Cu ions through antioxidant pathways. The S clone is also the only clone in which expression of genes encoding certain exoskeletal proteins and some digestive enzymes was significantly downregulated. Downregulation of genes in these categories could lead to impaired ability to reduce Cu body load through molting, as well as reduced ability to create energy from food, which is needed to fight the toxic effects of Cu.

The *metallothionein* gene was not significantly upregulated in the most resistant clone, clone K, but *glutathione transferase* was. The only genes that were found to be significantly differentially expressed only in the K clone currently do not have any known function. Taken together, our results suggest that sensitivity to Cu is controlled by many genes and that clones with different Cu tolerance regulate certain genes responsive to Cu stress differently from each other. Consequently, genetic diversity between *Daphnia* lineages should be taken into account in future ecotoxicogenomic studies.

Significance and Future Directions

This study primarily highlights the need to better understand the role of genetic background in patterns of gene expression. We have ample evidence in terms of physiology and fitness traits that large differences exist between clonal lineages of *Daphnia*, and thus, it is essential in the genomic era to understand these differences at the genetic level. Future work in ecotoxicology should include genetic variation as ecotoxicogenomic applications are far-reaching.

Using a non-targeted approach such as RNA-sequencing, we are able to obtain much more information than using a targeted approach based on candidate genes. This allows us to expand the library of responsive genes, some of which do not currently have a known function, and identify them for future investigation. We argue that RNA sequencing is an important aspect of this study as it offers many advantages over microarrays, such as better ability to make comparisons between studies, exceptional depth of sequencing for detection of genes expressed at low levels, and broad dynamic range to quantify expression. Future work should incorporate the use of genomic technologies with other physiological assays to create direct links between toxic mode of action and phenotypic outcomes, which can then be used to establish environmental regulations.

Lastly, *Daphnia* is an important species in ecotoxicology for many reasons including its ecological significance, decades of studies covering many fields, a reference genome, and clonal reproduction. Ecotoxicogenomic approaches can expose adverse gene expression consequences and thus prevent damage to sensitive species before they become visible in traditional ecotoxicological endpoints like growth and reproduction. Untangling the genetic basis of toxicity and tolerance to environmental contaminants is the key to ultimately preventing lasting damage to aquatic ecosystems.

APPENDICES

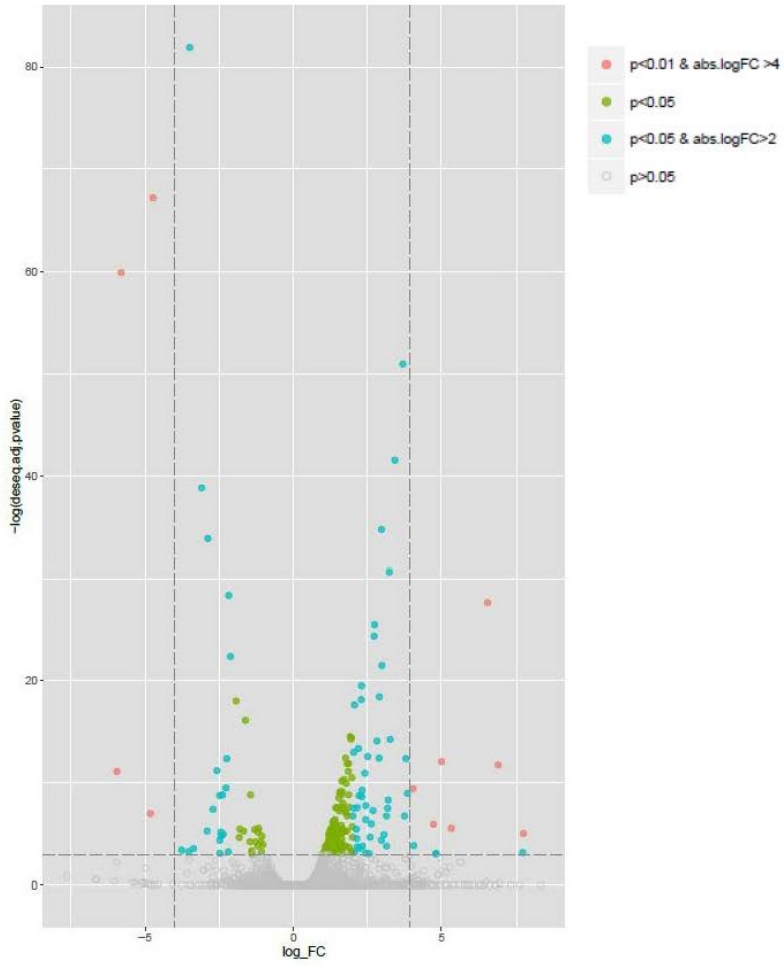


Figure S1: The expression in log fold change plotted against the negative log of the DESeq adjusted P value obtained from the differential expression (DE) analysis when all clones were considered together (n= 15) against all control samples (n=6) for all 17220 expressed genes. Thresholds include non-significantly DE genes with $p>0.05$ (grey), genes with $p<0.05$ (green), genes with $p<0.05$ and an absolute log fold change of greater than 2 (blue), and genes with a $p<0.01$ as well as an absolute log fold change of greater than 4 (pink). There are 11 pink highlighted genes that correspond to the genes in Table 1 in the main text.

Table S1: The Lethal Concentration, where 50% mortality occurs (LC50), to copper exposure in *Daphnia pulex* lineages from different habitats that were included in the 24 hr acute copper exposure experiment for subsequent gene expression analysis. LC50 was calculated using the *dose.p* function in the "MASS" package for R.

Habitat	LC50	SE	Classification	Location
Dump Pond 4	153.83	0.03017	Sensitive	Illinois, USA
Solomon Pond 7	169.98	0.005453	Sensitive	Michigan, USA
Kelly Lake 12	181.04	0.002298	Resistant	Sudbury, Canada

Table S2: Comparisons of LC50 values between genotypes of *Daphnia pulex* using the ratio test. Ratio estimate is defined by $LC50^a/LC50^b$ where *a* and *b* are two independent populations. The 95% Confidence interval was calculated for the ratio. If the 95% Confidence Interval does not contain 1, the null hypothesis (H_0; that the LC50 values of both populations are the same) would be rejected. All comparisons showed that the populations have significantly different LC50 values. The ratio test was calculated with the *comped* function for the "drc" package for R.

Comparison	**Ratio estimate**	**95% Confidence Interval**
Clone D with Clone S	0.9050	(0.8887-0.9212)
Clone D with Clone K	0.8497	(0.8494-0.85)
Clone S with Clone K	0.9389	(0.9387-0.9391)

Table S3: pH measurements taken from tanks used in *Daphnia pulex* 24 hour acute Cu exposures. pH was measured with a pH sensor while 100 ml sample was stirring continuously for six minutes to allow pH to stabilize. pH measurements of control and Cu spiked FLAMES without algae as well as Deionized (DI) water were taken at the same time.

Label	**Treatment**	**pH**
Tank A2	Control	5.97
Tank C3	Cu	5.83
Tank D2	Cu	6.00
Tank D3	Cu	5.72
Cu FLAMES	Cu	5.22
Control FLAMES	Control	5.97
DI Water	N/A	5.27

Table S4: Candidate and known responsive genes to metal stress in *Daphnia*. EC50 – concentration at which reproduction declines by 50%. LC50 - concentration at which 50% mortality occurs. NOEC- No Observed Effect Concentration – the highest concentration that does not cause a significant ($p<0.05$) reduction in survival after a 24 hr acute exposure. *Range of concentrations included 6.13, 20.24 and 36.79 µg/L Cd; relative LC50 value was not specified. See referenced study for additional concentrations.

Species	Metal	Concentration	Duration	Gene	Category	Direction	Life stage	Reference
Metal Binding and Transport								
D. magna	Cu	1/10 EC50	24 hrs	Metallothionein	Metal binding and transport	Up	Adult	Poynton et al., 2007
D. magna	Cu	1/10 EC50	24 hrs	Ferritin	Metal binding and transport	Up	Adult	Poynton et al., 2007
D. pulex	Cd	LC01	48 hrs	2-Domain hemoglobin protein subunit, Hemoglobin 1 and 2	Metal binding and transport	Up	Adults and neonates	Shaw et al., 2007
D. pulex	Cd	LC01	48 hrs	Metallothionein 1	Metal binding and transport	Up	Adults and neonates	Shaw et al., 2007
D. magna	Cu	1/10 EC50	24 hrs	No homology (MT)	Metal binding and transport	Up	Adult	Poynton et al., 2008a
D. magna	Cu	1/10 LC50	24 hrs	Ferritin, No homology (MT)	Metal binding and transport	Up	Adult	Poynton et al., 2008a
D. magna	Cu	NOEC	24 hrs	Heme binding protein, No homology (MT)	Metal binding and transport	Up	Adult	Poynton et al., 2008a
D. magna	Cu	1/10 LC50, NOEC	24 hrs	Metallothionein a	Metal binding and transport	Up	Adult	Poynton et al., 2008b
D. pulex	Cu	EC50	Chronic (8 days)	Metallothionein 1	Metal binding and transport	Down	Neonate	Asselman et al., 2013

D. pulex	Cu	EC50	Chronic (2 days and 8 days)	Metallothionein 2	Metal binding and transport	Up	Neonate	Asselman et al., 2013
D. pulex	Cu	EC50	Chronic (16 days)	Metallothionein 3	Metal binding and transport	Down	Neonate	Asselman et al., 2013
D. pulex	Cu	EC50	Chronic (2 days and 8 days)	Metallothionein 4	Metal binding and transport	Up	Neonate	Asselman et al., 2013
Digestion and nutrient absorption								
D. magna	Cu	1/10 EC50	24 hrs	Cellulase, Glucanase, Endo-mannanase, Amylase, Cubilin	Digestion and nutrient absorption	Up	Adult	Poynton et al., 2007
D. pulex	Cd	LC01	48 hrs	Endo-1,4-mannanase	Digestion and nutrient absorption	Down	Adults and neonates	Shaw et al., 2007
D. magna	Cu	1/20 EC50, 1/10 EC50, 1/10 LC50, NOEC	24 hrs	Cellulase, Amylase, Endo-mannanase, Hydrolase, Endo-glucanase, Cellobiohydrolase, Cubilin	Digestion and nutrient absorption	Down	Adult	Poynton et al., 2008a
D. magna	Cu	1/10 LC50, NOEC	24 hrs	Cellulase	Digestion and nutrient absorption	Down	Adult	Poynton et al., 2008b
D. magna	Cu	NOEC	24 hrs	Preamylase	Digestion and nutrient absorption	Down	Adult	Poynton et al., 2008b
Exoskeleton related proteins								
D. magna	Cu	1/10 EC50	24 hrs	Chitinase, Chitinase binding and metabolism	Exoskeleton related proteins	Up	Adult	Poynton et al., 2007

D. magna	Cu	1/10 EC50	24 hrs	Chitinase	Exoskeleton related proteins	Down	Adult	Poynton et al., 2007
D. pulex	Cd	LC01	48 hrs	Chitinase, Chitotriosidase, Cuticle proteins, Chitinase-like proteins, Gasp precursor, Chondroitinlycan	Exoskeleton related proteins	Up	Adults and neonates	Shaw et al., 2007
D. magna	Cu	1/10 EC50	24 hrs	Cuticle protein, Cuticle	Exoskeleton related proteins	Up	Adult	Poynton et al., 2008a
D. magna	Cu	1/10 LC50	24 hrs	Chitin binding	Exoskeleton related proteins	Down	Adult	Poynton et al., 2008a
D. magna	Cu	1/10 EC50	24 hrs	Chitinase	Exoskeleton related proteins	Up	Adult	Poynton et al., 2008a
D. magna	Cu	NOEC	24 hrs	Cuticle protein	Exoskeleton related proteins	Down	Adult	Poynton et al., 2008a
				Cell signalling				
D. magna	Cu	1/10 EC50	24 hrs	Inositol monophosphatase, Leucine rich protein phosphatase	Cell signalling	Up	Adult	Poynton et al., 2007
D. magna	Cu	1/10 LC50	24 hrs	Inositol monophosphatase	Cell signalling	Up	Adult	Poynton et al., 2008a
D. magna	Cu	NOEC	24 hrs	Cell division kinase	Cell signalling	Up	Adult	Poynton et al., 2008a
D. magna	Cu	1/10 LC50	24 hrs	Inositol monophosphatase	Monooxygenases	Up	Adult	Poynton et al., 2008b
				Immune Function				
D. magna	Cu	1/10 EC50	24 hrs	Lectin-like protein, Beta-glucan binding protein	Immune Function	Down	Adult	Poynton et al., 2007

D. magna	Cu	1/10 EC50	24 hrs	Beta-glucan binding protein	Immune Function	Down	Adult	Poynton et al., 2008a
D. magna	Cu	1/10 LC50	24 hrs	Beta-glucan binding protein, Macrophage lectin	Immune Function	Down	Adult	Poynton et al., 2008a
D. magna	Cu	NOEC	24 hrs	Beta-glucan binding protein, Macrophage lectin	Immune Function	Down	Adult	Poynton et al., 2008a
D. magna	Cu	1/1- LC50, NOEC	24 hrs	Lectin	Immune Function	Down	Adult	Poynton et al., 2008b
D. magna	Cd		24 hrs	Gram-negative bacteria binding protein	Immune System	Up	Neonates (< 24 hrs old)	Connon et al., 2008
				Monooxygenases				
D. magna	Cu	1/10 EC50	24 hrs	Monooxygenase	Monooxygenases	Up	Adult	Poynton et al., 2007
D. magna	Cu	1/10 EC50	24 hrs	Dopamine, Beta-hydroxylase	Monooxygenases	Down	Adult	Poynton et al., 2007
D. pulex	Cd	LC01	48 hrs	Similar monooxygenase	Monooxygenases	Down	Adults and neonates	Shaw et al., 2007
D. magna	Cu	1/10 LC50	24 hrs	Dopa-beta-hydroxylase	Monooxygenases	Down	Adult	Poynton et al., 2008a
D. magna	Cu	1/10 LC50	24 hrs	Retinol dehydratase, Monooxygenase X	Monooxygenases	Up	Adult	Poynton et al., 2008a
D. magna	Cu	NOEC	24 hrs	Acyl-coA dehydrogenase	Monooxygenases	Down	Adult	Poynton et al., 2008a
D. magna	Cu	NOEC	24 hrs	Monooxygenase X	Monooxygenases	Up	Adult	Poynton et al., 2008a
D. magna	Cu	1/10 LC50, NOEC	24 hrs	Monooxygenase	Monooxygenases	Up	Adult	Poynton et al., 2008b

Proteases/peptidases								
D. magna	Cu	1/10 EC50	24 hrs	Aminopeptidase, Trypsin precursor	Proteases	Up	Adult	Poynton et al., 2007
D. magna	Cu	1/10 EC50	24 hrs	Zinc metallopeptidase	Proteases	Down	Adult	Poynton et al., 2007
D. pulex	Cd	LC01	48 hrs	Carboxypeptidase A1	Peptidases	Down	Adults and neonates	Shaw et al., 2007
D. magna	Cu	1/20 EC50, 1/10 EC50, NOEC	24 hrs	Serine protease,	Peptidases	Down	Adult	Poynton et al., 2008a
D. magna	Cu	1/10 EC50	24 hrs	Serine protease	Peptidases	Down	Adult	Poynton et al., 2008a
D. magna	Cu	1/10 LC50	24 hrs	Zinc metallopeptidase	Peptidases	Down	Adult	Poynton et al., 2008a
D. magna	Cu	NOEC	24 hrs	Serine collagenase, chrymotrypsin, endopeptidase	Peptidases	Down	Adult	Poynton et al., 2008a
D. magna	Cu	1/10 LC50	24 hrs	Aminopeptidase	Peptidases	Up	Adult	Poynton et al., 2008a
Sulfotransferases								
D. magna	Cu	1/10 EC50	24 hrs	Sulfotransferase, Retinol dehydratase	Sulfotransferases	Up	Adult	Poynton et al., 2007
D. magna	Cu	1/10 LC50	24 hrs	Sulfotransferase	Other	Up	Adult	Poynton et al., 2008a
Developmentally related proteins								
D. magna	Cu	1/10 EC50	24 hrs	Posterior end mark	Developmentally related proteins	Down	Adult	Poynton et al., 2007
D. magna	Cu	1/10 EC50	24 hrs	Vitellogenin	Reproductive function and development	Down	Adult	Poynton et al., 2007

D. magna	Cu	1/10 LC50	24 hrs	Posterior end mark, CUB and sushi	Reproductive function and development	Down	Adult	Poynton et al., 2007
Lipid metabolism and Transport								
D. magna	Cu	1/10 LC50	24 hrs	Heart fatty acid binding	Lipid metabolism and transport	Down	Adult	Poynton et al., 2008a
D. magna	Cu	1/10 LC50	24 hrs	Fatty acid binding	Lipid metabolism and transport	Down	Adult	Poynton et al., 2008a
Metabolism								
D. magna	Cd	Range of concentrations *	24 hrs	Glycogen synthase, Glucose-6-Phosphatase, Endoglucanase 2	Carbohydrate and fat metabolism: glycolysis/gluconeogenesis, cellulose activity	Up	Neonates (< 24 hrs old)	Connon et al., 2008
D. magna	Cd	Range of concentrations *	24 hrs	GM2 ganglioside activator protein	Carbohydrate and fat metabolism: lipid metabolism	Down	Neonates (< 24 hrs old)	Connon et al., 2008
D. magna	Cd	Range of concentrations *	24 hrs	NADH dehydrogenase subunit 3, ATP synthase a chain	Energy metabolism: coenzymes	Up	Neonates (< 24 hrs old)	Connon et al., 2008
D. magna	Cd	Range of concentrations *	24 hrs	NADH dehydrogenase subunit 2	Energy metabolism: coenzymes	Down	Neonates (< 24 hrs old)	Connon et al., 2008
D. magna	Cd	Range of concentrations *	24 hrs	Cytochrome c oxidase subunit 1	Energy metabolism: electron transport, citric acid cycle	Up	Neonates (< 24 hrs old)	Connon et al., 2008
D. magna	Cd	Range of concentrations *	24 hrs	cytochrome b, succinate dehydrogenase	Energy metabolism: electron transport, citric acid cycle	Up	Neonates (< 24 hrs old)	Connon et al., 2008

D. magna	Cd	Range of concentrations *	24 hrs	carboxypeptidase A1, trypsin	Amino acid and polypeptide metabolism: oxidative deamination peptidases	Up	Neonates (< 24 hrs old)	Connon et al., 2008
D. magna	Cd	Range of concentrations *	24 hrs	Glutamate dehydrogenase, trypsin	Amino acid and polypeptide metabolism: oxidative deamination peptidases	Down	Neonates (< 24 hrs old)	Connon et al., 2008
D. magna	Cd	Range of concentrations *	24 hrs	Astacin (zinc metalloprotease)	Amino acid and polypeptide metabolism: chymotrypsin B2 metalloendopeptidase	Down	Neonates (< 24 hrs old)	Connon et al., 2008
Transcription and Translation								
D. pulex	Cd	LC01	48 hrs	Helix-loop-helix transcription factor	Transcription factor activity	Up	Adults and neonates	Shaw et al., 2007
D. pulex	Cd	LC01	48 hrs	Ribosomal protein	Ribosomal	Down	Adults and neonates	Shaw et al., 2007
D. magna	Cd	Range of concentrations *	24 hrs	16S rRNA, 28S rRNA	RNA	Down	Neonates (< 24 hrs old)	Connon et al., 2008
D. magna	Cd	Range of concentrations *	24 hrs	Histone H1	Nucleosome assembly	Down	Neonates (< 24 hrs old)	Connon et al., 2008
D. magna	Cd	Range of concentrations	24 hrs	Speckled-type POZ protein	mRNA processing	Down	Neonates (< 24 hrs	Connon et al., 2008

		*					old)	
D. magna	Cd	Range of concentrations *	24 hrs	Ubinuclein	Transcription factor binding	Up	Neonates (< 24 hrs old)	Connon et al., 2008
D. magna	Cd	Range of concentrations *	24 hrs	Stubarista	Ribonuclearprotein	Up	Neonates (< 24 hrs old)	Connon et al., 2008
D. magna	Cd	Range of concentrations *	24 hrs	Ribosomal protein S14E, Ribosomal protein L22, Ribosomal protein S3A, Ribosomal protein S20, Ribosomal protein L32	Ribosomal	Up	Neonates (< 24 hrs old)	Connon et al., 2008
D. magna	Cd	Range of concentrations *	24 hrs	Ribosomal protein L7, Ubiquitin-like/S30 ribosomal fusion protein	Ribosomal	Down	Neonates (< 24 hrs old)	Connon et al., 2008
D. magna	Cd	Range of concentrations *	24 hrs	Elongation factor 2	Translation elongation factor activity	Up	Neonates (< 24 hrs old)	Connon et al., 2008
D. magna	Cd	Range of concentrations *	24 hrs	rRNA intron-encoded homing endonuclease	Endonuclease activity	Up	Neonates (< 24 hrs old)	Connon et al., 2008
				Cellular processes				
D. magna	Cu	EC50	24 hrs	Glutathione-S-transferase	Oxidative stress	Up	Neonates (< 24 hrs old)	Watanabe et al., 2006
D. pulex	Cd	LC01	48 hrs	Similar to Glutathione-S-transferase	Oxidative stress	Up	Adults and neonates	Shaw et al., 2007
D. pulex	Cd	LC01	48 hrs	Myosin 2 light	Cell motility	Up	Adults	Shaw et

				chain			and neonates	al., 2007
D. magna	Cd	Range of concentrations *	24 hrs	Actin	Cell motility	Up	Neonates (< 24 hrs old)	Connon et al., 2008
D. magna	Cd	Range of concentrations *	24 hrs	Actin	Cell motility	Up	Neonates (< 24 hrs old)	Connon et al., 2008
D. magna	Cd	Range of concentrations *	24 hrs	Chitinase, Cuticular protein precursor, Cuticule extracellular matrix, Structural constituent, Cytochrome P450 monooxygenase	Endocrine system: molting	Up	Neonates (< 24 hrs old)	Connon et al., 2008
D. magna	Cd	Range of concentrations *	24 hrs	Low-density lipoprotein receptor domain class A, DD5 (structural constituent of cuticle)	Endocrine system: molting	Down	Neonates (< 24 hrs old)	Connon et al., 2008
D. magna	Cd	Range of concentrations *	24 hrs	Ferritin 1-like protein A, Hemoglobin 2	Inorganic ion transport and metabolism	Up	Neonates (< 24 hrs old)	Connon et al., 2008
D. magna	Cd	Range of concentrations *	24 hrs	Hemoglobin 1, Hemoglobin 2, Hemoglobin 3	Inorganic ion transport and metabolism	Down	Neonates (< 24 hrs old)	Connon et al., 2008
D. magna	Cd	Range of concentrations *	24 hrs	Glutathione peroxidase, Cu/Zn-superoxide dismutase	Cellular stress responses: oxidative stress	Up	Neonates (< 24 hrs old)	Connon et al., 2008
D. magna	Cd	Range of	24 hrs	Heat shock protein	Cellular stress	Up	Neonates	Connon et

		concentrations *		70	responses: chaperones and unfolded proteins		(< 24 hrs old)	al., 2008
D. magna	Cd	Range of concentrations *	24 hrs	Heat Shock Protein 20, T-complex protein 1, alpha subunit	Cellular stress responses: chaperones and unfolded proteins	Down	Neonates (< 24 hrs old)	Connon et al., 2008
D. magna	Cd	Range of concentrations *	24 hrs	Receptor for activated protein kinase C	Signal transduction	Down	Neonates (< 24 hrs old)	Connon et al., 2008
				Other				
D. magna	Cu	EC50	24 hrs	Serine protease inhibitor	Other	Up	Neonates (< 24 hrs old)	Watanabe et al., 2006
D. magna	Cu	EC50	24 hrs	Alcohol dehydrogenase	Other	Up	Neonates (< 24 hrs old)	Watanabe et al., 2006
D. magna	Cu	EC50	24 hrs	Lysosomal thiol reductase	Other	Up	Neonates (< 24 hrs old)	Watanabe et al., 2006
D. pulex	Cd	LC01	48 hrs	Compound eye opsin	Other	Up	Adults and neonates	Shaw et al., 2007
D. magna	Cu	1/10 EC50	24 hrs	Mitochondrial import inner membrane translocate	Other	Up	Adult	Poynton et al., 2007
D. magna	Cu	1/10 EC50	24 hrs	Fatty acid binding protein	Other	Down	Adult	Poynton et al., 2007
D. magna	Cu	1/10 EC50	24 hrs	Guanine nucleotide exchange, NADH	Other	Up	Adult	Poynton et al., 2008a

				dehydrogenase				
D. magna	Cu	NOEC	24 hrs	Serine protease inhibitor, ornithine decarboxylase	Other	Up	Adult	Poynton et al., 2008a
D. magna	Cu	NOEC	24 hrs	Glutamane synthetase-like, Guanine nucleotide exchange, Actin	Other	Down	Adult	Poynton et al., 2008a
D. magna	Cu	1/10 LC50, NOEC	24 hrs	Slit homolog	Other	Up	Adult	Poynton et al., 2008b

Table S5: Gene Ontology (GO) enriched terms of downregulated and upregulated genes found from using TopGO enrichment analysis on 207 differentially expressed genes identified when comparing all *Daphnia pulex* exposed samples to all controls. P-value for each GO term was corrected for multiple testing using the false discovery rate (FDR) for both Fisher's exact test and the weight test statistics.

Upregulated						
GO ID	**Term**	**Annotated**	**Significant**	**Expected**	**Fisher COR**	**Weight COR**
0006508	Proteolysis	775	48	13.65	3.12E-12	1.57E-10
0004252	Serine-type endopeptidase activity	264	23	3.61	3.28E-10	1.64E-09
0008061	Chitin binding	113	15	1.55	6.29E-09	2.20E-08
0006030	Chitin metabolic process	121	15	2.13	1.56E-06	1.28E-05
0004181	Metallocarboxypeptidase activity	38	7	0.52	8.59E-05	0.00031512
Downregulated						
GO ID	**Term**	**Annotated**	**Significant**	**Expected**	**Fisher COR**	**Weight COR**
0042302	Structural constituent of cuticle	303	10	1.68	0.00404999	0.00809999
0004089	Carbonate dehydratase activity	31	4	0.17	0.01107419	0.01661128
0006730	One-carbon metabolic process	32	4	0.15	0.02565011	0.02565011
0030246	Carbohydrate binding	127	6	0.71	0.02422175	0.03229567
0005201	Extracellular matrix structural constituent	47	4	0.26	0.02734256	0.04404503

Table S6: Quality and mapping information for the 21 RNA-sequencing libraries that were run on the Illumina Hi-Seq. Sequencing was 100 bp Paired-end reads. Libraries were prepared and barcoded using the NEXTflex™ RNA-seq kit (Bioo Scientific). Samples were run in the two lanes (indicated by run number). Average quality is in Phred score. Sample ID corresponds to tank (A2, control; D2, D3, C3, Cu exposed), individual ID number, and clone name (D, S, K).

Sample	Run	Group	Number of read pairs	Number of bases	Average quality	Coverage	Number of expressed genes	Percent mapped reads
13.D2-28D	2	Cu	13,913,712	2,782,742,400	34	13.91371	16931	91.3
8.D3-17S	2	Cu	20,419,349	4,083,869,800	34	20.41935	17798	90.1
9.C3-14D	2	Cu	16,994,445	3,398,889,000	34	16.99445	17687	90.9
15.D2-19S	2	Cu	21,680,702	4,336,140,400	34	21.6807	17448	91.1
14.D2-14K	2	Cu	20,323,016	4,064,603,200	34	20.32302	17869	89.2
12.C3-32S	2	Cu	13,455,769	2,691,153,800	34	13.45577	16864	92.6
10.C3-31K	2	Cu	15,385,836	3,077,167,200	34	15.38584	16900	92.2
11.C3-25S	2	Cu	13,229,077	2,645,815,400	34	13.22908	17047	90.9
5.D3-3D	2	Cu	15,932,432	3,186,486,400	34	15.93243	17568	88.9
6.D3-26K	2	Cu	19,866,455	3,973,291,000	34	19.86646	17799	88.7
7.D3-4S	2	Cu	17,358,612	3,471,722,400	34	17.35861	17516	89.59
11.D3-10K	1	Cu	13,991,948	2,798,389,600	35	13.99195	15134	84.6
14.C3-8D	1	Cu	15,101,356	3,020,271,200	35	15.10136	16454	85.0
12.D3-14D	1	Cu	12,746,931	2,549,386,200	35	12.74693	16545	85.9
13.C3-23K	1	Cu	15,271,661	3,054,332,200	35	15.27166	17369	88.4
4.A2_7S	2	Control	18,308,605	3,661,721,000	34	18.30861	17423	90.8
3.A2-16S	2	Control	15,723,304	3,144,660,800	34	15.7233	17245	89.7
2.A2-5K	2	Control	18,906,492	3,781,298,400	34	18.90649	17923	90.4
1.A2-22D	2	Control	13,434,101	2,686,820,200	34	13.4341	17199	87.5
1.A2-9K	1	Control	14,233,084	2,846,616,800	35	14.23308	16305	91.8

2.A2-11D	1	Control	14,560,655	2,912,131,000	35	14.56066	16727	89.7
		Total	245,671,301	49,134,260,200				
		Avg	16,230,359	3,246,071,829	34.3	16.378087	17128.6	89.3
		Min	12,746,931	2,549,386,200	34	12.746931	15134	84.6
		Max	21,680,702	4,336,140,400	35	21.680702	17869	92.6

Table S7: The 207 genes that were identified as differentially expressed (DE) when all clones were grouped together using a threshold of p <0.05 in both DESeq and EdgeR analysis. The gene ID indicates the Ensembl Metazoa ID. Log Fold change is shown for each analysis. The adjusted for multiple comparisons using the Bonferroni corrected P values for DESeq and EdgeR are shown for each clone compared to its control as well as the all grouping.

	D Clone			K clone			S Clone			All		
Gene ID	**Log FC**	**DESeq**	**EdgeR**	**logFC**	**DESeq**	**EdgeR**	**logFC**	**DESeq**	**EdgeR**	**logFC**	**DESeq**	**EdgeR**
319341	-5.076	0.012	0.0016	-6.153	1.40 E-05	3.30 E-07	-6.052	8.80 E-06	5.60 E-12	-5.918	1.50 E-05	3.10 E-21
104167	-5.672	0.00083	2.00 E-07	-5.976	8.80 E-17	8.70 E-16	-5.642	8.00 E-18	8.30 E-14	-5.775	9.40 E-27	3.90 E-36
109980	-3.41	0.22	0.067	-4.284	0.0047	0.0041	-5.812	3.20 E-05	5.60 E-09	-4.826	0.00094	1.00 E-12
225009	-5.378	1.30 E-06	2.00 E-07	-4.686	1.40 E-07	1.10 E-07	-4.457	5.70 E-09	4.70 E-09	-4.74	6.80 E-30	7.20 E-31
223070	-2.863	0.0023	0.072	-2.753	7.60 E-05	0.12	-4.21	7.20 E-18	4.00 E-06	-3.773	0.034	6.70 E-07
320065	-3.289	1	0.34	-2.253	0.92	0.56	-5.339	0.3	3.00 E-06	-3.526	0.039	5.20 E-06
221339	-3.456	6.90 E-11	2.50 E-07	-3.545	1.90 E-17	2.50 E-11	-3.482	4.00 E-11	5.90 E-08	-3.502	2.70 E-36	1.30 E-31
96378	-2.547	1	1	NA	NA	NA	-3.559	0.099	0.021	-3.369	0.029	0.00038
303054	-3.145	0.00039	0.00039	-3.053	7.90 E-05	0.00023	-3.099	2.50 E-05	0.00035	-3.096	1.40 E-17	1.70 E-17
310546	-1.515	1	0.79	-2.413	0.0044	0.0052	-3.839	7.30 E-11	4.20 E-07	-2.911	0.0052	9.90 E-10
226732	-3.046	0.0013	0.0045	-3.278	2.90 E-05	2.70 E-05	-2.491	0.0026	0.0094	-2.887	2.00 E-15	9.80 E-12
319643	-2.087	1	0.98	-2.91	0.28	0.067	-2.809	0.96	0.071	-2.713	0.00062	1.70 E-05
323732	-1.586	0.18	0.24	-3.505	0.57	8.80 E-06	-2.772	0.0017	4.60 E-06	-2.58	1.40 E-05	9.10 E-11

28846	-2.067	0.95	0.22	-2.269	0.043	0.0081	-2.752	1.20 E-05	0.0015	-2.487	0.00016	7.20 E-09
246487	-2.748	1	0.48	-2.236	1	0.69	-2.464	1	0.2	-2.486	0.013	1.70 E-05
253689	-2.872	0.19	0.027	-2.239	0.53	0.4	-1.988	1	1	-2.48	0.046	0.0012
274501	-2.712	0.49	0.28	-3.413	0.46	0.041	-1.791	1	1	-2.452	0.0058	0.0045
70722	-3.483	0.85	0.16	-4.107	0.72	0.032	-0.521	1	1	-2.443	0.0086	0.0076
311770	-1.636	1	1	-1.852	1	1	-3.376	0.016	0.00032	-2.392	0.00015	0.00013
15938	-1.253	1	0.73	-1.638	0.45	0.18	-3.393	1.90 E-08	1.70 E-07	-2.367	0.0071	6.80 E-10
332783	-2.757	0.031	0.022	-2.499	0.031	0.079	-1.962	0.053	0.11	-2.274	7.60 E-05	0.00011
246486	-1.79	0.13	0.33	-2.162	0.0057	0.0048	-2.512	2.70 E-05	0.021	-2.249	4.30 E-06	7.10 E-07
252511	-3.753	0.02	0.01	-2.286	1	0.29	-1.591	1	1	-2.196	0.04	0.022
49170	-2.434	5.80 E-05	0.056	-1.762	0.3	0.095	-2.535	7.60 E-06	8.00 E-04	-2.181	4.80 E-13	1.30 E-06
302343	-1.962	0.033	0.15	-2.477	0.00049	0.00036	-1.95	0.02	0.12	-2.122	1.90 E-10	1.40 E-08
305532	-2.012	0.04	0.056	-1.433	0.25	0.21	-2.313	0.00072	0.0023	-1.932	1.50 E-08	6.90 E-10
323470	-1.045	1	1	-2.933	0.13	0.12	-1.007	1	1	-1.827	0.0099	0.001
257184	-1.096	1	1	-1.304	0.22	0.21	-2.55	1.50 E-06	0.0013	-1.804	0.0043	5.10 E-05
234836	-2.016	0.002	0.13	-1.417	0.72	0.52	-1.617	1	1	-1.68	0.0053	0.013
223452	-2.004	0.0015	0.033	-1.617	0.0058	0.022	-1.324	1	1	-1.617	1.00 E-07	1.00 E-04
303404	-2.227	0.41	0.26	-1.44	1	0.92	-0.786	1	1	-1.465	0.015	0.037
320685	-1.778	0.12	0.26	-1.535	0.11	0.027	-1.015	1	1	-1.447	0.00015	0.00017
223524	-1.36	0.4	0.58	-0.75	1	1	-2.008	0.045	0.14	-1.405	0.036	0.014
308826	-1.32	0.96	0.48	-1.031	1	0.98	-1.533	0.55	0.35	-1.308	0.0045	0.00014
231870	-1.615	0.26	0.48	-1.046	1	1	-1.179	1	1	-1.271	0.015	0.029

47180	-0.906	1	1	-1.172	1	0.9	-1.427	1	0.93	-1.201	0.022	0.019
224885	-0.909	1	1	-0.956	1	0.76	-1.732	0.085	0.041	-1.189	0.0058	0.0011
226214	-1.596	0.12	0.26	-0.495	1	1	-1.535	0.22	0.24	-1.175	0.004	0.0041
25868	-0.328	1	1	-0.921	0.92	0.73	-1.793	0.0089	0.032	-1.101	0.015	0.01
48836	-0.517	1	1	-0.657	1	1	-1.805	0.011	0.012	-1.076	0.04	0.014
309195	-1.24	0.53	0.61	-0.678	1	1	-1.255	0.5	0.35	-1.062	0.0085	0.00094
306948	0.986	1	1	0.923	1	0.77	1.204	1	1	1.025	0.044	0.01
194537	1.457	0.56	0.56	1.272	1	0.47	0.516	1	1	1.044	0.045	0.018
303181	0.706	1	1	1.2	0.9	0.22	1.281	0.9	0.69	1.045	0.04	0.00031
220978	1.249	0.89	0.74	1.075	1	0.6	0.861	1	1	1.051	0.043	0.0029
321122	1.054	1	1	1.012	1	0.57	1.139	1	1	1.061	0.047	0.024
192571	1.767	0.22	0.28	0.585	1	1	0.985	1	1	1.07	0.045	0.0079
307754	1.286	0.96	0.61	1.173	0.87	0.37	0.751	1	1	1.071	0.037	0.0038
234105	1.195	1	0.88	1.118	1	0.63	0.924	1	1	1.08	0.04	0.018
442645	1.123	1	0.95	1.252	0.87	0.4	0.881	1	1	1.083	0.024	0.0024
238038	1.224	1	0.74	1.313	0.71	0.17	0.724	1	1	1.083	0.039	0.0089
303399	1.252	1	0.68	1.146	0.92	0.35	0.89	1	1	1.094	0.032	0.00035
304453	1.196	1	0.94	1.379	0.35	0.18	0.734	1	1	1.1	0.023	0.013
347770	1.077	1	1	1.728	0.28	0.086	0.654	1	1	1.111	0.023	0.0052
302482	0.866	1	1	1.27	1	0.4	1.188	1	0.81	1.111	0.026	0.0035
241365	1.367	0.96	0.56	0.717	1	1	1.347	1	0.87	1.129	0.041	0.0024
189897	1.164	1	0.86	1.205	0.72	0.35	1.05	1	1	1.136	0.022	0.0022
302654	0.996	1	1	1.464	0.28	0.21	0.974	1	1	1.139	0.027	0.0022
347753	1.348	1	0.92	1.397	1	0.49	0.722	1	1	1.149	0.029	0.023
97096	1.143	1	0.77	1.197	0.74	0.31	1.186	1	1	1.172	0.015	0.0011
303480	1.373	0.93	0.5	0.809	1	1	1.349	0.93	0.51	1.174	0.023	0.00017
347769	1.259	1	0.68	1.617	0.21	0.023	0.665	1	1	1.198	0.011	0.0036
231296	1.318	0.85	0.67	1.392	0.28	0.25	0.831	1	1	1.199	0.007	0.00096
117945	1.775	0.4	0.33	0.967	1	0.78	0.982	1	1	1.205	0.027	0.0019
347697	1.347	0.75	0.68	1.373	0.28	0.23	0.893	1	1	1.207	0.007	0.0011

322521	1.307	1	0.77	1.619	0.87	0.15	0.77	1	1	1.21	0.047	0.0097
302006	1.241	1	0.74	1.68	1	0.14	0.873	1	1	1.228	0.0055	0.0012
311788	1.29	0.85	0.73	1.405	0.25	0.27	0.993	1	1	1.236	0.04	0.011
308812	1.267	1	0.69	1.578	0.64	0.13	0.796	1	1	1.237	0.0073	0.00072
329543	1.219	1	0.85	1.232	1	0.53	1.278	1	0.92	1.24	0.027	0.00031
332162	1.493	0.55	0.48	1.49	0.26	0.11	0.694	1	1	1.253	0.0084	0.0079
196131	1.332	0.85	0.68	1.311	0.57	0.27	1.158	1	1	1.262	0.0068	0.0019
230174	1.609	0.35	0.48	1.458	0.21	0.18	0.782	1	1	1.274	0.004	0.0022
316719	1.526	1	0.68	1.11	1	0.66	1.224	1	1	1.284	0.032	0.013
227224	1.369	0.85	0.48	1.563	0.21	0.086	0.947	1	1	1.289	0.0045	0.00017
204216	1.528	0.47	0.48	1.044	1	0.77	1.4	0.86	0.69	1.3	0.0043	0.00031
225662	1.771	0.21	0.33	0.748	1	1	1.315	0.88	0.73	1.302	0.0057	0.0017
305365	1.732	0.26	0.32	1.536	1	0.33	0.766	1	1	1.303	0.004	0.0021
315258	1.688	0.35	0.22	1.802	0.067	0.011	0.772	1	1	1.326	0.0039	2.40 E-05
48805	1.471	0.55	0.5	1.323	0.37	0.25	1.208	1	1	1.333	0.0021	0.00023
96715	1.084	1	1	1.197	0.87	0.45	1.745	0.59	0.26	1.338	0.039	0.0021
240669	1.233	1	1	0.959	1	1	2.092	0.69	0.55	1.339	0.043	0.042
315267	1.854	1	0.49	0.971	1	0.81	1.21	1	1	1.341	0.024	0.0042
307582	1.62	0.89	0.48	1.582	1	0.41	0.901	1	1	1.349	0.014	0.0028
195271	1.84	0.37	0.31	0.984	1	0.79	1.275	1	1	1.364	0.002	0.00047
316663	1.277	1	0.7	1.21	1	0.48	1.766	0.38	0.35	1.365	0.003	0.0011
305376	1.203	1	0.89	1.91	0.1	0.033	1.055	1	1	1.379	0.0051	5.20 E-05
332854	1.832	0.53	0.37	1.181	1	0.8	1.152	1	1	1.387	0.0065	0.004
40490	1.467	0.85	0.44	1.24	0.87	0.37	1.469	0.67	0.39	1.39	0.0024	1.20 E-06
304469	1.656	0.37	0.24	0.964	1	0.68	1.669	1	0.56	1.402	0.0017	8.70 E-05
308564	1.598	0.87	0.59	1.067	1	0.84	1.699	1	0.8	1.411	0.0067	0.0021

103164	2.234	0.19	0.11	1.127	1	0.67	0.934	1	1	1.412	0.0024	0.00035
3545	1.604	0.95	0.48	1.074	1	1	1.498	1	1	1.414	0.012	0.0035
212927	1.12	1	0.93	1.749	0.87	0.13	1.359	1	0.91	1.426	0.01	0.00079
240849	1.95	0.33	0.16	0.876	1	0.98	1.417	1	0.93	1.429	0.037	0.0012
195011	1.959	0.07	0.24	1.497	0.13	0.13	0.941	1	1	1.432	0.00053	0.00028
221971	1.553	1	0.55	1.465	1	0.63	1.37	1	1	1.457	0.013	0.00046
231053	1.103	1	0.98	1.69	0.96	0.14	1.564	1	0.91	1.462	0.011	0.00089
22032	2.047	1	0.37	2.046	1	0.12	0.713	1	1	1.464	0.0095	0.0035
26734	1.638	0.41	0.27	1.556	0.21	0.12	1.212	1	1	1.465	0.00055	1.30 E-05
307755	1.492	0.55	0.54	1.569	0.13	0.12	1.331	0.9	0.84	1.468	0.00053	2.50 E-05
122715	1.844	0.85	0.51	1.876	1	0.6	0.895	1	1	1.468	0.02	0.01
46779	0.984	1	1	2.623	0.045	0.022	1.368	1	1	1.505	0.004	0.0056
347598	1.754	0.18	0.29	1.411	0.29	0.21	1.467	0.52	0.61	1.537	2.00 E-04	1.10 E-06
4284	1.474	1	0.95	1.275	1	0.98	1.989	0.71	0.35	1.55	0.021	0.0019
233234	1.871	0.41	0.29	1.563	1	0.38	1.281	1	0.81	1.554	0.00053	2.00 E-04
308963	1.319	1	0.7	2.208	0.048	0.011	1.102	1	1	1.558	0.00073	0.0048
231626	2.037	0.044	0.17	1.217	0.56	0.46	1.425	0.5	0.79	1.563	0.00012	1.20 E-05
119666	1.652	0.68	0.39	1.258	1	0.67	1.683	1	0.91	1.563	0.044	0.0021
322805	1.844	0.54	0.27	1.37	1	0.56	1.531	1	0.84	1.578	0.023	0.00059
241460	2.027	0.95	0.31	1.834	1	0.29	1.054	1	1	1.579	0.018	0.0014
333867	2.326	1	0.36	2.793	0.27	0.057	0.667	1	1	1.586	0.033	0.033
217034	1.825	0.16	0.28	1.117	1	0.61	1.809	0.1	0.28	1.589	0.00011	2.70 E-05
302511	1.555	1	0.61	1.623	1	0.35	1.663	1	0.59	1.605	0.0048	0.00019
300204	1.745	0.49	0.34	1.176	1	0.45	1.899	1	0.64	1.612	0.007	0.00029
95407	2.419	0.33	0.19	1.078	1	1	1.577	1	0.79	1.619	0.003	0.0077

299568	1.687	0.26	0.34	1.159	1	0.6	2.039	1	0.51	1.629	0.011	0.00027
320670	1.831	1	0.39	1.199	1	0.51	1.96	1	0.46	1.637	0.017	0.00087
233217	1.881	0.55	0.23	1.256	0.64	0.41	1.844	0.17	0.09	1.638	0.00011	3.80 E-07
304067	1.791	0.17	0.25	1.911	0.017	0.02	1.248	1	1	1.639	3.90 E-05	1.90 E-06
302859	2.252	0.012	0.067	1.627	0.049	0.086	1.165	1	1	1.639	0.00014	0.00014
306834	1.999	1	0.39	1.282	1	0.77	1.601	0.82	0.61	1.649	0.034	0.0011
321120	1.335	1	0.86	1.434	0.56	0.16	2.598	0.1	0.047	1.653	0.00053	0.00066
331148	2.208	0.55	0.23	2.075	0.85	0.19	0.939	1	1	1.656	0.0055	0.00039
228306	2.401	0.29	0.071	1.483	0.29	0.13	1.057	1	1	1.659	0.047	0.00091
306091	1.786	0.67	0.34	1.733	1	0.27	1.535	0.54	0.83	1.672	0.00034	0.00038
347751	2.03	0.48	0.31	1.861	0.53	0.1	1.172	1	1	1.675	0.0044	0.00056
220405	1.936	0.13	0.19	1.47	0.35	0.098	1.682	0.25	0.24	1.707	3.30 E-05	5.50 E-07
195644	2.41	0.56	0.19	1.727	1	0.16	1.316	1	1	1.728	0.0055	0.002
300414	1.776	1	0.48	1.196	1	0.78	2.405	0.092	0.078	1.741	7.00 E-04	3.70 E-05
309210	1.804	0.62	0.34	1.923	0.0068	0.0078	1.524	0.45	0.39	1.761	4.00 E-06	1.50 E-07
300342	2.018	0.54	0.24	1.188	0.9	0.5	2.06	1	0.64	1.772	0.023	3.00 E-04
316364	2.176	0.023	0.072	1.965	1	0.27	1.246	1	1	1.777	0.00092	0.00012
301598	1.934	0.27	0.17	1.942	0.14	0.021	1.501	0.69	0.47	1.784	4.90 E-05	1.20 E-10
46086	2.3	0.42	0.077	1.199	0.99	0.46	1.888	1	0.54	1.807	0.0052	5.00 E-05
223905	2.027	0.08	0.077	1.885	0.045	0.0044	1.545	1	0.51	1.817	7.00 E-06	6.40 E-09
303669	1.912	1	0.34	1.679	0.18	0.079	1.945	0.88	0.28	1.824	0.0012	1.20 E-05

223641	2.725	0.00077	0.008	2.29	1	0.04	0.952	1	1	1.855	1.50 E-05	0.00013
228686	1.209	1	0.85	3.103	0.35	0.0011	1.682	0.5	0.4	1.863	7.00 E-06	1.60 E-05
299926	1.963	0.9	0.34	1.348	0.72	0.4	2.328	0.0032	0.077	1.868	0.00015	2.10 E-05
347754	2.14	0.75	0.29	1.974	0.92	0.079	1.481	1	0.82	1.869	5.00 E-04	2.40 E-05
300203	1.953	1	0.31	1.474	1	0.29	2.227	1	0.61	1.894	0.027	0.00036
129101	2.17	0.024	0.07	2.008	0.0058	0.0022	1.595	0.24	0.45	1.92	4.90 E-07	2.30 E-10
305363	2.481	0.89	0.14	1.625	1	0.15	1.604	1	0.81	1.941	0.043	0.00023
307236	1.652	0.41	0.32	2.518	0.00044	0.00023	1.74	1	0.63	1.944	6.30 E-07	5.60 E-07
347757	2.592	0.0018	0.013	1.919	0.35	0.031	1.446	1	0.81	1.957	5.50 E-07	7.20 E-08
7769	2.013	1	0.48	1.458	1	0.99	2.636	0.2	0.037	1.966	0.04	0.0016
43425	2.202	0.02	0.077	1.503	1	0.41	2.423	0.73	0.17	1.975	2.80 E-05	1.40 E-05
223830	2.044	0.62	0.33	2.445	0.96	0.031	1.571	1	1	1.989	0.0034	6.40 E-05
272592	2.133	1	0.33	2.353	1	0.4	1.596	0.81	0.63	1.995	0.0096	0.00072
306981	2.037	0.26	0.25	1.796	0.64	0.098	2.492	1	0.11	2.007	0.0012	0.00025
226059	2.248	0.045	0.11	1.767	1	0.25	2.089	0.029	0.1	2.036	2.30 E-06	9.30 E-08
320635	1.039	1	1	2.67	0.036	0.11	2.559	0.62	0.14	2.04	0.00055	0.00063
305105	3.137	0.38	0.077	1.659	1	0.76	1.476	1	1	2.056	0.043	0.0027
49378	2.365	0.0047	0.024	2.519	0.00076	0.0012	1.487	1	0.93	2.063	2.20 E-08	6.40 E-08
302568	2.127	1	0.49	1.844	1	0.76	2.621	1	0.55	2.129	0.0044	0.0012
332859	2.37	0.95	0.52	1.959	1	0.76	2.035	0.5	0.45	2.145	0.011	0.0024
302256	2.852	0.28	0.13	1.187	1	1	3.032	0.18	0.079	2.151	0.025	0.0026

324577	1.571	1	0.95	2.425	0.37	0.19	2.882	0.67	0.35	2.158	0.00053	0.00077
326408	1.949	0.33	0.24	2.329	0.43	0.028	2.301	0.49	0.1	2.196	1.60 E-06	2.30 E-08
95750	2.603	0.0029	0.0054	3.065	1	0.0037	1.385	1	1	2.218	0.00016	1.20 E-05
316123	3.211	0.85	0.31	1.114	1	1	3.69	1	0.8	2.23	0.029	0.024
127566	2.377	0.0048	0.041	2.514	0.00018	0.00039	1.99	1	0.51	2.295	1.30 E-08	3.10 E-09
229417	2.591	0.0018	0.016	2.194	0.00091	0.0023	2.141	1	0.3	2.301	3.40 E-09	3.40 E-10
307274	3.207	0.12	0.019	1.836	0.087	0.033	1.872	0.57	0.32	2.311	0.00018	2.00 E-07
347760	2.875	0.028	0.016	2.761	0.74	0.015	1.473	1	0.98	2.319	9.50 E-05	1.10 E-06
305249	2.438	1	0.23	1.813	1	0.11	2.87	1	0.3	2.334	0.023	0.00015
347623	3.55	4.30 E-06	0.00015	2.909	1.30 E-06	5.90 E-06	0.851	1	1	2.414	1.80 E-05	2.40 E-05
226734	3.101	1	0.074	1.833	1	0.41	2.124	0.03	0.032	2.443	0.045	5.10 E-05
106953	1.749	1	0.65	3.53	0.075	1.50 E-05	3.154	1	0.51	2.444	0.0017	0.0035
226211	2.556	0.55	0.11	2.085	1	0.17	2.68	0.3	0.13	2.447	0.00043	4.30 E-06
64728	3.032	0.14	0.024	2.766	0.03	0.00036	1.837	0.099	0.27	2.511	3.50 E-06	7.20 E-09
300339	2.709	1	0.091	1.812	1	0.3	3.093	1	0.36	2.558	0.048	0.00026
238705	3.166	1	0.39	3.488	1	0.35	1.71	1	1	2.59	0.0096	6.50 E-05
57749	2.741	0.9	0.27	2.889	1	0.1	2.377	0.4	0.22	2.63	0.0026	0.00019
262612	3.749	0.0067	0.0039	5.208	0.019	0.00028	1.74	1	1	2.688	7.00 E-04	0.0014
307732	2.841	0.0013	0.005	2.819	0.22	0.01	2.477	0.0082	0.0028	2.726	2.60	3.80

											E-11	E-12
302821	3.079	0.006	0.0076	3.214	0.00049	8.80 E-06	2.137	0.057	0.042	2.741	8.40 E-12	2.70 E-14
331737	3.814	0.00013	0.00015	6.231	4.20 E-14	8.70 E-16	1.814	1	0.67	2.822	7.60 E-07	0.00011
303858	3.813	7.50 E-07	0.00011	1.688	0.9	0.2	3.774	0.73	0.012	2.896	4.10 E-06	3.20 E-07
325131	2.482	0.14	0.04	3.586	1.30 E-06	2.60 E-08	2.914	0.096	0.24	2.905	1.00 E-08	2.00 E-07
252435	3.606	1	0.68	1.458	1	1	4.505	0.19	0.041	2.97	0.013	0.0016
210571	3.028	8.90 E-05	0.0027	3.716	4.00 E-10	2.60 E-08	2.258	0.73	0.18	2.976	8.20 E-16	1.20 E-14
313427	3.226	0.85	0.11	2.663	0.00044	0.00043	3.12	0.00019	1.00 E-04	2.99	4.60 E-10	1.50 E-10
222354	3.499	0.97	0.031	2.808	1	0.036	2.747	1	0.12	3.056	0.0075	1.40 E-06
303491	2.819	1	0.092	3.775	1	0.01	3.199	0.039	0.011	3.144	0.0012	1.50 E-06
322264	3.625	1	0.026	2.699	0.022	0.00023	2.845	1	0.11	3.146	0.023	4.20 E-07
290503	3.176	0.85	0.25	3.16	1	0.41	3.183	1	0.35	3.185	0.00056	1.80 E-06
321422	3.257	0.38	0.056	3.296	1	0.15	3.049	0.25	0.0083	3.202	0.00025	2.80 E-06
229607	3.592	0.0011	0.00039	3.321	0.016	1.70 E-05	2.89	0.032	0.035	3.237	4.90 E-14	3.80 E-14
318320	3.947	0.0015	0.00026	2.535	1	0.19	3.776	0.52	0.021	3.268	6.50 E-07	2.80 E-07
319612	2.817	0.0011	0.0034	4.123	3.70 E-10	2.90 E-09	3.46	1.50 E-05	4.50 E-06	3.429	9.30 E-19	5.40 E-21
220200	4.068	5.30 E-08	9.50 E-06	3.23	1.00 E-06	2.20 E-06	3.942	2.80 E-08	4.20 E-06	3.698	7.40 E-23	1.10 E-28

000000 04628	5.589	1	0.39	3.662	1	0.27	3.187	1	0.82	3.748	0.0012	1.60 E-05
46683	3.106	0.0033	0.031	4.368	0.49	0.00017	4.297	1	0.04	3.799	4.30 E-06	1.90 E-08
327978	4.848	0.18	0.0045	3.23	1	0.24	3.417	0.011	0.0012	3.851	0.00013	5.60 E-07
247441	6.393	0.56	0.31	6.642	0.41	0.21	2.377	1	0.9	4.04	8.10 E-05	0.00011
324781	5.642	1	0.45	4.895	1	0.99	2.945	1	1	4.065	0.022	0.00067
317348	5.667	0.42	0.0045	4.323	0.77	0.00095	3.971	0.26	0.0065	4.731	0.0027	2.20 E-08
333097	5.322	1	0.0046	4.394	1	0.0044	4.21	0.69	0.18	4.816	0.049	2.90 E-06
337500	5.026	1	0.65	4.868	1	0.97	4.504	1	1	4.818	0.047	0.00037
313428	5.513	1	0.022	4.644	0.53	0.00013	4.921	0.0017	6.00 E-06	5.001	5.80 E-06	1.50 E-10
332119	5.396	1	0.57	4.999	1	0.98	5.507	1	0.51	5.33	0.004	0.00017
101472	5.259	0.5	0.016	8.327	0.00048	5.60 E-05	8.343	4.30 E-06	6.00 E-06	6.517	9.60 E-13	1.20 E-15
222529	8.169	0.021	0.00033	5.97	1	0.011	6.326	0.12	6.00 E-06	6.878	8.00 E-06	3.60 E-10
264563	8.301	1	0.0092	7.337	0.87	0.056	6.702	1	0.15	7.703	0.043	2.10 E-05
300382	8.63	0.33	0.015	5.843	1	0.04	8.147	0.64	2.60 E-05	7.728	0.0066	2.00 E-07