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Identifying conserved polychaete molecular markers of metal exposure:Comparative analyses using the *Alitta virens* (Annelida, Lophotrochozoa) transcriptome

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ABSTRACT

Polychaetes are vital for evaluating the effects of toxic metals in marine systems, and sensitive molecular biomarkers should be integral to monitoring efforts. However, the few polychaete markers that exist are inconsistent, even within the same species, failing to identify gene expression changes in metal-exposed animals incurring clear metabolic costs. Comparing previously characterised polychaete metal-responsive genes with those of another carefully selected species could identify biomarkers applicable across polychaetes. The ragworm *Alitta virens* (Sars, 1835) is particularly suited for such comparisons due to its dominance of fully saline coastal areas, widespread distribution, large biomass, and its phylogenetic position relative to other polychaete 'omic' resources. A transcriptome atlas for A. virens was generated and an RNASeq-qPCR screening approach was used to characterise the response to chronic exposures of environmentally relevant concentrations of copper and zinc in controlled mesocosms. Genes presenting dramatic expression changes in A. virens were compared with known metal-responsive genes in other polychaetes to identify new possible biomarkers and assess those currently used. This revealed some current markers should probably be abandoned (e.g. Atox1), while others, such as GST- Omega, should be used with caution, as different polychaete species appear to upregulate distinct GST-Omega orthologues. In addition, the comparisons give some indication of genes that are induced by metal exposure across phylogenetically divergent polychaetes, including a suite of haemoglobin subunits and linker chains that could play conserved roles in metal-stress response. Although such newly identified markers need further characterisation, they offer alternatives to current markers that are plainly insufficient.

1. Introduction

Copper and zinc are highly toxic metals (King *et al*., 2004; Reish and Gerlinger, 1997) that are of great concern for ecotoxicological risk management (Luoma and Rainbow, 2008; Walker *et al*., 2005) and have been ranked first and third most toxic aquatic pollutants respectively (Johnson *et al*., 2017). Traditionally, Cu and Zn are seen as contamination legacies (Walker *et al*., 2005) and environmental regulators assumed levels would decline due to lower industrial inputs and legislation (Rainbow *et al*., 2011), however, recent analysis has shown that coastal sediment concentrations are stable or increasing (Watson *et al*., 2018). This is possibly due to inputs from the use of these metals in new applications, such as nanoparticles (Gao *et al*., 2013), or renewed use, such as replacing TBT in antifouling paints (Srinivasan and Swain, 2007). Crucially, these metals are predicted to be more bioavailable under predicted ocean acidification scenarios (Millero *et al*., 2009).

Polychaetes are found in vulnerable marine benthic communities and their role as contaminant vectors, combined with their ecological importance, means they are often used to analyse ecosystem health (Dean, 2008). Sub-lethal molecular biomarkers are used extensively to study the effects of pollutants on exposed invertebrates (Schirmer *et al*., 2010), yet the selection of biomarkers can be complex (Hook *et al*., 2014). For example, genetic adaptations are known to underpin the evolution of metal tolerance (Grant *et al*., 1989) and different species, or even populations of the same species, can evolve independent mechanisms for coping with pollutants (Reid *et al*., 2016). Metal exposed polychaete species studied at the molecular level represent a wide phylogenetic range (McQuillan *et al*., 2014; Neave *et al*., 2012; Rhee *et al*., 2012), leading to uncertainty about the universality of existing molecular biomarkers. Polychaetes (and marine Lophotrochozoa more generally) remain underrepresented both genomically and transcriptomically (Mehr *et al*., 2015). A recent study illustrates the current issues regarding the lack of metal-response markers. Analysis of *Hediste diversicolor* suggests while exposure at a metal contaminated site induces metabolic costs that leads to reduced weight, current gene markers failed to identify any differential gene expression (Breton and Prentiss, 2019), this is despite the

application of biomarkers developed using *H. diversicolor* (McQuillan *et al*., 2014). However, characterising transcriptomic response to metal for a carefully selected polychaete species within the context of existing analyses (McQuillan *et al*., 2014; Neave *et al*., 2012; Rhee *et al*., 2007a, 2007b, 2012; Won *et al*., 2011) could help identify reliable and sensitive cross-species markers for monitoring diverse polychaetes.

Alitta virens (Sars, 1835) is an ideal species for ecosystem monitoring as it inhabits marine and estuarine environments in temperate regions of the northern hemisphere. It is also the dominant polychaete (and macrofaunal species more generally) in fully saline areas and has commercial importance (Kristensen and Kostka, 2005; Nielsen *et al*., 1995). As sediments are sinks for metals, the benthic existence of *A. virens* means it is continuously exposed to possible contaminants via sediment and porewater (Dean, 2008). *A. virens* is also phylogenetically well-placed to allow comparisons with the other polychaete transcriptomic data sets associated with metal responses; the closely related *H. diversicolor* and *Perinereis nuntia* (McQuillan *et al*., 2014; Santos *et al*., 2006; Rhee *et al*., 2012), and the more distantly related *Ophelina* (Neave *et al*., 2012; Rousset *et al*., 2007). Furthermore, toxicity experiments (Watson *et al*., 2018) associated with this *A. virens* transcriptomic analysis allows biomarker candidates to be anchored to measurable effects, as recommended to provide regulators with the tools to assess environmental risk (Hook *et al*., 2014).

Our first aim is to generate a comprehensive *A. virens* transcriptome to provide a rich resource for ecotoxicology. Our second is to use RNASeq-qPCR screening to characterise responses of *A. virens* to chronic (3 & 6 month) exposures of environmentally relevant concentrations of Cu, Zn and Cu-Zn using sediment-spiking. Sediment-spiking enables effects to be directly linked to test chemicals (U.S.EPA, 2005). This is particularly critical as other polychaete transcriptomic studies have compared animals from clean and contaminated sites (McQuillan *et al*., 2014; Neave *et al*., 2012; Rhee *et al*., 2012) where responses to a metal of interest may be obscured by acclimatisation and/or the influence of a multitude of other pollutants. Overall, we aim to identify potential biomarkers for metal-induced stress applicable across diverse polychaetes that enables comparative assessment of sensitivity and tolerance. Ultimately, this will help provide sensitive and widely applicable tools to identify and monitor vulnerable ecosystems.

2. Materials and methods

2.1. Mesocosm study exposure

Mesocosms and sample processing were as previously described (Watson *et al*., 2018). Briefly, sediment was spiked with seawater (control), $CuCl_2{\cdot}2H_2O$, $ZnCl_2$ or combined solutions and left for a week at 4 °C in the dark. Target bioavailable concentrations were based on UK sites (Pini, 2014), resulting in seven toxicogenomic treatments: control; low Cu; low Zn; low Cu-Zn; high Cu; high Zn; high Cu-Zn. *A. virens* (1–2 g) were added to each box (3 boxes per treatment per tank [3 tanks]) with each box connected to flowing seawater. Worms were fed fish pellets, 1–2% of starting biomass twice a week. One box per treatment per tank was destructively sampled in January and April 2013, sediment and porewater collected and worms depurated then weighed. Approximately 0.5 cm of gut tissue (~10 segments from head) was snap frozen in liquid nitrogen, homogenised in Tri-Reagent (Ambion, Life Technologies, Carlsbad, CA, USA) and stored at − 80 °C.

Presumed bioavailable (termed 'bioavailable' here) sediment and porewater concentrations were obtained using a BCR 3-stage sequential extraction protocol and measured by flame AAS as previously detailed (Pini, 2014). The metal distribution across three fractions was summed to give the bioavailable concentration. Recoveries for steps 1–3 were 98–111% (Cu) and 93–103% (Zn). Tissue samples were also processed with mean recovery percentages of 92% for Cu and 99.5% for Zn.

2.2. RNA isolation, library preparation and sequencing

Total RNA was extracted from the homogenised gut tissue in Tri-Reagent (see Table S1 for exposure conditions and animal numbers), purified (Direct-Zol, Zymo Research, Irvine, CA, USA), DNAse I treated (New England BioLabs, Ipswich, MA, USA), concentrated (RNA Clean & Concentrator-5, Zymo Research) and quantified (NanoDrop ND-100, Thermo Fisher Scientific, Waltham, MA, USA), before RNA integrity was checked using agarose gel electrophoresis. RNA was pooled to form two libraries, these comprised of equimolar amounts of RNA from control and exposed samples and included animals from each time point and/or exposure (Table S1). Sequencing libraries were prepared and indexed for paired-end multiplexed sequencing (TruSeq Stranded mRNA Library Prep Kit, Illumina, San Diego, CA, USA). The validated libraries (Agilent BioAnalyser 2100, Agilent Technologies, Santa Clara, CA, USA) were pooled (8pM) and sequenced (100 bp paired-end run with 1 lane of an Illumina HiSeq2000 flow cell v3). Raw data BCL files were converted to FASTQ files and quality information assessed. (library preparation and sequencing was performed by Source BioScience, Nottingham, UK).

2.3. Assembly, mapping, annotation and differential expression

Raw reads were quality trimmed using Trimmomatic (Bolger *et al*., 2014, v. 0.32) using the following settings: Illuminaclip 2:30:10, leading = 3, trailing = 3 sliding window = 4 bp, quality = 15, minimum length = 36 bp. The trimmed reads were assembled using Trinity (Grabherr *et al*., 2011, v. trinityrnaseq_r20140717), using the default parameters for paired-end reads with 'read normalisation', before assembled contigs were annotated using the Trinotate pipeline (Bryant *et al*., 2017) and a quantitative measure of

transcriptome completeness was performed with BUSCO (Kriventseva *et al*., 2015) (v. 3, odb10 metazoa gene set). Reads and assembled transcriptome were deposited in NCBI: Bioproject No. **PRJNA627092**. Reads associated with the control and exposed samples were mapped to the transcriptome assembly [RSEM, v. 1.2.13 (Li and Dewey, 2011)]. The original counts were normalised with weighted trimmed mean of M-values (TMM) to calculate relative expression levels. As the RNASeq has no biological replication, differentially expressed transcripts were calculated using the function exactTest implemented in edgeR [v. 3.16.5 (Robinson *et al*., 2010)] with a common dispersion factor of 0.1. This associated a p-value with each contig sequence. The cut-off for putatively differentially expressed genes was selected experimentally using a qPCR screen. Bio- logical and molecular function analyses were performed using Panther (Thomas *et al*., 2003) (v. 13.1). Enriched Gene Ontology (GO) and keyword terms associated with putatively upregulated genes were determined using DAVID (Huang *et al*., 2009) (v. 6.7) using the following parameters: a medium classification stringency, similarity term overlap 3, similarity threshold of 0.50, initial group membership 3, final group membership of 3, and multiple linkage threshold of 0.50. The GO terms associated with putatively up and downregulated genes was also analysed using ReviGO (Supek *et al*., 2011) and GOnet (Pomaznoy *et al*., 2018) software. For the ReviGO analysis, scored GO term lists were input (small similarity [0.5] with a SimRel semantic similarity measure). Specifically, separate scored GO term lists were created for upregulated and downregulated genes by summing fold-change levels linked to the contigs contributing to each list. GOnet analyses were performed using the log fold change values and their associated Uniprot accessions.

2.4. Orthologue determination, sequence alignment and phylogenetic tree building

Orthologous genes were determined using a Reciprocal Best Hit BLAST method (Moreno-Hagelsieb and Latimer, 2007) and sequence alignment, trimming and tree building were performed using Geneious (v. 8.1.9).

2.5. Determination of stable reference genes for qPCR validation and primer design

To develop a reliable qPCR assay, the expression of five candidate reference genes was determined using a comparative ΔCq method (Silver *et al*., 2006). Briefly, a mean ΔCq value and standard deviation (std. dv.) (R, v.3.3.0) was calculated for triplicate reactions performed for each gene in twelve independent cDNA samples (representing a range of control and exposed animals). A mean (std. dv.) was calculated to reflect the expression stability of all genes relative to the remaining four candidates (ggplot2 v. 1.0.1). The two genes presenting lowest mean std. dv. were then selected as reference genes. Putative differentially expressed genes were selected on the basis of differential expression (according to RNASeq screening) across control and exposed samples. Contig sequences representing candidate reference genes (*Elong-like*, *GAPDH-like*, *beta-actin-like*, *alpha-tubulin-like* and *Ubiquitin-like*) and differentially expressed genes (*Unk1-like*, *Vtg-like1*, *Actin-like*, *Calbind-like*, *GST- Omega-like*, *Unk2-like* and *GST-Mu-like*) were used in conjunction with Primer3 software (Rozen and Skaletsky, 1999) (v. 4.1.0) to produce suitable primer sets for qPCR (Table S2).

		Control	Low exposure	High exposure	Low CuZn	High CuZn
Copper	Nominal concentration (SQG =65, SQGH- 270)	-	70	120	70	120
	Sediment	6 ± 0.2	60 ± 8.8	125 ± 16	63 ± 6.5	81 ± 3.5
	Porewater	0.6 ± 0.2	0.9 ± 0.1	1.5 ± 0.3	1.0 ± 0.1	1.1 ± 0.1
	Tissue	9.4 ± 0.6	24 ± 4.6	79 ± 26	45 ± 13	57 ± 13
Zinc	Nominal concentration (SQG =200, SQGH- 410)	-	200	270	200	270
	Sediment	26 ± 1.4	180 ± 21	252 ± 40	163 ± 18	165 ± 23
	Porewater	2.2 ± 0.7	1.5 ± 0.5	1.6 ± 0.5	3.6 ± 0.9	3.8 ± 1.5
	Tissue	61 ± 7.7	108 ± 7.2	98 ± 20	88 ± 5.9	94 ± 6.4

Table 1 Copper and zinc concentrations (3 and 6 months combined) in Cu, Zn and Cu-Zn mesocosm exposures. Copper and zinc mesocosm nominal bioavailable concentrations in sediment (mg kg^{-1} dry weight) (Pini,2014), Bioavailable sediment (mg kg^{-1} dry weight); porewater (µg l^{-1}) and worm tissue (µg kg^{-1} dry weight) concentrations (mean ± SEM). SQG - Sediment Quality Guideline values (mg kg^{-1} dry sediment) SQGH - Sediment Quality Guideline High value (Simpson *et al*., 2007).

3.2. The A. virens transcriptome atlas

A representative transcriptomic atlas could be suitable for many applications. The completeness of the transcriptome can be estimated at the locus level by determining the number of near universal gene orthologues, and the extent such orthologues are present in complete or fragmented forms. The *A. virens* transcriptome atlas, produced using both control and exposed animals, was found to contain 99.2% (98.6% in complete form) of the 954 genes within the BUSCO metazoan gene set (Kriventseva *et al*., 2015) (transcriptome statistics in Table S3). Further analysis using Panther (Thomas *et al*., 2003) reveals that, despite originating from a single tissue, the sequencing depth has produced a functionally representative transcriptome when compared to the *Drosophila melanogaster* genome (Fig. S1) (Thurmond *et al*., 2019). The data provided here will enable like-for-like quality comparisons of different data sets, such as comparative transcriptomic analysis. This valuable catalogue of polychaete genes can be used for many applications, including the development of probes and primers for gene expression analysis, particularly useful considering the increasing use of *A. virens* in evolutionary developmental biology (Kostyuchenko *et al*., 2019).

3.3. RNASeq and qPCR screening of A.virens response to Cu-Zn exposure

We provide evidence for differentially expressed genes by comparing the control and metal exposed samples. A single repeat RNASeq method in conjunction with a suitable statistical approach is thought capable of identifying differentially expressed genes with low fold change thresholds (Al Seesi *et al*., 2014). We have taken a more conservative approach, limiting ourselves to identifying genes with pronounced differential expression patterns, where the cut-off for differential expressed genes is decided using qPCR. Similar approaches have provided reliable indications of pronounced gene expression differences (Al Seesi *et al*., 2014; Neave *et al*., 2012; Short *et al*., 2014; Wippler *et al*., 2016; Robertson *et al*., 2017). As we used qPCR to validate the RNASeq compar ison of control and exposed animals, it was important to identify reliable reference genes. Expression of candidate reference genes (Table S4) in twelve samples from across the experiment (control and exposed) was determined using a comparative ΔCq method (Silver *et al*., 2006). The Silver *et al*. (2006) approach has been shown comparable to other frequently used statistical approaches for determining stable reference genes (Feng *et al*., 2013). The stable expression suggested by the RNASeq experiment, indicated by broadly similar TMM values in control and exposed samples (Table S4) was supported by the qPCR (Fig. S2, Table S5). All five genes presented a std. dev. of less than 1 and could all be used as reference genes (Svingen *et al*., 2015). However, *GAPDH-like* and *Elong-like* had the greatest stability (indicated by a mean std. dev of 0.4755 and 0.5117 respectively, Table S5) and were chosen for further qPCR validations.

The expression of seven genes with a range of differential expression according to RNASeq (Table S6) were analysed by qPCR, using both *GAPDH-like* and *Elong-like* as reference genes (Fig. 1). The seven genes were selected on the basis of their association with a suitable range of p- values (determined using the exactTest) and because they represent a range of known and unknown functionality (Table S6). The qPCR experiment compared controls to biologically separate samples representing exposures to two concentrations of Cu, Zn and Cu-Zn for three and six months. Although variation is observed between some samples, the general expression pattern determined by qPCR agrees with the RNASeq in the p-value range $1.48e^{-11}$ to $2.7e^{-32}$.The *GST-Mu-like* gene, with the highest p-value ($3.7e^{-09}$), did not validate well, having lower expression than suggested by RNASeq (Fig. 1). Expression of both reference genes normalised to each other confirms their relatively stable expression (Fig. S3). Overall, of the 12 genes validated with qPCR, 11 correlate with the RNASeq data, 5 stable (Figs. S2 and S3) and 6 differentially expressed (Fig. 1). This suggests that any expression change in the RNASeq associated with a p-value $\leq 1.48e^{-11}$ represents a genuine expression difference.

The genes *Unk1* and *Vtg-like1* show a pattern of clear downregulation across both time points and all metal exposure scenarios, with the *GST-Omega-like* gene presenting an equivalent but upregulated expression pattern. This means the genes present strikingly similar patterns across twelve biologically independent samples, with each sample representing between 11 and 17 animals (Table S1). Therefore, it appears these gene expression responses are triggered by Cu, Zn or a Cu-Zn combination and appear to be maintained throughout the exposure period. For the *Unk2* and the cytoskeletal-associated *Actin-like* and *Calbind-like* genes, the consistent expression between the qPCR and RNASeq only holds if expression is averaged across qPCR experiments, as there is considerable variation across samples (Fig. 1). Furthermore, the expression variation does not obviously correlate with metal concentration or exposure time (Fig. 1). The response variation may reflect the inconsistent relationship between metal concentrations in sediments and tissues (Table 1). It is possible that some gene responses are strikingly non-proportional to metal concentration. For some genes (in this case associated with the cytoskeleton), the environmentally relevant concentrations of Cu/Zn (Table 1) may lead to varied responses if the varying metal levels in the organisms span a threshold that must be crossed to trigger cytoskeletal modifications. This hypothesis is supported by the strikingly similar pattern of variant expression across the equivalent qPCR samples for both cytoskeletal-associated genes. The similar expression pattern of *Unk2* and the *Actin-like* and *Calbind-like* genes (Fig. 1) suggests *Unk2* may also have cytoskeletal-functions and be induced in the same threshold dependent manner (note: the *Actin-like* gene is distinct from the *beta- actin-like* gene used in reference gene analysis).

Fig. 1. Comparative ΔΔCq qPCR expression analysis of seven *A. virens* genes associated with a range of differential expression values suggested by RNASeq. Each column represents the average expression (3 technical repeats) of each sample relative to controls, all normalised to both reference genes (*GAPDH-like* and *Elong-like*). Samples were performed in triplicate and error bars represent the standard deviation. Expression was determined at two concentrations (low and high) of Cu, Zn and Cu-Zn for three and six months (see Table S6 for details on gene annotations and RNASeq expression).

3.4. The challenge of finding consistent biomarkers

A range of biomarkers have previously been suggested for monitoring metal exposure in polychaetes, selected for their putative roles in metal handling (McQuillan *et al.*, 2014). However, these biomarkers have shown great variation, even within the same species (Breton and Prentiss, 2019; McQuillan *et al.*, 2014). Critically, our findings suggest that the existing markers fail to present any notable differential expression between control and exposed animals (summarised in Table S7). For example, *Atox1* binds and transports excess intracellular Cu to secretory pathways (Kim *et al.*, 2008). Studies of *Atox1* expression in polychaetes taken from heavily contaminated sites revealed significant but modest ~3× upregulation in *H. diversicolor* using qPCR (McQuillan *et al.*, 2014), and an even greater upregulation in *Ophelina*, using RNASeq and Western blot analysis (Neave *et al.*, 2012). However, although both *A. virens Atox1-like* genes possess the cysteine residues necessary for Cu binding (Rosenzweig *et al.*, 1999), the RNASeq suggests both genes have almost identical expression in control and exposed animals. Cu handling via mechanisms requiring increased *Atox1* may have evolved independently in different species. Given these findings, *Atox1-like* genes are probably not suitable as sensitive cross-species biomarkers, despite their functional role and increased expression in some metal exposed polychaetes.

3.5. Screening for biomarkers using GO term enrichment

Using the qPCR-determined cut-off, we created contig lists representing genes with apparent differential expression (DE) in exposed animals. Of the 696 and 141 contigs representing up and downregulated genes respectively, 371 and 114 could be annotated via BLAST analysis against the Uniprot database (E-value cut-off ≤$1e^{-10}$). The lists of Uniprot accessions were used to perform multiple analyses. Firstly, enriched Gene Ontology (GO) terms (separated into Biological Processes [BPs], Cell Components [CCs] and Molecular Functions [MFs]) associated with the accessions were determined using DAVID (Huang *et al.*, 2009) (Figs. S4 and S5). In addition, the GO terms associated with up and downregulated genes were scored by summing fold-change levels linked to all contributing contigs. This scored GO term list was then analysed using both ReviGO (Supek *et al.*, 2011) (Figs. 2 and S6) to reveal highly expressed GOterms and GOnet software (Pomaznoyetal., 2018) (Figs. S7 and S8) to reveal their various relationships.

The analysis of upregulated genes reveals clear enrichment of GO terms associated with cilia function (Figs. 2, S4, S7 and S8). These are largely represented by a suite of axonemal dynein genes, with a broad range of cilia-associated genes representing the remaining GO term 'cilium'. The modest enrichment of the KOG functional group 'cell motility' in the metal-exposed polychaete *Ophelina* (Neave *et al.*, 2012) suggests a somewhat similar response may occur across polychaetes. Assuming the modified cytoskeletal gene expression is adaptive, it is possible to speculate on its functional role. As the intestinal epithelium of polychaetes possesses many ciliated cell types (Kermack, 1955) that come into contact with contaminated sediment, the enrichment of cilium-associated GO terms could

represent attempts to replace damaged cilia. This response may also represent mechanisms that aid metal removal, as cilia in both invertebrates and vertebrates possess 'ciliary pockets' thought to be involved in vesicular trafficking (Benmerah, 2013). Indeed, gene expression suggests earthworms use a cilium-based system to sense and transport silver (Novo *et al*., 2015).

The analyses also provide evidence for upregulation/enrichment of GO terms broadly associated with cytoskeletal and microtubule-related functions (Figs. S4 and S8). Cytoskeletal-associated genes are enriched in both *Ophelina* exposed to Cu/Zn (Neave *et al*., 2012) and the earthworm *Lumbricus rubellus* exposed to cadmium (Owen *et al*., 2008). The cytoskeleton is an early target of oxidative damage (Dalle-Donne *et al*., 2001) and normal reactive oxygen species (ROS) concentrations are critical for regular cytoskeletal remodelling and function (Sakai *et al*., 2012; Wilson and González-Billault, 2015). Therefore, the metal- exposure may lead to dysfunctional changes to the cytoskeletal composition. However, the expression alterations may be adaptive to some degree, as cytoskeletal changes can act as a signalling mechanism for oxidative stress in diverse organisms (Clark *et al*., 2018; Farah and Amberg, 2007) and play critical roles in the repair of damaged cells (Abreu-Blanco *et al*., 2012). The cytoskeletal GO terms enriched in *Ophelina* and *A. virens* are not represented by the same gene sets (Neave *et al*., 2012). To some extent, this likely reflects the different tissues assayed (whole body versus gut tissue). It may also reflect the evolution of divergent response mechanisms, resulting from the multi-generation field exposure of *Ophelina* (Neave *et al*., 2012), compared to the exposed *A. virens* being taken from a relatively clean site. In support of this hypothesis, metal exposure of the polychaete *H. diversicolor*, taken from both metal-contaminated and clean sites, revealed animals originally from the contaminated-site possess more efficient strategies for sequestering and excreting metals (Mouneyrac *et al*., 2003). Specifically, the gut epithelium of exposed contaminated-site worms presented abundant Cu-containing lysosomes and extracellular granules, whereas exposed clean-site worms either lacked or possessed greatly reduced equivalent structures. Furthermore, while 'spherocrystals' were observed in the gut of both exposed clean and contaminated-site worms, these structures only act as a detoxified Zn store in the latter (Mouneyrac *et al*., 2003).

Enriched GO terms, associated with collagen metabolism and the extracellular matrix (Fig. S4), are dominated by a series of matrix metalloproteinases (MMPs) and these genes are also components of several prominent terms in the ReviGO analysis, such as 'Zinc ion binding' (Fig. 2). Further insight into the likely role of MMPs is given by their inclusion in the highly expressed terms 'proteolysis' (Fig. 2) and 'proteinaceous extracellular matrix' (Fig. S4), where they are combined with other ECM-related proteins including multiple collagen α-chains. Taken together, these provide clear evidence of increased ECM remodelling in exposed animals. Collagens dominate the ECM and MMPs are critical to their degradation and modification (Lu *et al*., 2011), with increased MMP expression and activity observed in vertebrates exposed to oxidative stress (Alge-Priglinger *et al*., 2009; Siwik *et al*., 2001). Although knowledge of ECM remodelling is primarily based on vertebrate systems, extrapolation is reasonable as major ECM components and MMP functionalities are conserved across metazoans (Özbeketal., 2010; Page-McCaw, 2008). On this basis, the presence of ECM and MMP GO terms may be explained if metal-induced ROS damage to intestinal cells induces repair mechanisms that involve substantial ECM remodelling. Not surprisingly, given the conservation of ECM-related genes, there is evidence of similar responses in other polychaetes. For example, even though direct comparisons of contributing genes are not possible, the KOG term 'extracellular structures' is also enriched in *Ophelina* from metal contaminated sites (Neave *et al*., 2012).

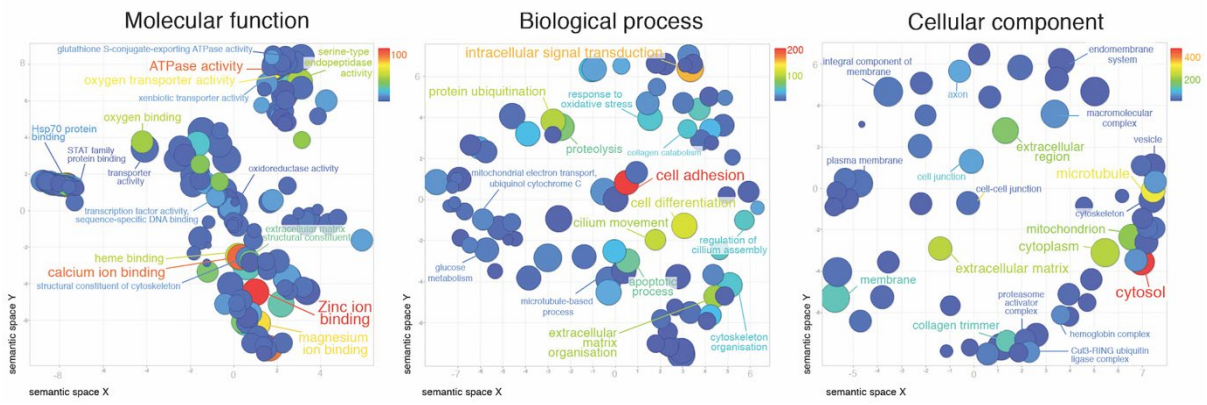

Fig. 2. Summarised ReviGO analysis of 'Molecular Function', 'Biological Process' and 'Cell Component' Gene Ontology (GO) terms associated with putatively upregulated genes. GO terms associated with relevant genes were scored by summing fold-change levels linked to contributing contigs. The scored GO term list was then subsequently analysed using ReviGO.

Other genes with established links to responses to cell damage. For example, *E3 ubiquitin ligase* that contributes to the GO term 'protein ubiquitination' (Fig. 2), has known roles in mediating response to oxidative stress (Wolyniec *et al*., 2013). Furthermore, the enriched 'Cul3-RING ubiquitin ligase complex' (Fig. 2) is dominated by highly conserved kelch-like proteins, which function as critical components of the ubiquitin-proteasome system (Gupta and Beggs, 2014). In addition, in mammalian cells, kelch proteins bound to metals can activate a battery of genes important for cell survival against oxidative and other stressors (He and Ma, 2010).

We observe high expression of genes associated with the GO terms 'oxygen binding', 'oxygen transporter activity' and 'heme binding' (Fig. 2). We also observe notable enrichment of GO terms 'oxygen transport' and hemoglobin complex' in the DAVID analysis (Fig.

S4). The term 'heme binding' includes a dual oxidase, a gene that, in vertebrates, responds to chemical-induced epithelial injury by promoting expression of genes critical to wound response, such as MMPs (van der Vliet *et al*., 2018). The increased expression of both dual oxidase and MMPs (see above) in exposed *A. virens* suggests a similar mechanism may be activated in the damaged gut epithelium of polychaetes. A suite of globin subunit and globin linker chains constitute the remaining genes in these GO terms. Although their role in metal-response is uncertain (considered in more detail below), it is striking that globin subunits in *Ophelina* from metal contaminated sites also present dramatically increased expression (Neave *et al*., 2012).

The GO term 'ATPase activity' is associated with highly upregulated genes (Fig. 2) and, although the contributing genes are functionally diverse, they all have plausible links to metal response. As well as cytoskeletal-related kinesin-like proteins, we observe upregulation of ATP-binding cassette sub-family E member 1 (ABCE1). This highly conserved gene, critical to protein synthesis, has particularly ROS-labile cofactors (Fe-S clusters) (Alhebshi *et al*., 2012). Interestingly, increasing ABCE1 expression in yeast rescues ROS toxicity, possibly because Fe-S sequestration into ABCE1 decreases the pool of ROS-sensitive Fe-S clusters (Alhebshi *et al*., 2012). The upregulation of ABCE1 suggests a similar mechanism in metal-exposed *A. virens*. The Multidrug resistance- associated protein 1 (MRP1) also contributes to this GO term. MRP1 is a membrane pump that mediates the efflux of a wide variety of xenobiotics, including glutathione conjugates, and is critical to protecting cells against a range of toxic compounds (Cole, 2014). The upregulation of MRP1 in concert with specific Glutathione S-transferases (GSTs) (see discussion of *GST-Omega* below) provides a metal-detoxification/efflux pathway. Interestingly, we also see upregulation of Unconventional myosin-XIX (Myo19), an ATPase critical to the ROS-induced mitochondria localisation to filopodia (Shneyer *et al*., 2016). Mitochondria play complex roles in stressed cells and can migrate within the cell to supply local ATP demand or provide a localised signalling platform (Shneyer *et al*., 2016; Tait and Green, 2012). If such mitochondrial re-localisation is occurring in *A. virens*, it may also explain some proportion of the upregulated cytoskeleton associated genes. The final contributing gene to this GO term is Heat shock cognate 71 kDa protein (Hspa8), a highly conserved molecular chaperone implicated in the protection of the proteome from metal-induced stress (Kwon *et al*., 2013), that has also been found upregulated in the polychaete *Perinereis nuntia* taken from a metal contaminated site (Rhee *et al*., 2012).

Enrichment of specific GO terms was also observed in the relatively fewer downregulated genes, although with higher enrichment p-values (Fig. S5). GO terms linked with cell junction/cell adhesion are a major feature of the DAVID and ReviGO output (Figs. S5 and S6). Downregulation of genes contributing to these groups might be related to ECM remodelling and wound-healing (supported by the upregulation of MMPs and other ECM-related genes). Furthermore, ROS can disrupt cell-cell junctions between epithelial cells (Narimatsu *et al*., 2013) and reductions of cell-junction associated proteins appears critical to normal wound-healing in *Drosophila* embryos (Hunter *et al*., 2015). Terms linked to ion channel/ion transport also appear in the various analyses (Figs. S5 and S6). The reason for this downregulation is unclear but oxidative stress is known to modulate ion channel activity (Kiselyov and Muallem, 2016), possibly the result of considerable 'cross-talk' between REDOX and calcium signalling (Hidalgo and Donoso, 2008).

3.6. Mining for conserved and new biomarkers

We further mined the validated gene list ($\leq 1.48e^{-11}$) to identify additional biomarkers, looking for genes known to be modulated by metal exposure in polychaetes and annelids (McQuillan *et al*., 2014; Mouneyrac *et al*., 2003; Neave *et al*., 2012; Novo *et al*., 2015; Rhee *et al*., 2007a, 2007b, 2012). We also include genes that have not been previously identified as polychaete biomarkers but, given their functional roles, plausibly represent reliable and sensitive indicators of heavy metal exposure (summarised in Table 2).

3.7. Glutathione S-transferases (GSTs)

GSTs conjugate glutathione to xenobiotic substrates (including metals) to facilitate detoxification via ATP-dependent pumps (Ishikawa, 1992). *GST-Omega-like* genes are associated with oxidative stress response (Kim *et al*., 2008) and are upregulated in several metal-exposed polychaete species, specifically *Hediste diversicolor* from metal contaminated sites (McQuillan *et al*., 2014), *Alitta succinea* exposed to cadmium (Won *et al*., 2011) and Cu-exposed *Perinereis nuntia* (Rhee *et al*., 2007a, 2012). Both the RNASeq and qPCR reveal upregulation of a *GST-Omega- like* gene in exposed *A. virens* (Table 2). However, sequence analysis reveals the closest *A. virens* homolog to the upregulated *A. succinea* and *P. nuntia* genes are not upregulated in *A. virens*. Furthermore, even within the same species, *GST-Omega* expression appears to vary dramatically between Cu contaminated sites (Breton and Prentiss, 2019; McQuillan *et al*., 2014). This divergence suggests independent evolution of *GST-Omega* response between polychaete species. Overall, the upregulation across polychaetes suggest *GST-Omega* genes play important roles in metal detoxification but, as the induced homolog varies between species, no single gene is an obvious cross-species biomarker.

Table 2 Summary of candidate polychaete biomarkers. Where qPCR expression is given, the values correspond to the three-month exposure. ‡No reads were present in the control sample, the fold-change value represents a minimum estimation (estimated using pseudo-counts). Coloured shading represents genes with related functional roles, references: 1 - Lu *et al.* (2011), 2 - Cole (2014), 3 - McQuillan *et al.* (2014), 4 - Breton and Prentiss (2019), 5 - Seehuus *et al.* (2006), 6 - Sun and Zhang (2015), 7 - Nakamura *et al.* (1999), 8 - Kim *et al.* (2008), 9 - Mouneyrac *et al.* (2003), 10 - Gutteridge (1986), 11 - Knapp *et al.* (2006), 12 - Neave *et al.* (2012), 13 - Bundy *et al.* (2008), 14 - van der Vliet *et al.* (2018), 15 - Wolyniec *et al.* (2013), 16 - Alhebshi *et al.* (2012), 17 - Kwon *et al.* (2013), 18 - Rhee *et al.* (2012).

Gene	*Av* Gene (contig/s)	Swiss-Prot Acc,	Differential expression (DE)		Putative function	Confidence as cross-species biomarker given expression in *A. virens* and other polychaetes
			RNASeq	qPCR: low-Cu, -Zn, -Cu-Zn		
GST-Omega-like	c59077_g1_i1	Q6AXV9 (2e-41)	‡ ≥ 1026x ↑	~4500x, 43x, 320x ↑	GST-Omega conjugates metal ions to glutathione for excretion via ATP-dependent pumps[1]. MRP1 is a membrane pump that mediates efflux of xenobiotics, including glutathione conjugates[2].	**Moderate**: *GST-Omega-like* genes up-regulated across polychaete species, but specific orthologues vary[3,4,18]. The co-expression of specific GST-Omega with MPR1 my increase confidence as a biomarker.
MRP1	c82198_g1_i3	P33527 (0)	‡ ≥ 56x ↑	Not tested		
Vit-like	c65732_g3_i1	Q90243 (3e28)	47x ↓	~7x, 1100x, 400x ↓	Defence against oxidative damage in metazoans[5,6]. It is thought that some Vtgs trap metal ions to protect critical cell components in *C. elegans*[7].	**Low:** The modulation of *Vit-like* genes could be conserved across polychaetes. However, more work is needed to compare expression in other polychaete species exposed to Cu/Zn.
Vit-like2	c46551_g1_i1	P18948 (5e-43)	46x ↑	Not tested		
Atox1-like	c43190_g1_i1 c75882_g1_i1	Q9TT99 (5e-11) O08997 (1e-11)	No DE No DE	Not tested Not tested	Binds excess intracellular Cu and transports to secretory pathway[8].	**Very low**: Large variation in response of *Atox1-like* genes to Cu/Zn between polychaete species suggests divergent regulation of these genes in response to metal.
Actin-like	c68928_g1_i1	Q2U7A3 (2e-81)	10643x ↑	~29,000x, 64x, 2x ↑	Unknown cytoskeletal role. Could be involved with sequestering metals into extracellular granules, observed using histology of exposed polychaetes[9].	**Low**: Metal induced expression changes to cytoskeletal genes occur across polychaetes but specific responses between species are divergent.
Calbind-like	84530_g1_i1	O18737 (2e-07)	4495x ↑	~9800x, 19x, 1.25x ↑		
Hb-subunits and *linker chains	c104830_g1_i1 c68421_g1_i2 c55755_g1_i1 c154766_g1_i1 c56032_g2_i1 *c120169_g1_i1 *c36018_g1_i1	P09966 (10e-82) P02219 (2e-30) P02219 (4e-43) P02220 (5e-72) P13578 (3e-83) P18207 (1e-91) P18208 (7-e128)	2970x ↑ ‡ ≥ 907x ↑ 2074x ↑ ‡ ≥ 5293x ↑ 2879x ↑ ‡ ≥ 3335x ↑ ‡ ≥ 4463x ↑	Not tested	Unknown. Cu can change Hb-O_2 affinity[10] or oxidise the haem group to form methemoglobin, which is unable to bind oxygen[11]. We speculate that metal induced Hb/linker chains may produce erythrocruorin molecules containing Hb resistant to Cu-induced conversion.	**High:** A specific set of haemoglobin subunits and linker chain genes is dramatically induced by Cu/Zn exposure in *A. virens*. Previous RNASeq data reveals orthologous haemoglobin subunits are induced by Cu/Zn in phylogenetically distant polychaetes[12]. Furthermore, microarray evidence using earthworms[13] suggests up-regulation of specific haemoglobin subunits in response to Cu/Zn may be conserved across annelids.
Dual oxidase	c85830_g10_i3	Q9VQH2 (0)	‡ ≥ 66x↑	Not tested	Dual oxidase responds to chemical-induced epithelial injury by promoting expression of genes critical to wound response/extracellular matrix remodelling, including MMPs[14].	**Moderate:** Conserved genes with known roles in oxidative stress response but would need testing across phylogenetically divergent polychaetes.
MMP9	c1858_g1_i1	O18733 (4e-28)	‡ ≥ 378x↑	Not tested		
E3 Ubiquitin ligase	c84381_g1_i4	Q5REG4 (3e-17)	‡ ≥ 85x↑	Not tested	Role in proteolysis and mediating responses to cell damage/oxidative stress[15].	**Moderate:** Established association with response to cell damage but, to date, not seen in other metal exposed polychaetes.
ABCE1	c79906_g1_i1	P61222 (2e-96)	204x↑	Not tested	Critical to protein synthesis but has particularly ROS-labile cofactors[16].	**Low:** This highly conserved gene has ROS-labile cofactors (Fe-S clusters)[16] and increasing its expression in yeast rescues ROS toxicity, possibly via Fe-S sequestration into ABCE1 decreases the pool of sensitive Fe-S clusters[16]. Existence of the same mechanism needs establishing in a range of polychaetes.
Hspa8	c57475_g2_i2	Q90473 (2e-35)	725x↑	Not tested	Highly conserved molecular chaperone implicated in the protection of the proteome from metal-induced stress[17].	**Moderate:** A highly conserved molecular chaperone known to help protect the proteome from metal-induced stress[17]. Possible that orthologous gene upregulated in other metal exposed polychaetes[18] but needs further testing to confirm.
MT-CYB	c85209_g5_i1	O03553 (1e-155)	6755x↑	Not tested	Mediates electron transfer from ubiquinol to cytochrome c, contributing to the generation of a proton gradient across the mitochondrial membrane.	**Moderate:** Cu/Zn exposure is associated with mis-regulation of mitochondrial-associated genes in other polychaetes[12] and *MT-CYB* is specifically upregulated in Cu exposed earthworms[13].

3.8. Vitellogenin

Our analysis reveals opposite expression responses of different *vitellogenin-like* (*Vtg-like*) genes in exposed animals, a clear downregulation of *Vtg-like* and contrasting up-regulation of *Vtg-like2* (Table 2). It is not clear whether these expression changes represent metal-induced dysfunction or an adaptive response mechanism to mitigate the effects of exposure. However, there is enough evidence to hypothesise a functional role, in addition to its role as a precursor of egg- yolk protein (Matozzo *et al*., 2008), as it is clear Vtg also protects cells from ROS damage across metazoa (Nakamura *et al*., 1999; Seehuus *et al*., 2006; Sun and Zhang, 2015). The oxidative stress associated with metal exposure may lead to the replacement of Vtg-like proteins with versions that offer better protection, perhaps with increased capacities to trap metal ions (Nakamura *et al*., 1999). No changes to *Vtg-like* gene expression were observed in the polychaete *Ophelina* from a Cu-Zn contaminated site (Neave *et al*., 2012). However, the relatively limited sequencing depth employed for *Ophelina* makes it unlikely the equivalent *Vtg* gene expression changes would be observed. Overall, further work is required to elucidate Vtg's role in polychaete metal/stress response and to determine if these metal-induced expression changes play a functional and conserved role across species.

3.9. Actin-like, Calbind-like and other cytoskeletal associated genes

The large number of cytoskeletal-associated genes upregulated in metal exposed *A. virens* and *Ophelina* (Neave *et al*., 2012) (Fig. S4) suggest these genes might be suitable biomarkers. However, the cytoskeletal-associated genes upregulated in *A. virens* and *Ophelina* are not orthologous and there is no evidence for universal markers. Furthermore, although the qPCR and RNASeq reveal upregulation of the *Actin-like* and *Calbind-like* genes in *A. virens*, the extent of upregulation is extremely variant across samples (Fig. 1). The interplay of factors contributing to cytoskeletal responses is not clear and could be linked to population exposure histories (Mouneyrac *et al*., 2003). Therefore, currently these genes are not suitable biomarkers of metal response (Table 2).

3.10. Complexes of the electron transport chain

Mitochondrial gene mis-regulation has been seen in Cu-exposed earthworms (Bundy *et al*., 2008) and there are established links between metal exposure and mitochondrial dysfunction (Sharpley and Hirst, 2006). The upregulation of the mitochondrial gene Cytochrome b (*CytB*) in *A. virens*, in a manner strikingly similar to that seen in Cu- exposed earthworms (Bundy *et al*., 2008), suggests a plausible biomarker for metal (or at least Cu) exposure (Table 2).

3.11. Haemoglobin (Hb) subunits and linker chains

The enrichment of the GO terms associated with 'oxygen transport' and the 'hemoglobin complex' (Fig. S4) is predominantly due to the upregulation of multiple haemoglobin (Hb) subunits and globin linker chains. Annelids, including *A. virens*, contain giant freely dissolved 'erythrocruorin' respiratory proteins (Hackert and Riggs, 2006; Weber, 1978). These proteins are assembled from multiple Hb subunits and linker chains (Knapp *et al*., 2006) and have been linked to polychaete metal response (e.g. Neave *et al*., 2012). The *Ophelina* transcriptome contained three Hb subunits that include the two paralogues *HbB1a* and *HbB1b*. Critically, while *HbB1b* was consistently expressed, HbB*1a* presented dramatic upregulation in animals from a contaminated site (Neave *et al*., 2012) (Figs. 3 and S9). The *A. virens* transcriptome contains eleven Hb subunit genes (5 paralogue pairs) and four Hb linker chain genes (2 paralogue pairs). These sequences likely represent distinct paralogous genes, as the amino acid identity between the most closely related sequences is ~90%, suggesting too much divergence for allelic variants. Furthermore, given the allelic homogeneity of the animals pooled for RNA samples, the sequences are unlikely to represent polymorphic alleles from divergent genotypes. Interestingly, in a manner strikingly similar to *Ophelina* (Neave *et al*., 2012), for each paralogue pair in *A. virens*, one is expressed consistently and the other is essentially only expressed in exposed animals (Fig. 3 and Table S8). Differences in the number of Hb subunits found in *Ophelina* by in Neave *et al*. (2012) and *A. virens* likely results from the considerable difference in sequencing depth (~650K reads for *Ophelina* (Neave *et al*., 2012) and ~390 million for *A. virens*, Table S3). In Cu-exposed earthworms, upregulation of a Hb subunit in a *Lumbricus rubellus* microarray study (Bundy *et al*., 2008) and increased total Hb levels in metal exposed *Lumbricus terrestris* (Calisi *et al*., 2011, 2013) suggest Hb upregulation may be a general annelid mechanism.

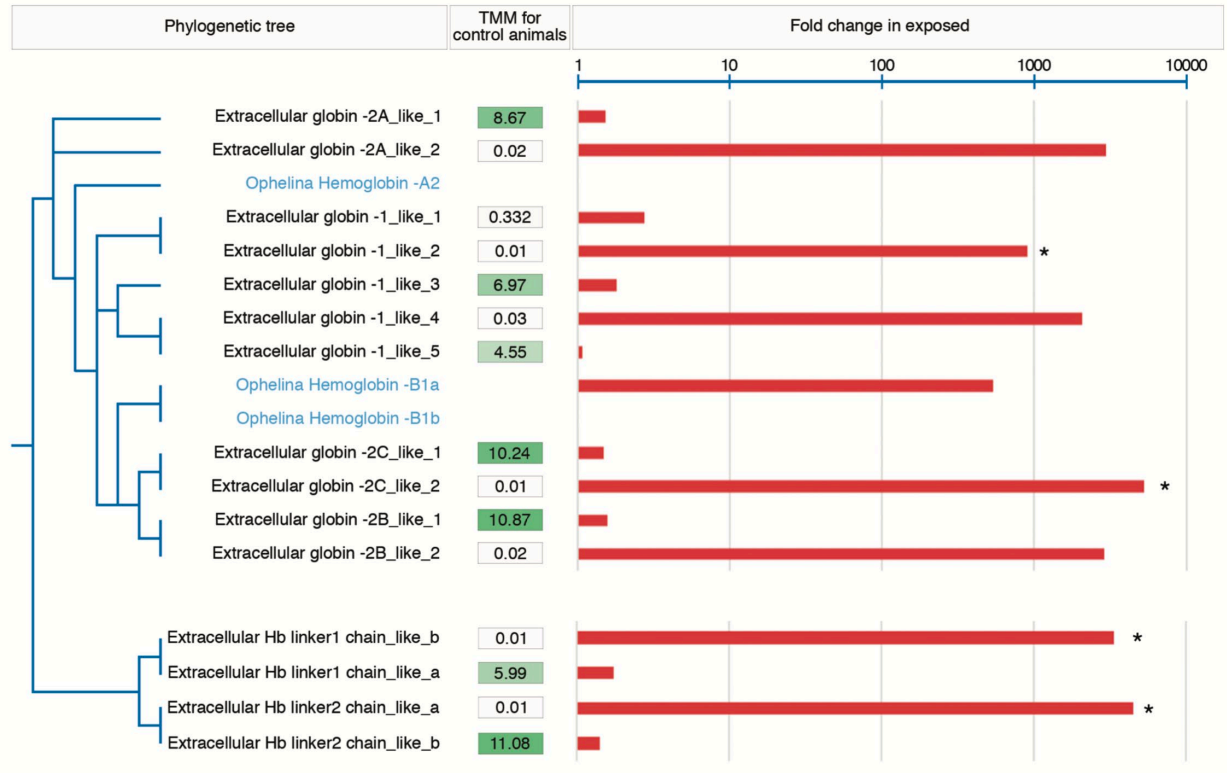

Fig. 3. Haemoglobin (Hb) subunit and linker chain expression changes in the polychaetes *Alitta virens* and *Ophelina* (Neave *et al*., 2012) exposed to metals. Expression observed in control animals is expressed as TMM (white to dark green shows low to high expression respectively). Expression of *Ophelina* genes A2 and B1b is not published but no differential expression was reported. *No reads were present in the control sample, the fold-change value represents a minimum estimation, *Ophelina* Haemoglobin-B1a is based on published read counts (Neave *et al*., 2012). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Assuming the upregulation of the Hb subunits and linker chains represents an adaptive response to stress, their exact role is uncertain. However, their conserved induction across polychaetes is consistent with an important function. It is possible the upregulated subunits and linkers may act in combination with the constitutive genes to produce modified erythrocruorins. However, the co-expression of a large repertoire of subunit and linkers could, at least in theory, lead to the production of entirely distinct erythrocruorins (Knapp *et al*., 2006; Weber, 1978). The subunit upregulation may reflect the need for a greater capacity to transport oxygen when under metal-induced stress, however, the negligible expression in unexposed animals (Fig. 3) suggests that their presence may be counter-productive under normal conditions. Hbs isolated from the blood of polychaetes binds to toxic heavy metals (Demuynck and Dhainaut-Courtois, 1993) and, despite being a critical component of annelid biology (Weber, 1978), Hbs are degraded by ROS and vulnerable to metal exposure (Gutteridge, 1986). Specifically, polychaete erythrocruorin oxygen affinity is altered by Cu/Zn (Everaarts *et al*., 1979) and Cu exposure can oxidise earthworm Hb to produce methaemoglobin, a form that is unable to bind oxygen (Calisi *et al*., 2011). We propose that erythrocruorin produced using the induced genes are resistant to some combination of ROS damage, harmful changes to oxygen affinity, or Hb-methaemoglobin conversion. The hypothesis that modified Hbs can mitigate toxicity is supported by evidence that free cysteines on specific Hb subunits bind hydrogen sulphide in sulphide-rich environments (Bailly *et al*., 2002; Chabasse *et al*., 2006). This capacity may extend beyond polychaetes, as a transcriptomic survey suggests that divergent Hb subunits, in the oligochaete *Olavius algarvensis*, are able to reduce the harmful effects of hydrogen sulphide produced by bacterial symbionts (Wippler *et al*., 2016). Further evidence comes from the polychaete *Amphitrite ornata* that possesses a dehaloperoxidase, evolved from an oxygen carrying haemoglobin, capable of breaking down volatile metabolites secreted as repellents by organisms cohabiting the same ecosystem (Lebioda *et al*., 1999; Barrios *et al*., 2014). Overall, the use of modified Hbs by polychaetes to mitigate environmental stress may be more of a rule than an exception. To determine if the Hb induction is as conserved as suggested by the *A. virens* and *Ophelina* data, the upregulation of Hb subunits and linkers needs to be confirmed in other polychaete species. Also, Hb induction needs to be measured following exposure to specific metals and a range of other stressors, as such data should reveal insights into whether the induced subunits are critical to mitigating the specific effects of metal exposure, or play more general roles in stress response. So, although more work needs to be done, independent evidence of dramatic Hb subunit upregulation in relatively divergent polychaetes, exposed in field and mesocosm scenarios, suggests the Hb subunits could be a conserved and sensitive marker of metal exposure.

Notes

The authors declare no competing financial interest. All sequencing data (both sequencing reads and the transcriptome assembly) will be made available through Genbank (NCBI), BioProject number: **PRJNA627092**.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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