Exposure to low pH induces molecular level changes in the marine worm, *Platynereis dumerilii*

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ABSTRACT

Fossil fuel emissions and changes in net land use lead to an increase in atmospheric CO_2 concentration and a subsequent decrease of ocean pH. Noticeable effects on organisms' calcification rate, shell structure and energy metabolism have been reported in the literature. To date, little is known about the molecular mechanisms altered under low pH exposure, especially in non-calcifying organisms. We used a suppression subtractive hybridisation (SSH) approach to characterise differentially expressed genes isolated from *Platynereis dumerilii*, a non-calcifying marine polychaeta species, kept at normal and low pH conditions.

~~Of the partial~~Several gene sequences have been identified as differentially regulated. ~~several~~ These are involved in processes previously considered as indicators of environment change, such as energy metabolism (NADH dehydrogenase, 2-oxoglutarate dehydrogenase, cytochrome c oxidase and ATP synthase subunit F), while others are involved in cytoskeleton function (paramyosin and calponin) and immune defence (fucolectin-1 and paneth cell-specific alpha-defensin) processes. This is the first study of differential gene expression in a non-calcifying, marine polychaete exposed to low pH seawater conditions and suggests that mechanisms of impact may include additional pathways not previously identified as impacted by low pH in other species.

Keywords:

pH, acidification, polychaete, gene expression

Highlights

- Differentially expressed genes were isolated from *P. dumerilii* kept at low pH conditions.
- Transcripts indicating change in energy metabolism, cytoskeleton and immunity were observed.
- The results indicate that non-calcifying organisms are impacted at low pH exposures.

1. Introduction

The ocean and atmosphere exchange large amounts of carbon dioxide (CO_2) with the atmospheric concentration of CO_2 increasing by 40% since pre-industrial times (IPCC, Climate Change 2013). For the past 200 years the oceans have absorbed approximately one-third of the CO_2 produced by human activity (Sabine et al., 2004) resulting in a measurable decrease of ocean pH. The ocean's surface pH has dropped by 0.1 pH-units which is equivalent to a 26% increase in hydrogen (H^+) ion concentration (IPCC, Climate Change 2013) leading to a current average pH of ocean surface water at around pH 8.1 (IGBP, Ocean Acidification Summary for Policymakers, 2013). Furthermore, global ocean surface pH is predicted to decrease to ~7.8 by 2100 (IPCC, Climate Change 2013). For the last 800 000 years the CO_2 concentration in the atmosphere has thus remained relatively stable (172-300 ppmv) (Lüthi et al., 2008) as compared to the predicted values expected for the end of the century (1071 ppmv) (Plattner et al., 2001).

Rapid changes in water chemistry, termed ocean acidification, are predicted to lower the stability and productivity of ecosystems by loss of biodiversity and keystone sensitive species (Barry et al., 2011). The precise mechanisms are predicted to include many different physiological processes such as photosynthesis, calcification, acid-base homeostasis, respiration / gas exchange and metabolic rate (Melzner et al., 2009; Gattuso et al., 1999; Seibel and Walsh, 2003). To date, the main focus of research has resided on calcifying organisms and molecular-level gene expression impacts following short term low pH exposure (O'Donnell et al., 2010; Todgham and Hoffmann 2009; Stumpp et al., 2011; Martin et al., 2011; Parker et al., 2011; Tomanek et al., 2011; Wong et al., 2011; Moya et al., 2012; Dineshram et al., 2012, 2013; Pespeni et al.. 2013; Vidal-Dupiol et al., 2013). Recent work also suggests that non-calcifying organisms, such as fish, may also be impacted by acidification (Fabry et al., 2008; Wittmann and Pörtner, 2013). Even where taxa are able to tolerate high CO_2 water exposure, there are still apparent impacts in terms of altered energy requirements for basic biological functions, enzyme activities and metabolic shifts (Pörtner et al., 2000;

Seibel and Welsh, 2003; O'Donnell et al., 2010; Wong et al., 2011; Pespeni et al., 2013; Vidal-Dupiol et al., 2013). Specifically, genes involved in energy metabolism and biomineralisation in Japanese rice fish (*Oryzias latipes*), larval sea urchins (*Strongylocentrotus purpuratus*) and oyster (*Crassostrea hongkongensis*) have been shown to be down regulated (Tseng et al., 2013; Todgham and Hofmann, 2009; Dineshram et al., 2013), while genes for ion regulation and acid-base balance pathways have been reported as up-regulated (O'Donnell et al., 2010) in organisms kept at low pH relative to control pH exposure conditions. The data is inconsistent however in that up regulation of energy metabolism genes have also been reported in sea urchin (*S. purpuratus*) and coral (*Pocillopora damicornis*) studies under similar exposure conditions (Stumpp et al., 2011; Vidal-Dupiol et al., 2013). Further biological processes that are potentially impacted by acidification include cell signalling and cytoskeleton assembly (Dineshram et al., 2012). An experimental exposure using oysters (*C. virginica* and *C. hongkongensis*) exposed to high pCO_2 conditions for two weeks, revealed up-regulation of several proteins involved in cytoskeleton and oxidative stress (Tomanek et al., 2011; Dineshram et al., 2013). It is therefore clear that changes in seawater pH have an impact on aquatic organisms, both calcifying and non-calcifying, and that multiple cellular processes may be affected.

In order to understand the molecular-level impacts of acidic pH exposure on the polychaeta species, *P. dumerilii*, differentially - regulated pH-specific mRNA transcripts from worms maintained at control (8.2) and acidified (7.8) pH levels were isolated and identified. *P. dumerilii* is a model organism used for the study of molecular development, evolution, neurobiology, ecology and toxicology (Hardege, 1999; Hutchinson et al., 1995; Tessmar-Raible and Arendt, 2003) and, relevantly, can be found in, and perhaps adapted to, naturally occurring acidified habitats (Cigliano et al., 2010; Calosi et al., 2013). The experimental conditions used in this study represent a pH stress scenario predicted to occur in the surface ocean water column within the next century (Gattuso and Lavigne, 2009).

2. Methods

2.1. Animals and experimental exposure

P. dumerilii (mean mass ± SEM: pH 8.2 worms 1 week: 13 ± 1.55 mg, n=8; pH 7.8 worms 1 week: 10.94 ± 1.76 mg, n=8) from the laboratory culture supplied by EMBL Heidelberg (Germany) were used for the experiment. Atokus worms, a sexually immature stage of the worm, were used to reduce natural variation linked to maturation, reproduction and sex. Specimens were raised in filtered natural seawater (~pH 8.2). For the low pH exposure regime, 8 worms were kept in closed plastic containers (2000 cm^3) with approximately 800 ml filtered natural seawater of pH 7.8 (salinity 35 ppt) at a light regime of 16 hrs light/ 8 hrs dark at 18 °C simulating summer conditions. A further 8 worms were kept at pH 8.2 and in the same experimental conditions as for controls. A complete water change was conducted each day. Hydrochloric acid (1M) and sodium hydroxide (1M) were used to maintain pH levels. Although not identical to the seawater carbonate chemistry changes expected for 2100, the procedure is based on the Le Chatelier's principle and the carbon equilibrium system. Adjusting pH with CO_2 or mineral acid (in a closed system) leads to the same partial pressure of CO_2 and the same CO_2 concentration (Gattuso et al., 2010). After 7 days, chosen to reflect an acclimation time point used in a number of ocean acidification studies (Tomenek et al., 2011; Parker et al., 2011; Moya et al., 2012), each single worm was immediately immersed in 800 µl of RNALater solution (Sigma-Aldrich Company Ltd., Gillingham, U.K.) and stored at -80^oC until molecular analysis.

2.2. Total RNA isolation

Total RNA was extracted from individual worms kept at normal and low pH using High Pure RNA Tissue (Roche, Burgess Hill, U.K.) reagents using the protocol described in the manufacturer's procedures. The RNA concentration was measured using a Qubit® Fluorometer (Life Technologies, Paisley, U.K.) and the integrity of the RNA checked using a standard ethidium bromide stained 1% formaldehyde agarose gel.

2.3. Suppressive Subtractive Hybridization (SSH), sub-cloning and sequencing

The SSH technique was used to isolate and enrich differentially expressed genes between *P. dumerilii* kept at normal and low pH conditions. Equal amounts of RNA were pooled from each worm (n=8 worms/group), with a total of 2.5 µg RNA for each pool. cDNA was synthesised using the SMARTer^{TM} PCR cDNA Synthesis Kit reagents and manufacturer's protocol (Clontech, Saint-Germain-en-Laye, France). The forward and reverse subtracted libraries were produced using PCR-Select cDNA Subtraction reagents (Clontech, Saint-Germain-en-Laye, France) according to the manufacturer's protocol. The differential PCR products generated by SSH were inserted in a pCR^{R}2.1 linearized vector (Life Technologies, Paisley, U.K.) and the constructs were transformed into competent DH10B-T *E.coli* (Life Technologies, Paisley, U.K.). Two hundred randomly selected colonies from each subtracted library were inoculated in LB broth and screened by PCR for inserts using vector-based primers. Fifty two clones were sent for commercial sequencing (Macrogen Europe, Amsterdam, Netherlands). Sequence identities were obtained by BLAST searches against the NCBI nucleic acid and protein databases as well as the PLATYpopsys database (http://hydra.cos.uni-heidelberg.de/pps/). Using the BLAST search results, sequence reads with an *E*-value $<10^{-5}$ were discarded.

2.4. Quantitative RT-PCR (qRT-PCR) validation

Target mRNAs, identified using SSH, were selected for validation using qRT-PCR according to MIQE guidelines (Bustin et al., 2009). Total RNA was isolated from 8 individual worm tissues per exposure group using High Pure RNA Tissue Kit reagents (Roche, Burgess Hill, U.K.) and treated with RNA-free DNase I (Qiagen, Manchester, U.K.). Template RNA was then removed using RNase H enzyme and 10 × buffer (Thermo Fisher Scientific, Loughborough, U.K.) with a 45 min incubation at 37˚C. The RNA concentrations were measured with the Quant-iT RNA assay kit and Qubit® fluorometer (Life Technologies, Paisley, U.K.). Reverse transcription of 140 ng of total

RNA from each individual worm sample (based on the typical yield from each RNA extraction) was carried out using SuperScript VILO cDNA Synthesis reagents (Life Technologies, Paisley, U.K.) and following the manufacturer's instructions. qRT-PCR reactions were performed in duplicate, in a final volume of 20 µl containing 10 µl of qPCR Fast Start SYBR Green Master Rox (Roche, Burgess Hill, U.K.). 1 µl of cDNA and the corresponding primers (see concentrations used in Table 1) were used. Amplification were performed in a CFX96 Real Time PCR Detection System (Bio-Rad, Hemel Hempstead, U.K.) and using the following conditions: after 2 min at 95˚C, 45 cycles at 95˚C for 10 sec, 60˚C for 1 min and 72˚C for 1 min. To generate the melt curve, a heating step gradient from 5 sec at 60˚C to 5 sec at 95˚C, was added to the end of the PCR run. For each of the *target* mRNAs, the melting curve, gel picture and sequences were analysed in order to verify the specificity of the amplified products. The amplification efficiency of each primer pair was calculated using a ten times dilution series of cDNA. Additionally, the crossing point (Cp) was detected for each *target* mRNA and normalised to the reference genes *α-tubulin* (*Tub*), and *18S rRNA* (*18S*) (Zheng et al., 2011; Won et al., 2011).

2.5 Statistical analyses

Statistical analyses were carried out using GraphPad InStat v3 (GraphPad Software Inc., La Jolla, U.S.A.). All data were tested for normality of residuals and the homogeneity of variance. Significance for relative gene expression was tested using an unpaired *t*-test. Outliers were identified, and removed, if they differed by more than twice the standard deviation of the mean. For *paramyosin* (pH 8.2) one value was identified as an outlier (as defined by the MIQE guidelines, Bustin et al., 2009) and excluded from the statistical analysis. Statistical significance was accepted at $p<0.05$.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)	Primer concentration (nM)
18S rRNA	GCGCATTTATCAGCACAAGA	CTTGGATGTGGTAGCCGTTT	239	50
α-Tub	CTTCAAGGTCGGCATCAACT	TGGCAGTGGTATTGCTCAAC	101	100
Calponin	GGAGCCAGTGTGCTTGGT	AGCCTGTCCAGACTTGTCCA	126	100
Paramysin	AGAACGCTGAGGGTGAATTG	CGAGCTGGAGCCTGTCGGCA	183	80
Cytochrome c oxidase	GCGCAGATGTTCGTATGCTA	GAGCCTACTCGGCATCTGTC	197	100
NADH	CGAACCGGATTATGGCTTTG	GGGAATTTGTCCCGTCTGCA	147	100

Table 1. *Target* and *reference* gene primer sequences, amplicon sizes and primer concentrations used for qPCR.

3. Results

3.1. SSH analysis

A total of fifty-two differentially expressed mRNA sequences were isolated and then compared with sequences in the NCBI GenBank database and PLATYpopsys database (http://hydra.cos.uni-heidelberg.de/pps/styled-2/) using the blastn and blastx algorithms. 32% of the sequences libraries matched to genes from different organisms, predominantly invertebrates (Table 2). The remaining sequences showed either similarity to unidentified hypothetical proteins or showed no similarity with the sequences available on the database.

Clone accession no.	Gene identity	Length (bp)	Homolog species	*E*-value	GenBank accession no.	Functional category
	Up-regulated in pH 7.8:					
JZ820677	Paramyosin*	380	*Mytilus galloprovincialis*	0.0	O96064.1	Cytoskeleton
	Calponin*	50	*Echinococcus granulosus*	$4E^{-143}$	CDJ18009.1	Cytoskeleton
KP640621	ribosomal protein L34 (Rpl34)	230	*Cerebratulus lacteus*	$1E^{-15}$	KJ526218.1	Protein synthesis
JZ820678	DNA replication complex GINS protein PSF3	765	*Saccoglossus kowalevskii*	$8E^{-13}$	NM_001184840.1	DNA replication
	Down-regulated in pH 7.8:					
JZ820679	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 7-like	292	*Ceratitis capitata*	$3E^{-7}$	XM_004520945.1	Energy metabolism
JZ820680	Cytochrome c oxidase subunit 6A	489	*Poecilia formosa*	$4E^{-14}$	XM_007557010.1	Energy metabolism
JZ820681	2-Oxoglutarate dehydrogenase*	316	*Pseudopodoces humilis*	0.0	XP_005532951.1	Energy metabolism
JZ820682	ATP synthase F chain	350	*Ixodes scapularis*	$4E^{-6}$	XM_002399310.1	Energy metabolism
KP640622	16S ribosomal RNA	500	*Hediste diadroma*	$8E^{-150}$	AB703100.1	Protein synthesis
JZ820683	Serine protease 55*	168	*Pteropus alecto*	$2E^{-22}$	XP_006906396.1	Hydrolysis of peptide bonds
JZ820684	Ferritin	571	*Perinereis aibuhit-ensis*	$1E^{-173}$	KJ784305.1	Iron storage
JZ820685	WNK lysine deficient protein kinase 1 (WNK1)	234	*H. sapiens*	$3E^{-05}$	NG_007984.2	Ion transport
JZ820686	Calcium-binding and coiled-coil domain-containing protein 2*	363	*Peromyscus maniculatus bairdii*	$1E^{-6}$	XP_006971971.1	
JZ820687	IQ and ubiquitin-like domain-containing protein	711	*S. purpuratus*	$7E^{-64}$	XM_789342.3	Predicted: cilia formation
JZ820688	fucolectin-1	323	*Anguilla japonica*	$1E^{-05}$	AB037867.1	Immune defense
JZ820689	Paneth cell-specific alpha-defensin	366	*Equus caballus*	$5E^{-5}$	NM_001166074.1	Host defence

Table 2 - Differentially expressed (subtracted) mRNAs in *P. dumerilii* maintained at normal or low pH conditions. * indicates hits with PLATYpopsys only and corresponding *E*-value represents the re-blast result of the matched sequence.

3.2. Validation of differentially expressed mRNA transcripts

Four *target* mRNAs were selected to validate the SSH differential transcription results by qRT-PCR (Figure 1). *Calponin* and *paramyosin*, were significantly (p=0.0440; p=0.0386) up-regulated in

worms kept at low pH compared with worms kept at normal pH (Figure 1A-1B). *Cytochrome c oxidase* and *NADH dehydrogenase* were down-regulated in worms kept at pH 7.8 compared with worms kept at pH 8.2 (Figure 1C-1D), though the results were not significant (p=0.2615; p=0.2757).

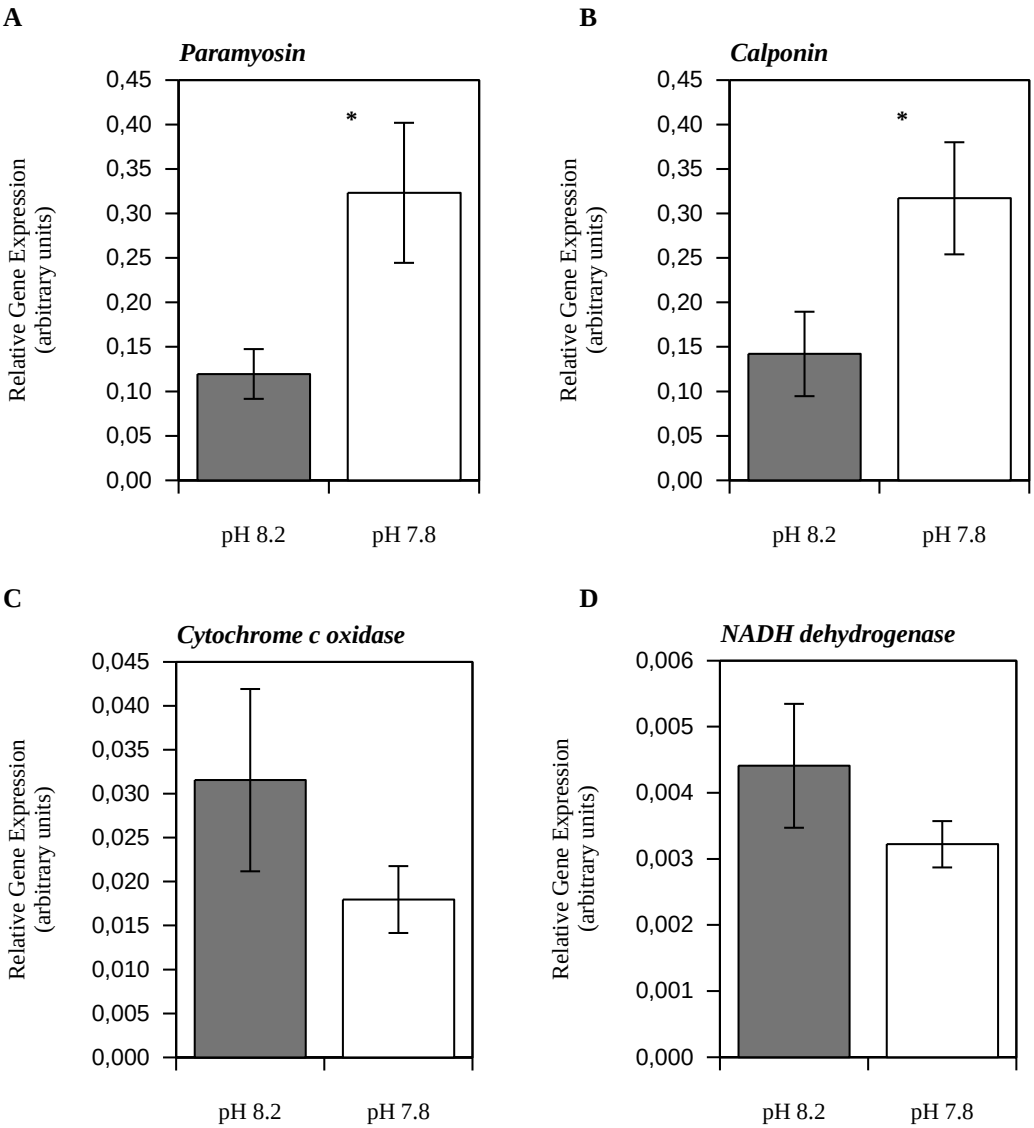

Figure 1. qPCR analysis of selected transcripts identified by SSH as differentially expressed in worms maintained at low pH seawater conditions relative to normal pH conditions. n=8, (with the exception of n=7 for paramyosin at pH 8.2), *p<0.05.

4. Discussion

Using the SSH approach we generated libraries enriched for genes that vary between normal pH

(pH 8.2) and simulated acidification (of pH 7.8) conditions from the marine polychaete worm *P. dumerilii*. In the subtractions reported here two separate libraries were constructed using: a) cDNA from worms exposed to normal pH for one week as driver (reverse library), and b) cDNA from worms exposed to acidified pH conditions for one week as tester (forward library). From these libraries, 33% of the sequences obtained were identified, which is comparable to similar studies using invertebrates (6–44%, Boutet et al., 2008; Craft et al., 2010; Ciocan et al., 2011; Ciocan et al., 2012).

Several mRNA transcripts were identified and validated as up regulated in *P. dumerilii* kept under low pH seawater conditions (Table 2; Figure 1). Amongst these transcripts were proteins involved in metabolism, cytoskeleton function (paramyosin and calponin), protein synthesis (Ribosomal protein L34 and 16S ribosomal RNA), and DNA replication (GINS protein Psf3) functions. For calponin, the sequence isolated is short (at 50 bp) and, as such, only a tentative identity can be assumed. In every case, further work is merited that would ideally seek full sequence lengths and additional functional protein studies.

Changes to metabolic rates have often been observed in organisms under stress conditions. Down regulation of *2-oxoglutarate dehydrogenase*, *NADH-dehydrogenase*, *cytochrome c oxidase*, and *ATP synthase*, collectively representing genes involved in the Krebs cycle and the mitochondrial electron transport chain, indicate a reduction in oxidative metabolism and capacity to generate ATP and NADPH. Similar down regulation of such metabolism-related genes has been reported in coral (*A. millepora*) and sea urchin (*S. pupuratus*) larvae after 28 days high pCO_2 exposure and 40 hours post hatching with medium level CO_2 exposure treatment respectively (Kaniewksa et al., 2012; Todgham and Hoffmann, 2009). In a study using the scleractinian coral, *P. damicornis*, *2-oxoglutarate dehydrogenase* was observed to be slightly up regulated at pH 7.8 and subsequently down-regulated at pH 7.4 (Vidal-Dupiol et al., 2013). Also, in contrast to our findings, Vidal-Dupiol et al. (2013) reported an enrichment of genes involved in oxidative phosphorylation (among them NADH dehydrogenase) at pH 7.8 and 7.2 in coral. Oyster (*C. hongkongensis*) and sea

urchin (*Lytechinus pictus*) larvae behaved similary to the worms however, with an apparent down regulation of *cytochrome c oxidase* and *NADH dehydrogenase* at low pH (Dineshram et al., 2013; O'Donnell et al., 2010).

Of the cytoskeleton function transcripts identified, *paramyosin* is primarily a muscle thick filament protein that is common in invertebrate species (Hooper et al., 2008). Unusually, paramyosin has roles in both muscle contraction as well as immunoregulation (Gobert et al., 2005). Nematode muscles consist of a contractile part made up of fibres with myosin, actin, tropomyosin and paramyosin, and a non-contractile part supplying energy requirements (Hooper et al., 2008). Related to this is another of the isolated transcripts, *calponin*. Calponin (calcium- and calmodulin-binding troponin T-like protein) is a calcium-binding protein that inhibits ATPase activity of myosin in smooth muscle (Castagnone-Sereno et al., 2001). An up regulation of *paramyosin* and *calponin* transcripts following acidic pH seawater exposure is consistent with other investigations reported in the literature. Adult oysters (*C. virginica*) and oyster larvae (*C. hongkongensis*) exposed to pH 7.9 – pH 7.5 displayed increased calponin-2 and myosin (light chain) expression respectively (Tomanek et al., 2011; Dineshram et al., 2013).

In addition to muscle microfilament upregulation, there was also an apparent increase in protein synthesis related (ribosomal protein L34) and DNA replication (GINS protein Psf3) processes (Table 2). Such changes are common following stress conditions and consistent with findings for other invertebrate species, including oysters (*C. virginica*) exposed to low pH (Tomanek et al., 2011). Specifically, Psf3, is a protein from the Psf family, involved in cell cycle, and has been observed herein as up regulated in worms kept at low pH (7.8) similarly to the *Psf2* transcript identified in sea urchin (*S. purpuratus*) larvae kept under a moderate level of acidification conditions (Todgham and Hofmann, 2009). In contrast, the marine coccolithophore, *Emiliania huxleyi*, responded to low pH, with a down-regulation in the chloroplastic 30S ribosomal protein S7 (Jones et al., 2013).

Down regulation of the immune-related transcripts *fucolectin* and *paneth cell-specific alpha defensin* was observed in worms kept at low pH conditions (Table 1). Fucolectins are fucose binding proteins that have a pathogen recognition role in fish (Honda et al., 2000), while alpha defensins are microbiocidal and cytotoxic peptides involved in host defence (Szyk et al., 2006). Defensins have previously been isolated from invertebrate species such as the horseshoe crab (*Tachypleus tridentatus*, Kawabata, 2010) and oyster (*C.gigas*, Rosa et al., 2011), but, to our knowledge, this represents the first such isolation from a polychaete species (Smith et al., 2010). Immunosuppression upon contaminant exposure is well documented for marine vertebrates (de Swart et al., 1996). To what extent pH changes will similarly impact the immune system of invertebrates is less extensively studied. Bibby et al. (2008) reported that several components of mussel (*Mytilus edulis*) immune response, including suppressed phagocytosis, were modulated following (32 day) exposure to acidified seawater. A similar trend in immune suppression has also been reported in the star fish *Asterius rubens* (Henroth et al., 2011). Calcium ion concentrations have been suggested as the mechanism by which acidification causes suppression of the immune system (Bibby et al., 2008).

The other genes down regulated in worms exposed to low pH include (IQ ubiquitin-like domain-containing protein (IQUB), ferritin and WNK1 (Table 1). IQUB is a putative cilia protein (Lai et al., 2011) previously observed as down regulated in larvae hatched from gastropod egg masses that had been co-exposed to temperature and UVB stresses (Fischer and Phillips, 2014). Ferritin stores iron and protects cells from iron-induced redox damage while also controlling its' release for different enzymatic reactions (Theil, 1987). In an investigation of the interactive effects of acidification (using elevated CO_2 levels for a duration of 4 to 5 weeks) and metal exposure in oysters (*C. virginica*), Goetze et al. (2014) reported pCO_2 potentiation of metal-induced ferritin expression. WNK1 down regulation in worms exposed to low pH is also interesting in that WNK1 in vertebrates is a serine-threonine kinase expressed in kidneys that phosphorylates synaptotagmin 2, leading to activation of the epithelial sodium channel and also stimulates endocytosis of

ROMK1, an ATP dependent potassium channel (Kahle et al., 2006). The observed differential expression of the WNK1 gene is therefore interesting because no common sodium, calcium nor carbonate transporter protein transcripts are among those differentially expressed in the worms kept at low pH in this study, and as such WNK1 may represent an alternative mechanism of ion transport and balance in this worm relative to other species.

5. Conclusion

Using an SSH transcriptomic approach, differentially expressed genes were identified in worms kept a low pH seawater conditions. Such worms represent a non-calcifying marine species. Particularly novel findings are differentially regulated transcripts involved in cytoskeleton processes and the immune system. These findings demonstrate that non-calcifying organisms, as well as calcifying organisms, are also at risk of potential future ocean acidification impacts.

Conflict of Interest Statement

None declared.

Acknowledgements

We would like to thank the Arendt Group (EMBL Heidelberg) for access to the PLATYpopsys database. We would also like to thank Tomas A. Larsson (Department of Chemistry & Molecular Biology, University of Gothenburg) for conducting the laboratory exposure experiment.

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