**Transcriptome-wide analysis of the response of the thecosome pteropod *Clio pyramidata* to
short-term CO₂ exposure**

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Abstract

Thecosome pteropods, a group of calcifying holoplanktonic molluscs, have recently become a research focus due to their potential sensitivity to increased levels of anthropogenic dissolved CO₂ in seawater and the accompanying ocean acidification. Some populations, however, already experience high CO₂ in their natural distribution during diel vertical migrations. To achieve a better understanding of the mechanisms of pteropod calcification and physiological response to this sort of short duration CO₂ exposure, we characterized the gene complement of *Clio pyramidata*, a cosmopolitan diel migratory thecosome, and investigated its transcriptomic response to experimentally manipulated CO₂ conditions. Individuals were sampled from the Northwest Atlantic in the fall of 2011 and exposed to ambient conditions (~380 ppm) and elevated CO₂ (~800 ppm, similar to levels experienced during a diel vertical migration) for ~10 hrs. Following this exposure the respiration rate of the individuals was measured. We then performed RNA-seq analysis, assembled the *C. pyramidata* transcriptome *de novo*, annotated the genes, and assessed the differential gene expression patterns in response to exposure to elevated CO₂. Within the transcriptome, we identified homologs of genes with known roles in biomineralization in other molluscs, including perlucin, calmodulin, dermatopontin, calponin, and chitin synthases. Respiration rate was not affected by short-term exposure to CO₂. Gene expression varied greatly among individuals, and comparison between treatments indicated that *C. pyramidata* down-regulated a small number of genes associated with aerobic metabolism and up-regulated genes that may be associated with biomineralization, particularly collagens and C-type lectins. These results provide initial insight into the effects of short term CO₂ exposure on these important planktonic open-ocean calcifiers, pairing respiration rate and the gene expression level of response, and reveal candidate genes for future ecophysiological, biomaterial and phylogenetic studies.

Keywords: acidification, climate change, gastropod, lectin, mollusc, RNA-seq, transcriptomics

Introduction

The dissolution of anthropogenic carbon dioxide (CO₂) into seawater, or ocean acidification (OA), stands to impact a broad variety of marine organisms, especially those that secrete calcium carbonate (CaCO₃) shells or skeletons (Fabry et al., 2008). As CO₂ equilibrates into seawater, pH and the concentration of carbonate ions decrease, causing CaCO₃ to dissociate and affecting the ability of calcifying animals to create and maintain calcareous structures (Gattuso et al., 1999; Riebesell et al., 2000). Furthermore, decreasing pH in the water alters the acid-base balance of the intra- and extracellular fluids of marine organisms (Miles et al., 2007; Seibel and Fabry, 2003; Seibel and Walsh, 2001). Since CO₂ is produced naturally as a byproduct of respiration, all organisms have physiological mechanisms for maintaining internal cellular pH. These compensatory mechanisms may, however, cease to function if internal acidosis becomes too severe over short time scales and generally fail over extended periods of internal pH imbalance. Classical organismal-level metrics (e.g., metabolic rate, calcification, mortality) have revealed a complex pattern of intra- and inter-specific variation in responses to CO₂ exposure (reviewed in Hendriks et al., 2010; Kroeker et al., 2013; Kroeker et al., 2010). The variability in these responses, and their dependence on duration of exposure or co-varying stressors, suggest that there is an energetic cost associated with compensating for high CO₂ (e.g., Cummings et al., 2011; Langenbuch and Pörtner, 2004; Stumpp et al., 2011b).

One group thought to be particularly sensitive to OA is the thecosomatous (i.e., shelled) pteropods. These holoplanktonic gastropods, related to terrestrial snails and also known as sea butterflies, produce thin shells made of aragonite, a highly soluble form of CaCO₃. In temperate and polar seas, thecosomes can become a numerically dominant member of the zooplankton community (Hunt et al., 2008). This causes them to be significant consumers of primary production, serve as a key food item for commercially important fish, seabirds and whales, and substantially contribute to carbon flux to the deep ocean (reviewed in: Bednaršek et al., 2012a; Lalli and Gilmer, 1989; Manno et al., 2010). Studies of thecosome biomineralization have revealed that increasing CO₂ levels result in decreased calcification and shell degradation (Bednaršek et al., 2014; Bednaršek et al., 2012b; Comeau et al., 2009; Lischka and Riebesell, 2012; Manno et al., 2012). It remains unclear whether the decreases in calcification documented in thecosomes at high CO₂ concentrations are a result of their inability to form carbonates, or rather are a result of a re-allocation of energy, as has been described in other species (e.g., Melzner et al., 2013; Stumpp et al., 2012).

The impact of CO₂ on the metabolic rate and mortality of thecosome pteropods has been shown to be variable, and highly dependent upon the presence of co-varying stressors such as salinity, temperature and feeding history (Comeau et al., 2010a; Lischka and Riebesell, 2012; Maas et al., in review; Manno et al., 2012; Seibel et al., 2012). In contrast to laboratory findings concerning the effects of under-saturation on pteropods, Maas et al. (2012) found that some species of thecosomes regularly experience conditions of high CO₂ and aragonite saturation states <1 during diel vertical migrations into an oxygen minimum zone in the eastern tropical North

Pacific, and do not respond to short-term laboratory CO₂ exposure with a change in metabolic rate. This suggests that in some thecosome species there is a potential adaptation or acclimation to high CO₂, at least over short periods. Supporting this idea, more recent work comparing thecosomes from the Northeast Pacific and Northwest Atlantic revealed that the metabolic rates of multiple thecosome pteropod species are not affected by short-term exposure to CO₂ at the levels predicted for the coming century (800 ppm; Maas et al., in review). Exploring the physiological mechanisms these species use to cope with short-term CO₂ exposure may provide insight into what duration and level of exposure to anthropogenic CO₂ thecosomes can tolerate. Elucidating these mechanisms will also provide a means to characterize the physiological condition of pteropods within natural populations and enable development of molecular biomarkers indicating responses to elevated CO₂ exposure.

An increasing number of studies have employed molecular methods to disentangle the complex picture associated with CO₂ response, revealing changes in expression of genes with a known or predicted role in acid-base balance, apoptosis, biomineralization, development, protein synthesis, energetic metabolism, and stress responses (Hüning et al., 2013; Kaniewska et al., 2012; Kurihara et al., 2012; Moya et al., 2012). For example, Moya et al. (2012) showed that coral larvae respond to CO₂ through changes in gene expression that were consistent with suppressed metabolism and changes in calcification. They also discovered an unexpected lack of response in ion transport proteins and a down-regulation of carbonic anhydrase genes. Relatively few studies have matched observations of altered gene expression with organismal-level observations such as developmental delays or changes in metabolic rate (e.g., Stumpp et al., 2011b). Many of the previous studies have used quantitative PCR (qPCR), a targeted approach which involves picking a small number of genes *a priori* such as studies with mussel (Hüning et al., 2013), abalone (Zippay and Hofmann, 2010), urchin (Stumpp et al., 2011a), and oyster (Liu et al., 2012), or using microarrays, which have historically only been done with well-studied organisms such as urchins (Evans et al., 2013; O'Donnell et al., 2010; Todgham and Hofmann, 2009) and corals (Kaniewska et al., 2012).

Despite the benefits of using gene expression studies for understanding the response to CO₂ at a mechanistic level, there have been no published studies of pteropod gene expression to date. High throughput RNA sequencing (RNA-seq) methods enable quantitative characterization of gene expression patterns across the transcriptome (Ekblom and Galindo, 2011; Vijay et al., 2012). When a reference genome is not available, a transcriptome can be assembled *de novo* from millions of short reads. The reads from individual samples can then be mapped onto this reference transcriptome for a comprehensive assessment of differential gene expression. Furthermore, once a transcriptome has been assembled, it can serve as a resource for targeted analyses of individual genes. In the context of ocean acidification, these targeted analyses may include the identification of pteropod homologs of genes associated with calcification in other mollusc species.

A number of recent papers have focused on the characterization of biomineralization proteins and genes in molluscs (Gardner et al., 2011; Jackson et al., 2010; Joubert et al., 2010; Shi et al., 2012; Zhang and Zhang, 2006; Zhao et al., 2012a), while other studies have increased the number of available molluscan transcriptomes to include pearl oysters (Huang et al., 2012; Zhao et al., 2012a; Zhao et al., 2012b), sea hares (Fiedler et al., 2010; Heyland et al., 2011), freshwater snails (Sadamoto et al., 2012), abalone (Franchini et al., 2011; Picone et al., 2015), scallops (Artigaud et al., 2014), mussels (Freer et al., 2014), and clams (Clark et al., 2010; Sleight et al., 2015). These studies confirm that molluscan biomineralization is a complex process with some conserved pathways shared between distantly related animal groups, and other proteins and compounds that are taxonomically restricted (Jackson et al., 2010), as has been shown in more distantly related animal groups (Jackson et al., 2007; Moya et al., 2012; Moya et al., 2008). Comparing the transcriptomic underpinnings of shell accretion in thecosomes with those in other molluscs may thus provide a useful contrast because of the different ecological pressures of a holoplanktonic lifestyle and the structures and processes associated with building a calcium carbonate shell principally (> 50%) comprised of aragonite (Bé and Gilmer, 1977). Beyond understanding the mechanism of calcification to explore the specific effects of climate change on shell formation, biomineralization in the molluscan lineage may thus also be interesting from a material properties and evolutionary standpoint (Jackson et al., 2010; Xie et al., 2011).

In this study, we focus on *Clio pyramidata* (Linnaeus, 1767), a common circumglobal species that has a multilayered aragonite shell, and is known to migrate vertically on a diel basis into regions of high CO₂ in some ocean basins (Maas et al., 2012). As in most thecosomes, the growth sequence and processes of biomineralization for this species are poorly-characterized and no gene sequences beyond the barcoding regions were known. In this study, we have assembled and annotated a *de novo* transcriptome for *C. pyramidata*. To characterize the transcriptome, and to gain insight into the effects of short-term CO₂ exposure (similar to what would be experienced during a diel migration), we then exposed *C. pyramidata* to ambient or elevated CO₂ concentrations, measured respiration rate, and used RNA-seq to test for changes in gene expression. By pairing these two metrics we hoped to shed light on the lack of a significant effect of CO₂ exposure on *C. pyramidata* oxygen consumption rates that has been observed previously in similar metabolic experiments in the Pacific (Maas et al., 2012). Specifically, transcriptional profiling was used to investigate whether this lack of response to short term CO₂ exposure is due to an absence of physiological stress, or is rather a product of the redistribution of energetic resources.

Materials and Methods:

Animal Collection and Experimental Setup:

C. pyramidata were collected from the R/V *Oceanus* in August 2011 at an off-shelf station east of the Grand Banks in the Northwest Atlantic (44.94°N, 42.00°W). Animals were captured using

a 1 m diameter, 150 μm mesh, Reeve net. *Clio pyramidata* are small, relatively rare, difficult to capture without damaging, and hard to maintain in shipboard conditions, limiting the numbers available for experimentation. Individuals for this experiment were collected from the same net tow to minimize pre-existing environmental variability and handling differences between tows (hydrographic data and tow details in Supplementary File 1). *Clio pyramidata* from this tow were placed in open jars of locally collected filtered seawater (33 psu) at 15°C in temperature-controlled waterbaths at densities < 15 individuals L^{-1} for 12 h (± 30 min) to allow for gut clearance. This temperature and salinity were chosen as individuals of this species had recently been sampled under these conditions.

CO₂ Exposures and Respiration Experiments

After the gut clearance period, healthy individuals (swimming and with intact shells) were selected for closed chambered respiration experiments using a Clarke-type oxygen electrode method (Maas et al., 2012; Marsh and Manahan, 1999). Individuals were placed in separate 20 mL air-tight glass chambers with 0.2 micron filtered seawater. The experimental water (33 psu) had previously been bubbled in 1 L batches for 45-60 minutes with ambient air (~ 380 ppm CO₂) or a certified gas mixture containing 800 ppm CO₂ ($\pm 2\%$, Corp Brothers; 21% oxygen, balanced with nitrogen). This treatment was applied to simulate projected levels for the open ocean in the year 2100 (A2 emissions scenario, I.P.C.C., 2007) and is a level that *C. pyramidata* is known to currently experience in other portions of its distribution (i.e., the North Pacific). The water contained 25 mg L^{-1} each of streptomycin and ampicillin to prevent bacterial growth, a procedure that has been shown to have no effect on the respiration rate of thecosome pteropods (Howes et al., 2014).

For every 3 experimental glass chambers, another control glass chamber was set up with the same water but without an animal to enable monitoring of background bacterial respiration. Respiration chambers were maintained at temperature in a waterbath during experimental incubations, after which the oxygen saturation in the chamber was measured. Duration of exposure varied slightly due to the sequential measurement of O₂ concentration in successive chambers at the beginning and end of the experiments, but CO₂ exposures were between 9.5-10 hours for the high treatment and 10.25-10.75 h for the ambient treatment (to replicate diel vertical migratory periods). For each measurement, an aliquot of water was withdrawn using an airtight 500 μL Hamilton syringe and injected past a Clarke-type microcathode (part #1302, Strathkelvin Instruments, North Lanarkshire, United Kingdom), which was attached to an O₂ meter (part #782) in a water-jacketed injection port (part #MC100), and the reading was allowed to stabilize for at least 30 seconds prior to being recorded. At the conclusion of the experiments, oxygen in the chambers remained between 73-96% of saturation, above the critical oxygen partial pressure typical of marine animals (Childress and Seibel 1998). The animals were then removed from the chamber, blotted dry, and frozen in liquid nitrogen. Total handling time of

organisms during the final O₂ measurements was ~10 min per respiration experiment, with less than a minute between removal from the chamber and preservation to minimize handling stress.

The same individuals used in the study of oxygen consumption were stored at -80°C for transcriptomic analysis. Prior to RNA extraction, individuals were quickly weighed on a microbalance (± 0.0001 g) to allow for the calculation of their mass specific O₂ consumption rate ($\mu\text{moles g}^{-1} \text{ h}^{-1}$ wet weight). Tissue was then dissected from the shell to avoid both the possible pH interference due to the calcium carbonate of the structure and contamination by other taxa trapped in the shell. Statistical analysis of respiration rate was conducted using a general linear model in SPSS on log-transformed oxygen consumption, with treatment as the random factor and log-transformed wet mass as a covariate.

Carbonate Chemistry

DIC measurements were taken after each experiment from control syringes that had been allowed to come to room temperature (>6 h) before analysis. DIC samples were analyzed on a DIC auto-analyzer (AS-C3, Apollo SciTech, Bogart, USA) via acidification, followed by non-dispersive infrared CO₂ detection (LiCOR 7000: Wang et al., 2013a; Wang and Cai, 2004). Samples were measured a minimum of three times to obtain replicate measurements. The instrument was calibrated with certified reference material (CRM) from Dr. A.G. Dickson at the Scripps Institution of Oceanography and has a precision and accuracy of $\pm 2.0 \mu\text{mol kg}^{-1}$. Best practices for calculating carbonate chemistry (Riebesell et al., 2010) include measurement of two of the CO₂ system parameters. Due to the small volumes of water in the experimental chambers it was not possible to measure both DIC and total alkalinity simultaneously from the control syringes, and instead total alkalinity of batches of water (collected weekly) was measured using an Apollo SciTech alkalinity auto-titrator, a Ross combination pH electrode, and a pH meter (ORION 3 Star) based on a modified Gran titration method (Wang et al., 2013b). These batches of water were then used for multiple experiments over the course of several days. Unfortunately, the alkalinity changed over time, likely due to microbial activity, and measurements proved not to have been taken at sufficient temporal resolution to constrain the carbonate chemistry within this experiment using total alkalinity. Subsequent diffusion calculations, however, indicated that the 45 - 60 min bubbling was sufficient to fully achieve the targeted $p\text{CO}_2$ levels. As such we used the manufacturer's certification of $p\text{CO}_2$ within the gas mixture as our second parameter to characterize the carbonate system. Other carbonate system parameters, including aragonite saturation state, were calculated using the CO₂SYS software (Pierrot et al., 2006), the carbonic acid dissociation constants by Mehrbach et al. (1973), refitted by Dickson and Millero (1987), and the KHSO₄ dissociation constant from Dickson (1990). The $\pm 2\%$ error in CO₂ concentration reported by the gas supplier was incorporated into the calculations of the carbonate chemistry to characterize uncertainty.

RNA Extraction and Illumina Sequencing

RNA was extracted from individuals with either the Aurum Total RNA Fatty and Fibrous Tissue Kit (BioRad) or the E.Z.N.A. Mollusc RNA Kit (Omega Biotek) and eluted in 25-40 μ L molecular biology grade water. Both methods yielded one sharp band of RNA with no signs of degradation on a denaturing agarose gel and on a Bioanalyzer profile. This indicates that pteropods, like some other protostomes, likely have a delicate 28S ribosomal RNA strand that breaks during extraction, making a traditional quality score relatively uninformative (Gayral et al., 2011; Winnebeck et al., 2010).

RNA extracts from four individuals per treatment (8 individuals in total, A-D exposed to elevated CO₂, E-H exposed to ambient) were chosen based on RNA quality and yield. RNA was submitted to the Genomic Sequences Laboratory of Hudson Alpha for library construction and sequencing. Libraries were constructed using Tru-Seq RNA Sample Prep Kits, multiplexed, and sequenced in duplicate on two lanes of the Illumina HiSeq platform (v. 1.9) as 50 base pair (bp) paired-end reads.

Assembly and Annotation

Raw sequences were quality filtered and assembled into transcripts *in silico*. The resulting transcripts were annotated to indicate their similarity to genes with known function. To accomplish this, adapter sequences and bases with a phred quality score below 30 were first removed using Trimmomatic (Lohse et al., 2012). *De novo* transcriptome assembly was performed with Trinity (version r2013-02-25: Grabherr et al., 2011) using a pooled file of all reads from both treatments and the default parameters (minimum contig length: 200, k-mer length 25). The assembled transcripts were imported into Blast2GO (Conesa et al., 2005), where those > 200 bp were compared to the SWISS-PROT database using BLASTX with an e-value <1.0 e⁻⁶ to remain relatively consistent with other recent non-model marine transcriptomes (i.e., Burns et al., 2013; Craft et al., 2010; de Wit and Palumbi, 2013; Harms et al., 2013). These transcripts were further annotated using the Blast2GO program that assigned Gene Ontology (GO) terms for those with an e-value <1.0 e⁻¹⁰ to reduce the probability of mis-annotation (du Plessis et al., 2011).

Analysis of Biomineralization Genes

To specifically search for transcripts associated with biomineralization, we utilized the recent annotation of the oyster genome, which includes both a set of genes that were either highly or differentially expressed in shell mantle tissue and a comparison of oyster genes with sequences known to be associated with shells in other molluscs (Zhang et al., 2012). We assembled a BLAST database using the Genbank accession numbers for these mantle and biomineralization proteins. We then conducted a TBLASTN search to find putative homologs of these genes within the *C. pyramidata* transcriptome (e-value <1.0 e⁻⁶). Transcripts of interest, such as those that showed homology to perlucin sequences in the molluscan biomineralization database were again used to query the NCBI non-redundant database (nr) using BLASTX as implemented in

Blast2GO. The sequences exhibiting greatest similarity to annotated perlucin sequences (five sequences with lowest e-values) were translated *in silico* using the ExPASy translate tool (Artimo et al., 2012). Stop codons were trimmed, and then these sequences were aligned with annotated perlucin protein sequences from other molluscs using MUSCLE (Edgar, 2004). These included the perlucin sequences that have been shown to be associated with biomineralization in the triangle sail mussel (*Hyriopsis cumingii*, GenBank KC436008: Lin et al., 2013), the abalone (*Haliotis laevigata*, P82596.3: Mann et al., 2000), and from the disk abalone (*Haliotis discus discus*, ABO26590.01: Wang et al., 2008). Also included in the alignment was the C-type lectin from the bay scallop that appears to be associated with immune response (*Argopecten irradians*, ADL27440.1 Mu et al., 2012), as well as sequences inferred *in silico* such as the perlucin sequences associated with the Pacific oyster (*Crassostrea gigas*, EKC39512.1: Zhang et al., 2012) and the abalone (*Haliotis diversicolor*, AEQ16377.1) and the C-type lectins from the pearl oyster (*Pinctada fucata*, ACO36046.1) and the clam (*Ruditapes philippinarum*, ACU83213).

Differential Expression

The reads from each library were pooled and separately mapped to our reference transcriptome using Bowtie (Langmead et al., 2009) via the RSEM script that is associated with the Trinity package set with default parameters (Haas et al., 2013). To compare overall gene expression patterns among individuals prior to identifying differentially expressed genes, we used the statistical package Primer (Clarke and Gorley, 2006) to construct a Bray-Curtis similarity matrix of the standardized edgeR normalized (TMM, trimmed mean of M-values; Robinson and Oshlack, 2010) fragments per kilobase per million reads mapped (FPKM) transcript counts of all genes (Trinity components) expressed in at least two individuals (to reduce variability caused by rare transcripts). The edgeR package was then used to identify genes (Trinity components) that exhibited significant differences in expression between the two CO₂ treatments (Robinson et al., 2010). Results were reported as significant when the change in log₂-transformed gene expression was greater than two (corresponding to a four-fold change in expression) and the p-value and false discovery rate were < 0.05. These differentially expressed genes were re-annotated using a BLASTX against the NCBI non-redundant (e-value < 1.0 e⁻³) and Interpro (Hunter et al., 2011) databases.

Results

Consistent with experiments conducted in other ocean basins (Maas et al., in review; Maas et al., 2012), wild caught *C. pyramidata* from the Northwest Atlantic that were exposed to either ambient (~380 ppm) or elevated (~800 ppm) CO₂ for ~10 h, simulating the timing of exposure associated with diel vertical migration, showed no significant difference in respiration rate. Using these same experimental individuals, libraries were sequenced from eight pteropods (4 per treatment) using Illumina HiSeq technology. These libraries were used to build a reference transcriptome for *C. pyramidata* that revealed a number of biomineralization-associated genes similar to those found in other molluscan lineages. Only a small number of genes exhibited

significant changes in expression in association with short-term exposure to moderately elevated CO₂.

Respiration Experiments and Carbonate Chemistry

The experimental CO₂ exposure achieved an aragonite saturation state (Ω_{Ar}) of 2.44 and pH of 8.06 in the ambient treatment (380 ppm CO₂) and an Ω_{Ar} of 1.41 and pH of 7.78 (800 ppm CO₂) in the elevated treatment (Table 1). Incorporating the uncertainty in pCO₂ based on the manufacturer's certification of the gas mixture, we calculated a potential error of ± 0.03 in Ω_{Ar} and ± 0.01 in pH for the 800 ppm treatment; thus our treatments were near the targeted values and achieved the desired result of acutely different exposure conditions. There were no significant differences in the respiration rate of individuals from the two treatments; this also remained true when considering a larger sample size of individuals from more sample locations in the Atlantic as well as from the Pacific Ocean (Fig. 1). There were no significant differences between the treatments within the Atlantic ($F_{1,14} = 0.037$, $p = 0.850$), nor when individuals from the Pacific were included in the analysis ($F_{1,42} = 0.540$, $p = 0.466$).

Assembly and Annotation

Sequencing resulted in ~50 million reads per individual after quality trimming. The assembled transcriptome contained 30,800 components ("genes") and 45,739 transcripts ("isoforms") greater than 200 bp, an average transcript length of 618 bp, and an N50 of 852 bp. We were able to annotate 9,280 of the transcripts (20%) based on similarity to genes within the SWISS-PROT database using an e-value cut-off of 1.0×10^{-6} . The percent of annotated sequences was positively correlated with transcript length; however, we were still unable to identify 70% of the longest sequences (>2,000 bp). Gene ontology (GO) terms were assigned to 7,989 of the transcripts with significant BLAST hits (86%).

Candidate biomineralization-associated transcripts

A number of genes that have previously been shown to be involved with biomineralization have potential homologs within the pteropod transcriptome. Queries of the pteropod transcriptome with 45 biomineralization-associated genes from other molluscs resulted in identification of 831 *C. pyramidata* transcripts. These included 13 transcripts that were annotated as carbonic anhydrase precursors, 59 as calmodulin or calmodulin-like proteins, 10 chitin synthases, and surprisingly 282 transcripts annotated as perlucin (Supplementary File 2). In addition, we identified 56 transcripts through queries with 23 sequences that were up-regulated in oyster mantle (Zhang et al., 2012). Transcripts that showed homology to perlucin sequences in the molluscan biomineralization database were BLASTed against the nr database to help provide a list of the most likely perlucin candidates. When translated, the top five matches (based on e-value) aligned well with previously identified perlucin transcripts from various other molluscs (Fig. 3), revealing the six highly conserved cystine residues, the residues putatively involved in calcium-dependent carbohydrate binding (Mann et al., 2000) and the motifs proposed to

determine carbohydrate-binding specificity for galactose (QPD) binding (Iobst and Drickamer, 1994).

Differential expression

There was a high degree of variability in overall transcript expression patterns among individuals (20-80% similarity in pairwise comparisons, Fig. 2). Between treatments 29 genes were differentially expressed, four of which were down-regulated in high CO₂ and the rest of which were up-regulated (Table 2). Of the four genes that were down-regulated in individuals exposed to high CO₂, two were annotated: both were associated with the mitochondrial inner-membrane complex (cytochrome c oxidase subunit I and III), however there was high variability in their expression levels within treatments. Of the 29 genes that were up-regulated only six could be annotated through BLASTX searches of the NCBI non-redundant database and an InterPro Scan. Two of these were associated with carbohydrate binding of the C-type lectin family (conglutinin and a lactose-binding lectin that was also identified as being a perlucin homolog), one was associated with protein binding, another with the regulation of transcription via DNA binding, another with hydrolase activity in an extracellular region, and the final was a chymotrypsin-like elastase (Table 2).

Discussion

The sequences generated by this *de novo* transcriptome assembly provide a first glimpse into the gene complement of a thecosome pteropod, revealing a number of transcripts that are putative homologs of genes involved in biomineralization in other molluscs. The respiration rate experiments show no effect of short term CO₂ exposure, a finding that is also consistent with the very small number of candidate genes that were identified in the differential expression analysis. The candidate genes that may respond to pH conditions replicating exposures and durations comparable to a diel vertical migration include those associated with biomineralization and aerobic metabolism, although these results should be interpreted cautiously and require validation with a larger dataset.

Assembly and Annotation

Comparison of the *de novo* thecosome pteropod transcriptome with the SWISS-PROT database resulted in annotation of ~20% of the 45,739 transcripts. This relatively low percentage of identified transcripts is within the range of other recent assemblies of transcriptomes from non-model marine invertebrates, all of which were annotated using the NCBI non-redundant database (19-37%, spider crab (Harms et al., 2013), coral (Meyer et al., 2011), krill (Clark et al., 2011), amphipod (Zeng et al., 2011), abalone (de Wit and Palumbi, 2013), brittle star (Burns et al., 2013). Use of SWISS-PROT, a highly-curated and more restricted database, rather than the NCBI non-redundant database, which has greater species coverage, was selected to achieve more informative annotation. Thus, as in other studies, low identification is likely due to the relatively

small available database of annotated genes from closely-related species, typical in the study of non-model organisms.

Biom mineralization

Queries of the pteropod transcriptome with bivalve biom mineralization transcripts revealed a large number of potential homologs including dermatopontin, calmodulin, calponin, chitin synthases and carbonic anhydrase precursors, providing a useful resource for future exploration of these gene families. There were also a number of genes for which the annotation corresponded to as-yet undescribed transcripts associated with shell mantle expression in oyster. The highest number of transcripts found via our search, however, belonged to a group of C-type lectin domain proteins that are similar to perlucin. We identified five likely perlucin homologs, which would be the best first choices to further explore this gene family in pteropods. Perlucin, a water-soluble non-acidic protein with a calcium-dependent carbohydrate binding domain has been shown to be important for shell biom mineralization in the nacreous (aragonitic) layer of the abalone *Haliotis laevis* (Mann et al., 2000), and in the freshwater pearl mussel *Hyriopsis cumingii* (Lin et al., 2013). Perlucin-like genes have also been found to be strongly expressed in the outer mantle tissue of the mussel *Mytilus edulis* (Freer et al., 2014; Hüning et al., 2012). The abalone perlucin protein has been described through functional studies as a promoter of calcium carbonate precipitation, as the site of nucleation for calcium carbonate crystallization, and as a modifier of crystal morphology (Blank et al., 2003; Weiss et al., 2000). A recombinant perlucin gene from the abalone *Haliotis discus discus* has been shown to promote the growth of calcium carbonate crystals in *E. coli* (Wang et al., 2010). The C-type lectin domain proteins also can have an immune function in molluscs (Mu et al., 2012; Yang et al., 2011), and it has been hypothesized that some perlucin-like sequences may have an immune function (Venier et al., 2011; Wang et al., 2008). If the pteropod perlucin-like sequences are associated with shell healing or repair, their induction could be an important physiological pathway for pteropods in the response to high CO₂.

Interestingly, as was found during annotation of the oyster genome, a number of different transcripts were frequently identified as putative homologs of a single sequence. This was particularly evident for perlucin, where all 282 genes were identified as being homologs of only 4 abalone sequences, each which were described as either perlucin or perlucin-like genes. Since the perlucin-like C-type lectins appear to have diversified within the lineage leading to *C. pyramidata*, further investigation of the evolution of C-type lectins within pteropods should be a priority for further study. Similarly, the large number of paralogous biom mineralization genes in the oyster genome was suggested by Zhang et al. (2012) to be a consequence of rapid diversification and strong selection pressure, which is thought to underlie the evolutionary history of shell related genes. It is interesting that rapid diversification may have occurred independently in the highly derived holoplanktonic pteropods, emphasizing the usefulness of this species for comparative studies of biom mineralization.

Differential Expression

We found high variability in gene expression among individual pteropods, likely due to the fact that samples were generated from wild-caught individuals, which may have included differences in genetic background, physiological condition and size. It is also important to note that, because these are small animals, analysis was done with whole organisms, rather than specific tissues, which may dampen signals from specialized tissues such as the biomineralizing mantle. In the context of this high variability, the limitation of whole animal extractions, and the low number of biological replicates, the small subset of genes that were differentially expressed as a consequence of exposure to high CO₂ should be considered with caution and requires further confirmation, including further exploration of gene expression patterns with qPCR. Due to the rarity and small size of the organisms, we did not have enough remaining material from these experiments to validate our findings concerning differential expression reliably through qPCR measurements. In combination with a lack of effect on respiration rate (Maas et al., in review; Maas et al., 2012), however, the low number of differentially expressed genes suggests a lack of substantial physiological response to the experimental conditions.

Despite the limitations of the differential expression analyses, the present results nonetheless provide insight into transcripts that may be potentially influenced by short duration CO₂ exposure. The small number of annotated down-regulated genes in the high CO₂ treatment were highly variable in expression pattern and were associated with the mitochondrial inner membrane complex. Changes in mitochondrial inner membrane complex transcript abundance were also observed in urchins exposed to CO₂ in a longer duration developmental study (40 h) conducted by Todgham and Hofmann (2009) and in coral exposed to CO₂ for three days by Moya et al. (2012) and interpreted as being associated with a reduction in oxidative metabolism. Subunits of the downstream enzyme in the oxidative metabolism pathway, ATP synthase, have also been documented to be down-regulated in invertebrate models (mussels, Hüning et al., 2013; urchins, Todgham and Hofmann, 2009), emphasizing oxidative metabolism as an important molecular response to high CO₂. Those genes that were up-regulated had annotations that corresponded with the extracellular region and gene families known to be associated with biomineralization. These included putative homologs of perlucin, and conglutinin, which is a calcium-dependent collagen-containing lectin. Both conglutin and perlucin are C-type lectins. This diverse family is known to be associated both with innate immune response and with the biomineralization protein matrix (Mann et al., 2000; Mu et al., 2012; Wang et al., 2008). Moya et al. (2012) also found up-regulation of collagens and lectins in coral that had been exposed to elevated CO₂ conditions that were not corrosive to CaCO₃ structures (saturation state remained > 1). Other studies have seen patterns of down-regulation of transcript expression of collagens in urchins (Todgham and Hofmann, 2009) and both down- and up-regulation of transcript expression of C-type lectins, such as SM30 in urchins (Kurihara et al., 2012; Stump et al., 2011a). A qPCR-based study with species more closely related to pteropods (mussels) found no significant changes in a specific

perlucin-like C-type lectin (Hüning et al., 2013). Clearly, the complexity and diversity of the protein biomineralization matrix and its response to CO₂ exposure still bears further investigation across a diversity of taxa, including thecosome pteropods.

Conclusions

The species studied is a strong diel migrator that experiences conditions of high CO₂ in portions of its natural distribution, and was shown here to have no significant respiratory response to short-term, moderate CO₂ exposure. In conjunction with this lack of organismal-level response, the molecular response to this degree of exposure appeared to be limited to a small portion of the *C. pyramidata* transcriptome, with some of the identified transcripts being associated with the mitochondrial inner membrane complex, carbohydrate binding and the extracellular matrix/biomineralization. The analysis of biomineralization associated genes suggests a number of similarities with those of other molluscan calcifiers. The C-type lectin family, and the perlucin-like homologs in particular, merit further exploration both as a consequence of their abundance in the transcriptome and their likely role in calcification. The sequences and potential responses identified here thus pave the way for a detailed exploration of thecosome pteropod biomineralization. They also provide groundwork for further experimentation with larger scale experiments of individual- and population-level responses to longer term CO₂ stress at the molecular level, complementing the growing body of literature exploring metabolic, ecological, and calcification responses of these sentinel open-ocean calcifiers.

Data Accessibility

The NCBI Bioproject number for the data associated with this project is PRJNA231010. Raw sequence data was submitted to the NCBI Short Read Archive (SRA) under accession numbers GSM1283048–GSM1283055. This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GAWL000000000. The version described in this paper is the first version, GAWL010000000. Gene expression data was submitted to the NCBI Gene Expression Omnibus (GEO) under the accession number GSE53151.

List of Abbreviations

base pair (bp), Basic Local Alignment Search Tool (BLAST), calcium carbonate (CaCO₃), carbon dioxide (CO₂), fragments per kilobase per million reads mapped (FPKM), Gene ontology (GO), General Linear Model (GLM), high throughput RNA sequencing (RNA-seq), National Center for Biotechnology Information (NCBI), ocean acidification (OA), quantitative polymerase chain reaction (qPCR), trimmed mean of M-values (TMM)

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Author Contributions

Study design was initiated by all three authors. The animal collection and experimental manipulations were conducted by AEM and GLL. RNA processing and bioinformatics analysis were done by AEM and AMT. The initial draft was prepared by AEM, and all co-authors collaborated on data interpretation and manuscript revision.

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Figure Legends:

Figure 1: Oxygen consumption rates ($\mu\text{mol g}^{-1} \text{h}^{-1}$) of the individual pteropods used in the transcriptomic study that were exposed to ambient CO_2 (~380 ppm, dark red circles) and high CO_2 (~800 ppm, dark blue circles). There was no significant difference between the treatments even when the dataset was expanded to include more individuals from the Atlantic (light colored circles) and individuals from the Pacific (light colored diamonds) (datapoints from Maas et al., in review).

Figure 2: Heat map comparing the gene expression patterns among eight individual pteropods. Depicts a Bray-Curtis similarity matrix of the standardized edgeR normalized (TMM, trimmed mean of M-values; Robinson and Oshlack, 2010) fragments per kilobase per million reads mapped (FPKM) transcript counts for all genes that were expressed in at least two of the eight study individuals. Letters A-D correspond to individuals exposed to high CO_2 , and letters E-H correspond to individuals exposed to ambient CO_2 ; analysis constructed without grouping individuals by treatment.

Figure 3: Alignment of the conserved domain of the C-type lectin domain family proteins. The six highly conserved cysteine residues are labeled with stars, open circles label the residues putatively involved in calcium-dependent carbohydrate binding (Mann et al., 2000) and grey boxes contain the motifs proposed to determine carbohydrate-binding specificity for galactose (Iobst and Drickamer, 1994). Amino acid residue that consist of over 75% of the sequences are documented as identical (dark grey shading) or similar (light grey shading) overlaying the sequence.

Tables:

Table 1: The carbonate chemistry parameters for the experiments including temperature (T.) salinity (S.), and measured dissolved inorganic carbon (DIC). These were used, along with the manufacturer's certification of the gas concentration (ppm calculation), to calculate the aragonite saturation state (Ω_{Ar}) and pH of the treatments using CO2SYS. The calculations were also made incorporating one standard deviation away from the nominal gas concentration ($\pm 2\%$) to characterize the certainty of the treatment (ppm calculation $\pm 2\%$), as well as by using the measured TA of the batch of water (2307.3; TA calculation, grey box). The TA measurements are likely not representative of conditions during the actual experiments as a consequence of ongoing biological activity in the batch of water between the point at which it was sampled for TA and when it was used for respiration experiments (see text). Irrespective of the choice of parameters, the calculations resulted in similar estimates of the pH and Ω_{Ar} and suggest that the two treatments were distinctly different.

Treatment	T. °C	S. psu	DIC $\pm$ SD ($\mu\text{mol kg}^{-1}$)	ppm calculation		ppm calculation $\pm 2\%$			TA calculation		
				Ω_{Ar}	pH	$p\text{CO}_2$ (μatm)	Ω_{Ar}	pH	$p\text{CO}_2$ (μatm)	Ω_{Ar}	pH
380 ppm	15	33	2036.3 $\pm$ 57.9	2.44	8.06	372.4 - 387.6	2.49 - 2.40	8.07 - 8.05	350.6	2.66	8.10
800 ppm	15	33	2194.9 $\pm$ 19.9	1.41	7.78	797.2 - 829.7	1.43 - 1.38	7.77 - 7.79	830.1	1.38	7.77

Table 2: Transcripts that were identified as differentially expressed ($p < 0.05$) in the full dataset are listed with their putative annotation via BLASTX against the nr database (BLASTX ID) along with GO annotation, the e-value and percent similarity (% sim.) of the transcript to the top match. Also reported is the log base 2 fold change (Log FC), log counts per million (Log CPM) and the false discovery rate (FDR) of the analysis reported by edgeR.

Transcript ID	BLASTX ID	e-value	% sim.	Log FC	Log CPM	FDR	GO Annotation
GAWL01006673	cytochrome c oxidase subunit iii	1.90E-13	76.9	-10.34	9.88	0.010	P:aerobic electron transport chain; C:integral to membrane; C:mitochondrion; F:cytochrome-c oxidase activity
GAWL01040808	cytochrome c oxidase subunit i	2.40E-22	87.8	-10.04	9.25	0.010	P:aerobic respiration; C:mitochondrial inner membrane; C:respiratory chain; F:electron carrier activity; F:iron ion binding; P:oxidative phosphorylation; F:heme binding; C:integral to membrane; P:electron transport chain; F:cytochrome-c oxidase activity
GAWL01040684	venom allergen 5-like	2.60E-23	53.8	7.07	2.27	0.040	F:hydrolase activity; C:extracellular region
GAWL01040977	lactose-binding lectin l-2-like, BAA19861.1 (perlucin)	5.70E-14	51.0	7.27	1.31	0.041	F:carbohydrate binding
GAWL01006926	transcription factor glial cells missing	9.90E-60	69.1	7.62	1.23	0.035	P:regulation of transcription, DNA-dependent; F:DNA binding
GAWL01040816	conglutinin 1	2.10E-08	46.9	8.80	0.12	0.040	F:carbohydrate binding
GAWL01040704	chymotrypsin-like elastase family member 1-like	7.80E-13	52.1	11.08	0.25	0.008	P:single-organism process; P:regulation of cellular process; F:serine-type endopeptidase activity; P:proteolysis
GAWL01040711	polycystic kidney disease protein 1-like 2-like	6.30E-06	54.8	11.29	0.38	0.040	F:protein binding

FIGURES

Figure 1

Figure 2

Figure 3