High CO₂ alters the hypoxia response of the Pacific whiteleg shrimp (Litopenaeus vannamei) transcriptome including known and novel hemocyanin isoforms


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High CO₂ alters the hypoxia response of the Pacific whiteleg shrimp (Litopenaeus vannamei) transcriptome including known and novel hemocyanin isoforms. Physiol Genomics 47: 548–558, 2015. First published September 1, 2015; doi:10.1152/physiolgenomics.00031.2015—Acclimation to low O₂ in many organisms involves changes at the level of the transcriptome. Here we used high-throughput RNA sequencing (RNA-Seq) to explore the global transcriptomic response and specific involvement of a suite of hemocyanin (Hc) subunits to low O₂ alone and in combination with high CO₂, which naturally co-occurs with low O₂. Hepatopancreas mRNA of juvenile L. vannamei exposed to air-saturated water, low O₂, or low O₂/high CO₂ for 4 or 24 h was pooled, sequenced (HiSeq 2500) and assembled (Trinity: 52,190 contigs) to create a deep strand-specific reference transcriptome. Annotation of the assembly revealed sequences encoding the previously described small Hc subunit (HcS), and three full-length isoforms of the large subunit (HcL-1,-3). In addition to this, a previously unidentifed full-length Hc subunit was discovered. Phylogenetic analysis demonstrated the subunit to be a β-type Hc subunit (denoted HcB), making this the first report of a β-type hemocyanin subunit in the Penaeoidea. RNAs of individual shrimp were sequenced; regulated genes identified from pairwise comparisons demonstrated a distinct pattern of regulation between prolonged low O₂ and low O₂/high CO₂ treatments by GO term enrichment analysis (Roff-Bentzen, P < 0.0001), showcasing the stabilization of energetically costly translational machinery, mobilization of energy stores, and downregulation of the ubiquitin/proteasomal degradation machinery. Exposure to hypoxia for 24 h resulted in an increase in all of the full-length hemocyanin subunits (HcS, HcL1, HcL2, HcL3, and HcB). The addition of CO₂ to hypoxia muted the transcriptomic response of all the Hc subunits to low O₂ except for the β-type subunit.

COASTAL AND ESTUARINE ENVIRONMENTS are highly productive and dynamic systems that regularly experience drastic fluctuations in levels of dissolved oxygen (O₂) and carbon dioxide (CO₂) driven largely by tidal and seasonal cycles, but amplified by agricultural and industrial activities due to the steadily expanding population in the coastal regions (10). Anthropogenically derived CO₂ has been projected to decrease open ocean pH by 0.2–0.7 pH units within this century (11, 19, 68); however, estuarine systems already experience daily fluctuations in excess of 1.5 pH units over a single tidal cycle (http://cdmo.baruch.sc.edu). It remains largely unclear how organisms that inhabit these environments will cope with the predicted elevation of CO₂ in an already dynamic estuarine habitat.

Litopenaeus vannamei, the Pacific whiteleg shrimp, has become an important model species for studying the effects of a wide range of environmental stressors (64). A native to the Pacific coast, ranging from northern Mexico on the Gulf of California down to central Peru, L. vannamei has become the most extensively cultured crustacean species globally, with US imports worth upwards of $5 billion annually (http://www.fao.org/fishery/culturedspecies/Litopenaeus_vannamei). Although L. vannamei is a successful aquaculture species due to its high yield and tolerance of low salinities (16), animals raised in high-density farming conditions also experience wide variations in dissolved O₂ and CO₂.

While low O₂ and high CO₂ have complex and independent effects on the physiology of estuarine organisms, we know little about the organismal response to hypercapnic hypoxia (HH, simultaneous low O₂ and high CO₂) and its molecular underpinnings (10). Crustaceans have adapted physiological strategies for responding to low O₂ pressures namely by maintaining internal O₂ pools through increased ventilation rate and cardiac output (30, 47, 48, 56). In the presence of severely low and sustained O₂ tensions, anaerobic energy production pathways may be engaged to maintain the metabolic rate (8, 60). Many crustaceans can also exhibit a metabolic depression in response to hypoxia (H; low O₂), whereby protein synthesis rates are reduced for energy conservation (28). Recently, it has been shown that HH specifically decreases fractional protein synthesis rates in the hepatopancreases of L. vannamei and appears to do this through a reduction in RNA translational efficiency, and not RNA capacity (29). Like many other O₂-regulating crustaceans, L. vannamei is also equipped to maintain O₂ uptake via changes in its respiratory pigment, hemocyanin (Hc). Hc, like other respiratory pigments, functions by maximizing O₂ pressure gradients between the ambient environment and the hemolymph at the gill surface, which in turn maximizes O₂ transport (reviewed in Ref. 10). Hc production is inducible in many species, and structural changes in the Hc molecule can result in a pigment with higher O₂ affinity, a compensatory mechanism that can result in acclimation and adaptation to declining environmental O₂ (18). Conversely, crustaceans respond to elevated CO₂ levels in water largely through ionic exchanges between the blood and the ambient environment. Resulting blood acidosis during hypercapnia can affect general metabolic processes and impair oxygen transport, thermal tolerance, and growth (12, 36, 49, 57, 58). Using a custom cDNA microarray, Rathburn et al. (61)
assessed changes in the hepatopancreas transcriptome of *L. vannamei* exposed to H or HH. Among the genes most sensitive to combined low O2 and high CO2 (HH), gene-ontology (GO) categories of mitochondrial energetics, translation, protein maturation, and immune function were highly represented. Furthermore, this analysis suggested that the addition of CO2 to a low dissolved O2 environment could impair or even reverse the shrimp’s transcriptional response to H alone. A noteworthy finding from this study (61) was the identification of two Hc probes that were significant in discriminating H and HH at 24 h, suggesting that the delivery of O2 to tissues in H might be impaired by the addition of high CO2.

The artificial neural network approach used to analyze the microarray output in Rathburn et al. (61) identified transcripts that were sensitive for the discrimination of H- and HH-treated animals from normoxic controls. RNA-Seq, or the direct sequencing of transcripts by high-throughput sequencing technologies, has demonstrated considerable advantages since it does not rely on prior sequence information or annotation for probe selection, thus allowing for greater discovery of novel transcripts and isoforms, and their subsequent direct quantification (21). Using an RNA-Seq approach, the current study generated a comprehensive transcriptome and described a more complete Hc transcript repertoire. The expression changes in the suite of novel and previously known Hc transcripts are discussed in the context of the overall transcriptomic response to sublethal environmental H and HH compared with normoxia (N; air-saturated water) in juvenile *L. vannamei*.

**METHODS**

**Animal care.** Specific pathogen-free shrimp were obtained from Waddell Mariculture Center in Bluffton, SC. Subadult shrimp were held in four stock tanks at the Hollings Marine Laboratory. Stock tanks containing recirculating UV-sterilized seawater were kept on a 12 h light-dark cycle. Shrimp were fed commercial shrimp pellets (Shrimp PL 40-9 and Shrimp Grower 35; Zeigler Bros., Gardeners, PA) daily. Salinity and temperature were recorded daily and kept between 29.5 and 30.5 ppt and 24.0 and 26.0°C, respectively. Two weeks prior to the start of the experiment, shrimp were transferred into a raceway tank (2 m x 60 cm x 60 cm) and held at the same salinity and temperature. Shrimp were fed commercial shrimp pellets daily [Shrimp Grower SI (semi-intensive), Zeigler Bros.]. Shrimp used in the experiments consisted of only those in the intermolt stage of the molt cycle (2–5 days postmolt). Molt stage was controlled by methods developed by Rathburn (62).

**Experimental exposures.** Nine intermolt shrimp (3–13 g) were chosen at random and transferred to three 42 l aquaria (n = 3/aquarium) for each treatment and time. Shrimp were fed prior to transfer and then held for 24 h prior to treatment in air-saturated (N) UV-sterilized seawater at the same salinity, temperature, and pH as stated previously. After 24 h acclimation, tanks were assigned to one of three sterilized seawater at the same salinity, temperature, and pH as stated and then held for 24 h prior to treatment in air-saturated (N) UV-sterilized seawater (YSI model 58, Yellow Springs, OH) for each treatment and time. Shrimp were fed prior to transfer in air-saturated seawater containing UV-sterilized seawater were kept on a 12 h light-dark cycle. Shrimp were fed commercial shrimp pellets (Shrimp PL 40-9 and Shrimp Grower 35; Zeigler Bros., Gardeners, PA) daily. Salinity and temperature were recorded daily and kept at the same salinity and temperature. Shrimp were fed commercial shrimp pellets daily [Shrimp Grower SI (semi-intensive), Zeigler Bros.]. Shrimp used in the experiments consisted of only those in the intermolt stage of the molt cycle (2–5 days postmolt). Molt stage was controlled by methods developed by Rathburn (62).

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**Tissue harvest.** Tissues were collected from the hepatopancreas and preserved separately in 1.7 ml RNase-free microcentrifuge tubes containing RNA later (Qiagen). Samples were refrigerated at 4°C for 24 h at which time the RNA later was removed from the microcentrifuge tubes by pipetting and the tissue was transferred to −80°C.

**RNA extraction.** RNA was extracted from the hepatopancreas using RNeasy Mini Kits (Qiagen) following the manufacturer’s protocol for “Purification of Total RNA from Animal Tissues” with the addition of the optional on-column DNase digestion. RNA yield and quality were analyzed with a NanoDrop ND 1000 (Thermo Scientific, Waltham, MA) and a 2100 Bioanalyzer (Agilent, Santa Clara, CA).

**Illumina sequencing for stranded transcriptome assembly.** High quality total RNA from 52 *L. vannamei* hepatopancreas samples from N, H, and HH (4 and 24 h) exposed animals (outlined in Experimental exposures) was prepared for strand-specific next generation RNA sequencing using the NEBNext Ultra Directional RNA Library Prep Kit and NEBNext Poly(a)mRNA Magnetic Isolation Module for Illumina according to the manufacturer’s protocol (New England Biolabs, Ipswich, MA). The pooled stranded library was single-end (SE) sequenced (100 bp reads) on the Illumina HiSeq 2500 platform (Illumina, San Diego, CA).

**De novo transcriptome assembly and annotation.** The 100 bp SE raw sequences generated from the pooled *L. vannamei* stranded hepatopancreas cDNA library were assessed for quality and adapter contamination with FastQC (4). Preprocessing of raw reads was performed with the removal of quality-reads and trimming of adapter sequences by the flexible trimming tool Trimomatic (44). TruSeq adapters were trimmed; an average quality score of 30 was required on a sliding window of 20 bases, and a minimum length of 36 bases after trimming was enforced. The high-quality reads were assembled using the RNA-Seq de novo assembly program Trinity (26) using a 200 bp minimum contig length in the reverse-forward strand-specific option. To obtain assembly statistics for the number of reads that could be mapped back to transcripts (RMBT), the short read aligner Bowtie2 was used in the end-to-end sensitive mode (38). The assembled transcriptome was annotated against the nonredundant (nr) database using BLASTX (e-value threshold: 10−3) (http://www.ncbi.nlm.nih.gov), and GO categories were assigned using Blast2GO (15).

**Illumina sequencing for RNA-Seq experiment.** Individual RNA samples (n = 6/treatment x time) from the following experimental exposures were prepared for strand-specific next-generation RNA sequencing using the NEBNext UltraDirectional RNA and NEBNext Poly(a)mRNA Magnetic Isolation Module according to manufacturer’s protocol (New England Biolabs): normoxia 4 h (N4), n = 6; normoxia 24 h (N24), n = 6; hypoxia 4 h (H4), n = 6; hypoxia 24 h (H24), n = 6; hypercapnic hypoxia 4 h (HH4), n = 6; hypercapnic hypoxia 24 h (HH24), n = 6.

**Differential expression analysis.** To identify differentially expressed genes (DEGs), the rounded effective counts for each individual were calculated by xExpres (65). DEGs were identified by the DESeq2 package in the R software (3) using an adjusted P value of 0.05 as the threshold in the treatment pairwise comparison analysis. Due to the fact that analyzing the suite of Hc was of interest, and these
transcripts were found in such high abundance in the hepatopancreas, we chose to confirm the DESeq2 findings with Cufflinks (69). Cufflinks is a statistical package that is more suited for analysis of RNA-Seq data with a genome reference; however, it can more accurately quantify highly expressed transcripts compared with CuffDiff (65). Briefly, Cufflinks (v2.1.1) was used to estimate the relative transcript abundance following quartile normalization, bias correction, and multiread correction for each Bowtie2 alignment. Individual Cufflinks assemblies were merged using Cuffmerge (v1.0.0). Replicate samples (n = 6) per treatment group were used to estimate confidence intervals around expression estimates of each transcript using a false discovery rate control and Benjamini-Hochberg multiple test correction (Cuffdiff v2.1.1). Comparisons to time-matched N controls were used to define the DEGs. CummRbund (Biconductor) in R Studio was used for subsequent data analysis and visualization. The direction and magnitude of expression change for the set of Hc transcripts were compared between DESeq2 and Cufflinks. The Roff-Bentzen (1989) χ² analysis was used to test for significant enrichment of the most abundant GO terms assigned to the DESeq2 gene list of each treatment group compared with the Trinity assembly. This χ² method uses a Monte Carlo technique to overcome the problem of small sample sizes that are associated with using traditional Fisher’s exact test or χ² contingency tables for transcriptome applications. Data for the resulting DEGs are presented as log-ratio values for each treatment × time combination (H4, HH4, H24, HH24) versus the time-matched control (i.e., H4:N4, HH4:N4, H24:N24, HH24:N4). Fold-change values are also presented for brevity throughout the text and reflect the same comparisons as described above for log-ratio values.

Multiple sequence alignment and phylogenetic tree analysis. The protein sequences of the L. vannamei Hc contigs obtained from the Trinity assembly were predicted using OrfFinder (National Center for Biotechnology Information), and putative signal peptides were identified with SignalP 4.0 (59) with standard parameters. A multiple sequence alignment was built using the ClustalO algorithm (67) with CLC Sequence Viewer 7.5 (Qiagen Aarhus) and included 48 Hc sequences from 20 crustaceans, with the complete list of analyzed sequences given in Table 2. For phylogenetic analysis, signal peptides were removed prior to tree generation. To evaluate the evolutionary relationships of the five full-length L. vannamei Hc, a phylogenetic tree was inferred and visualized using PhyML available as part of the Seaview alignment software using the WAG model with invariable sites and across-site variations optimized with four rate categories (24).

The best of NNI and SPR tree searching operations was used, and the starting topology was an optimized BioNJ tree. Bootstrapping analysis with 100 replications tested the robustness of the tree.

RESULTS

Transcriptome assembly and analysis. A total of 1.44 × 10⁸ 100 bp SE strand-specific sequences from the pooled hepatopancreas mRNAs from 52 L. vannamei exposed for 4 h or 24 h to N, H, or HH were analyzed on an Illumina HiSeq2500. More than 96% of the raw reads passed quality control (QC) metrics, and were assembled using the de novo assembler Trinity. A total of 52,190 contigs were assembled (mean length, 870 bp; N50, 1,680; length range, 200-18,722 bp; Table 1). Of the 52,190 contigs derived from the Chrysalis stage of Trinity, 50,214 “Trinity genes” were identified by Butterfly, of which 8,389 reported evidence for multiple isoforms. The quality of the assembled transcriptome was further assessed by mapping the cleaned raw reads back to the Trinity assembled contigs and resulted in 96.03% RMBT.

Transcriptome annotation. All Trinity contigs were compared with the NCBI nr protein database for functional annotation using BLASTX with an e-value cutoff of 10⁻³, among which 38.15% showed significant matches. Functional annotation of the contigs was assessed using Blast2GO to assign GO terms. A total of 19,612 contigs were assigned at least one GO term (38%), with 15,172 mapped to biological process (BP) terms, 16,461 molecular function (MF) terms, and 8,707 cellular component (CC) terms. GO Slim terms were assessed across levels to define the categories that contained at least 50 sequences per category (10% sequence filter) and resulted in 40 BP, 35 MF, and 14 CC categories represented (Fig. 1). The biological process GO categories of signal transduction, cellular protein modification, and cellular amino acid metabolism processes, the molecular function GO category of ion binding, and the cellular component GO category protein complex represented the top five categories present in the transcriptome (Fig. 1).

RNA-Seq experiment. Individual directional libraries were sequenced from the hepatopancreas mRNAs of L. vannamei, with n = 6 per treatment (N, H, or HH) × time (4 or 24 h) combination. The average number of reads produced for each animal was ~20 million to 25 million reads post-QC and trimming, with individual RMBT% values ranging from 90 to 98%. Individuals were mapped to the assembled and annotated transcriptome and a total 919 DEGs were identified using xExpress/DESeq2 pipeline (adj. P value < 0.05). Pairwise comparisons between treatments at each time identified 4 (H4:N4), 7 (HH4:N4), 142 (H24:N24), 862 (HH24:N24), 17 (HH4:H4), and 1 (HH24:H24) DEGs from each designated treatment comparison. GO terms were assigned to 77.5% of the DEGs. Enrichment analysis detected a significant shift in the distribution of GO Slim terms assigned to the DEGs compared with that of the annotated transcriptome (Roff-Bentzen, P <

Table 1. Summary statistics of L. vannamei transcriptional transcriptome sequencing and assembly

<table>
<thead>
<tr>
<th>L. vannamei study</th>
<th>Hypoxia &amp; Hypercapnic Hypoxia (current)</th>
<th>Development (39)</th>
<th>Taura Virus Syndrome (71)</th>
<th>White Spot Syndrome Virus (13)</th>
<th>Nitrite Stress (25)</th>
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<tr>
<td>Sequencing platform</td>
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<td>Illumina</td>
<td>454</td>
<td>Illumina</td>
<td>Illumina</td>
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<td>raw reads</td>
<td>1.4 × 10⁸</td>
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<td>2 × 10⁵</td>
<td>6.4 × 10⁵</td>
<td>5.8 × 10⁵</td>
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<tr>
<td>Assembly</td>
<td>Trinity</td>
<td>SOAP denovo</td>
<td>Newbler</td>
<td>Trinity</td>
<td>Trinity</td>
</tr>
<tr>
<td>Contigs, n</td>
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<td>15,004</td>
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<td>42,346</td>
</tr>
<tr>
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<td>507</td>
<td>676</td>
<td>561</td>
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<tr>
<td>N50, bp</td>
<td>1680</td>
<td>478</td>
<td>539</td>
<td>982</td>
<td>736</td>
</tr>
<tr>
<td>RMBT, %</td>
<td>96.03%</td>
<td>not reported</td>
<td>not reported</td>
<td>85.80%</td>
<td>not reported</td>
</tr>
</tbody>
</table>

The sequencing platform, depth (number of raw reads), de novo assembler software, number of assembled contigs, contig mean length, N50, and percent of reads mapped back to the transcriptome (RMBT%) for the current study (boldfaced column) are compared with other L. vannamei RNA-Seq studies related to development (40), Taura virus syndrome (72), white spot syndrome (13) and nitrite stress (25).
Fig. 1. Functional categorization of the assembled transcriptome (light bars) and differentially expressed genes (DEGs) (dark bars) in response to hypoxia and hypercapnic hypoxia in the hepatopancreas of *Litopenaeus vannamei* based on Gene Ontology (GO) distribution. GO-Slim terms are shown on the y-axis. The percentage distribution of genes present in GO terms that contained >50 genes/GO for biological process (BP), molecular function (MF), and cellular component (CC) are shown. *Significantly enriched GO terms (Rolf-Bentzen, *P* < 0.0001).
The DEGs enriched for BP GO categories included carbohydrate metabolic processes, cell wall biogenesis, generation of precursor energy and metabolites, ribosome biogenesis, and translation. Several BP GO categories were underrepresented including cell cycle, cell death, cell differentiation and proliferation, transmembrane transport, and tRNA metabolic process. The MF categories hydrolyase activity acting on glycosyl bonds, peptidase activity, and structural constituent of ribosome were enriched, while ion binding, the most prevalent category in the transcriptome was completely missing from the DEGs results. Along with enrichment in the ribosome cellular component, the extracellular region was also enriched within the CC terms of the DEGs (Fig. 1).

Genes regulated in H or HH compared with N. The majority of DEGs (97.8%) were identified at the 24 h time point (summarized in Fig. 2, full list in Supplemental Table S1). Among the 142 DEGs identified in H24 animals, 105 were also significantly changed in HH24. For 43% of these shared DEGs, expression in HH was reduced or reversed, compared with H alone, indicating that the addition of CO2 antagonized the transcriptional response to low O2 levels. In comparison, CO2 antagonized the expression of 100% of the 37 DEGs unique to hypoxia alone but only 2% of the DEGs unique to HH24 (Fig. 2). Among the DEGs shared between H24 and HH24, some have been previously associated with crustacean H responses, including 12 arginine kinases and several triacylglycerol lipase transcripts (Fig. 2, Supplemental Table S1). Other families of DEGs that were shared by the H24 and HH24 treatment groups included polyadenylate binding proteins, chitinase precursors, and chitin binding proteins, all of which showed overall increased abundance. Most striking was the distinct transcriptional signature of DEGs in shrimp exposed to HH for 24 h. Components of protein folding and degradation machinery were altered at HH24 (Supplemental Table S1).

Among the 142 DEGs identified in H24 animals, 105 were also significantly regulated in the HH24 (Fig. 2, Supplemental Table S1). Several targets of the hypoxia inducible factor (HIF)-dependent pathway were induced, including a collagen specific prolyl-4-hydroxylase delta-subunit, MAT2B, and TRAC1. Target of rapamycin complex 2 subunit mapkap1 isoform 4 was significantly downregulated in HH24 only (Fig. 2, Supplemental Table S1). Transcripts associated with antioxidant stress response and cell death such as glutathione s-transferase, thioredoxin, HSP70, HSP90, cyclophilin, bax inhibitor, ras and ef hand domain proteins, senescence-associated protein, and autophagy-related proteins were differentially expressed (Fig. 2, Supplemental Table S1). Several targets of the hypoxia inducible factor (HIF)-dependent pathway were induced, including a collagen specific prolyl-4-hydroxylase alpha-subunit, MAT2B, and TRAC1. Target of rapamycin complex 2 subunit mapkap1 isoform 4 was significantly downregulated at HH24 (Supplemental Table S1).

Hc transcript assembly and differential expression. A total of 27 L. vannamei Hc contigs were identified from the transcriptome library by reciprocal BLAST searching. The resulting contigs were further assembled into five full-length sequences and eight partial sequences. Of the full-length contigs, one represented the penaeid small subunit class (HcS) and shared 100% identity with a previously sequenced HcS subunit (AHN85635). The base mean count from the DESeq2 analysis for HcS (2,778,544 counts) represents 12% of the transcriptome.

Fig. 2. Overview of differential expression at 24 h. Venn diagram indicates the 794 unique DEGs of L. vannamei hepatopancreas in pairwise comparisons among hypoxia (H) and hypercapnic hypoxia (HH) treatments vs. the time-matched normoxic control (N) at the 24 h time point. In total, 862 DEGs were identified in the hypercapnic hypoxia (HH24)-treated animals, 142 in hypoxia only (H24), with 105 DEGs significantly changing in both H24 and HH24. DEGs were identified using DESeq2/eXpress analysis (adj. P < 0.05). The percentage of transcripts demonstrating an antagonistic expression pattern (muted or reversed) upon exposure to the addition of elevated carbon dioxide is denoted in parentheses. Summary of DEGs at 24 h are listed to the right of the Venn Diagram. Expression profiles unique to hypoxia (H24), hypercapnic hypoxia (HH24), and DEGs shared between H24 and HH24 are shown. Up and down arrows indicate the direction of relative expression compared with the normoxic control (N24). Categories with individual DEGs demonstrating both increased and decreased expression patterns compared with N24 are depicted by a Δ symbol.

1 The online version of this article contains supplemental material.
tome and identified HcS as the most highly expressed transcript in the hepatopancreas (Fig. 3). Three full-length sequences represented distinct isoforms of the large subunit (HcL) and share 91.5% identity with each other, with the majority of the amino acid differences present in the N and C domains (Fig. 3). HcL1 shares 99% homology with the large variant L1 (AHY86471.1), while HcL2 shares 97% with AHY86472.1, and HcL3 shares 95% similarity with AHY86473.1 (Fig. 3). Together, HcL1-3 constitute over 4% of the transcriptome.

Most notable to the Hc sequence assembly data was the discovery of a previously unidentified full-length subunit (denoted HcB). BLASTX results identified the sequence to share 66% similarity with *Pacifastacus leniusculus* β-type Hc subunit (AAM81357.1). Multiple sequence alignment verified the presence of the six conserved histidines required for oxygen binding in HcB (data not shown). The HcB mean base count value (1355) represents only 0.006% of the transcriptome and over 2,700-fold lower expression than the four full-length subunits together (Fig. 3).

A phylogenetic tree was reconstructed from 48 Hc sequences from 20 crustacean species to further classify the *L. vannamei* hemocyanin (LvHc) subunit types (Fig. 4, Table 2). The resulting maximum likelihood tree rooted at the *H9252*-subunit branch generated general clade arrangements that agreed with other decapod crustacean Hc evolutionary trees (55), and like those, distinguished the decapod Hc subunits into α-, β-, and γ-types, with the Peracarida and Phyllocarida each forming additional separate clades. In agreement with the BLAST results and multiple alignment similarities, HcS, HcL1, HcL2, and HcL3 belong to the γ-type subunit, while the newly identified full-length subunit belongs to the β-type subunit family (denoted LvHcB, Fig. 4) and represents the first report of a β-type Hc subunit from the Penaeoidea.

Exposure to H for 24 h induced the expression of many of the Hc transcripts identified in this study. The addition of elevated CO₂ generally antagonized Hc transcript expression compared with H alone (Fig. 3). This pattern was consistent for all full-length sequences and partial contigs, except for HcB (Fig. 3). However, none of these changes were significantly different from time-matched controls except for c24171_g1_i1, which was significantly induced in both H24 and HH24 (ExPReSS/DESeq2 analysis, adj. P < 0.05). Three of the partial sequences followed a different pattern of expression, showing decreased expression after 24 h in either H or HH compared with N (c27512_g1_i1, c5591_gi_i1, c6637_gi_i1). However, the base mean values for these contigs were below 100 counts, a read cutoff value that is still difficult in RNA-Seq analyses to observe biologically relevant changes (6). Due to the overall high expression profiles of HcS and HcL1-3, these differential expression changes were confirmed with the Cufflinks package (Supplemental Table S2). The Cufflinks package analysis agreed with the direction of change but across the board estimated larger fold changes than those of the DESeq2 method, a general pattern that was also observed across the whole transcriptome (data not shown). As did the DESeq2 method, Cuffdiff identified c24171_g1_i1 as significantly induced in both H24 and HH24 and followed the antagonistic expression pattern (Supplemental Table S2). DESeq2 results demonstrated that the expression of the γ-types, HcS and HcL1-3, increased in H24-treated shrimp compared with N.

<table>
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<th>Sequence Length (aa)</th>
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<th>% ID</th>
<th>Domain</th>
<th>Base Mean Value</th>
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<tr>
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<td>Litopenaeus vannamei (HcL3)</td>
<td>69</td>
<td>-/-/C</td>
<td>245</td>
</tr>
<tr>
<td>c51402_g1_i1</td>
<td>Litopenaeus vannamei (HcS var.)</td>
<td>74</td>
<td>-/-/C</td>
<td>119</td>
</tr>
<tr>
<td>*c24171_g1_i1</td>
<td>Litopenaeus vannamei (HcS var.)</td>
<td>94</td>
<td>/-/C</td>
<td>809</td>
</tr>
</tbody>
</table>

**Fig. 3.** *L. vannamei* hemocyanin (Hc) sequences and differential expression during 4 h and 24 hypoxia and hypercapnic hypoxia exposure. Differential expression profiles for the full-length (HcS, HcL1, HcL2, HcL3, and HcB) and partial Hc contigs are depicted for each treatment × time combination vs. the normoxic time-matched control (H4:N4, HH4:N4, H24:N24, HH24:N24). Log2 ratio values are displayed in columns with downregulated transcripts in yellow and upregulated transcripts in blue. Sequence length, top BLAST hit description, species and accession number, percent identity to the top BLAST hit, and present domains are given for each Hc sequence. The quality-controlled pooled library was aligned to the assembled transcriptome using Bowtie2, and the total number of reads aligned to each sequence (base mean value) are presented.
controls (1.65- to 1.98-fold change), but this increase in expression was muted with concurrent exposure to hypercapnia (1.32- to 1.54-fold change). This is in contrast to the \( HcB \)-type subunit that demonstrated a synergistic expression profile, where the increased expression of \( HcB \) in \( H24 \) shrimp (1.73-fold induction) was mildly enhanced by the addition of elevated \( CO_2 \) (1.9-fold change). To confirm the expression profiles of the two previously described \( Hc \) microarray probes (61), fold-change values were calculated versus the time-matched control for the RNA-Seq and microarray experiments. Both microarray probes (MGID388401 and MGID405503) align to a conserved portion of \( HcL2 \) and \( HcL3 \) in the current transcriptome assembly. MGID388401 expression was induced by 1.8-fold during \( H24 \) (\( H24:N24 \)) and was muted (1.19-fold) with the addition of elevated \( CO_2 \) (\( HH24:N24 \)). Similarly, MGID405503 was induced 1.97-fold (\( H24:N24 \)) and concomitantly muted (1.32 fold, \( HH24:N24 \)). Thus results obtained by Rathburn et al. (61) for both of these microarray probes agree in magnitude and direction of change with the \( HcL2 \) and \( HcL3 \) isoforms reported in the current study (Fig. 3).

**DISCUSSION**

The present study identified transcriptomic changes in the hepatopancreas of shrimp exposed to \( H \) (low dissolved \( O_2 \)) alone or in combination with elevated \( CO_2 \) (\( HH \)). We used deep, directional RNA sequencing to assemble a more complete repertoire of transcripts relevant to \( O_2 \) and \( CO_2 \) sensing, response, and tolerance than has been reported previously. The transcriptome reported herein displays much stronger assembly metrics including mean length, N50, and RMBT values (Table 1) compared with other published shrimp transcriptomes that used the Illumina sequencing platform and Trinity assembler (13, 25, 40). Improved mapping quality (RMBT) was likely achieved in the current study due to the long, directionally sequenced reads used in the transcriptome assembly (39). It was surprising that annotation performance did not greatly improve along with these metrics, as the percentage of transcripts with a BLAST hit was only 38%, an annotation value that falls well within other reported studies with lower assembly metrics (13, 25, 40, 72). Along with the other \( L.\ vannamei \) transcriptomic studies, we too suggest that this will not greatly improve until a quality genome is publicly available for this species.

The eXpress/DESeq2 differential expression analysis employed in the current study defined that the overall transcriptional response to \( H \) was antagonized (muted or reversed) by concurrent exposure to hypercapnia (Fig. 2), a phenomenon first identified in the \( H \) and \( HH \)-based \( L.\ vannamei \) transcriptome study using microarray technology (61). The current study revealed a set of transcripts differentially expressed only in \( H24 \) that were antagonized by the addition of carbon dioxide, while the suite of transcripts unique to \( HH24 \)-only were in fact synergized (further upregulated or further down-regulated) compared with \( H \) alone at 24 h, highlighting a clear and striking difference in the engaged oxygen-sensing pathways in \( H \) alone compared with \( HH \) at 24 h (61).

\( H \) alters cell physiology largely through three \( O_2 \)-sensing pathways: the mammalian target of rapamycin (mTOR) kinase pathway, the unfolded protein response (UPR), and the HIF-dependent pathway (5, 7, 22). The mTOR pathway, which is inhibited during hypoxia, integrates many different kinds of signals, and works through a wide range of downstream pathways to influence cell survival and growth through changes in translation, ribosome biogenesis, autophagy, and metabolism under N conditions (71). The current analysis identified a striking upregulation of ribosome biogenesis factors and translational machinery after 24 h exposure to \( HH \), which was not present in \( H \) alone. Ribosomal biogenesis is a major consumer of ribonucleotides. It is not surprising that the addition of elevated \( CO_2 \) (\( HH \)) resulted in much greater expression of \( HcB \) (Fig. 1) than observed in \( H \) alone, as the unfolded protein response (UPR), which is activated during hypoxia, is a major regulator of ribosome biogenesis (7).

Fig. 4. Circular representation of the evolutionary tree of malacostracan crustacean \( Hc \) from the amino acid sequences of 48 \( Hc \) subunits of 22 crustacean taxa. The tree was rooted with the \( \beta \)-type subunit clade and was obtained with the maximum-likelihood procedure implemented in phyML with the WAG substitution model. The positions of the full-length \( Hc \) sequences from \( L.\ vannamei \) hepatopancreas identified in the present study are shown with arrows. Refer to Table 2 for list of species abbreviations and accession numbers.
of energy, and its regulation occurs primarily at the level of transcription (54). Transcription of these genes can account for >90% of total cellular transcriptional activity when resources, energy, and health status are favorable (54, 70). The concerted activation of this suite of ribosomal biogenesis components suggests that adding hypercapnia to the hypoxia regime may alleviate mTOR inhibition and implies that the transcriptome is poised for cellular survival and growth during HH.

Simultaneous with the reawakening of the translational machinery, a marked decrease in the ubiquitin-proteasomal degradation machinery was observed in L. vannamei during HH at 24 h. This protein degradation machinery interfaces with, and in some ways can regulate, the UPR pathway (71). Proteasome-mediated degradation of unfolded or misfolded proteins can reduce endoplasmic reticulum (ER) stress, which is often a product of H stress, but in some tissue-specific contexts, proteasome inhibition can lead to increased cell survival, a finding that has become increasingly recognized in relation to hypoxic tumor microenvironments (2, 35). The UPR resolves ER stress partly by the direct production of chaperone proteins; however, concomitant with the marked decrease in the ubiquitin-proteasome pathway, L. vannamei showed a distinct reduction in ER-localized chaperones (peptidyl prolyl cis-trans isomerase, prefoldin, and cyclophilin). In tumor hypoxic microenvironments, the UPR pathway can engage the autophagic degradation pathway, a catabolic process to promote cellular survival through the formation and fusion of autophagosomes with the lysosome for cellular component breakdown via specific resident hydrolases (32). ATG13, a key component of the scaffolding that recruits other ATG complex members to the scaffolding that recruits other ATG complex members to the lysosome for cellular component breakdown via specific resident hydrolases (32). ATG13, a key component of the scaffolding that recruits other ATG complex members to

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initiate autophagosome formation, was significantly induced in HH24-exposed *L. vannamei*. Because autophagy involves dramatic subcellular membrane remodeling, the secretory pathway can be greatly affected. In shrimp exposed to HH24, VSP45, synaptobrevin, and members of the actin-related (Arp) 2/3 complex were reduced, while resident hydrolases showed marked induction. The current study makes clear that the signaling pathways involved in regulating the H and HH response can signal through multiple avenues, and direct interrogation of mTOR and UPR constituents is both warranted and now possible given the increased depth of transcriptomic information now available in this shrimp species.

Members of the HIF-dependent oxygen-sensing pathway appeared to be largely insensitive to the addition of CO₂. HIF-1α, the well-studied oxygen-related master regulator, is a gene product principally controlled on the posttranscriptional level, therefore the absence of transcript level changes of HIF-1α in the current study was not surprising. Classic energy-related transcriptional targets of the HIF pathway were induced in the current study and within the same timeframe as other H-related shrimp studies (42, 43). For example, phosphoenolpyruvate carboxykinase (PEPCK), an enzyme involved in increased glucose production during hypoxic events, was slightly induced in H24 shrimp but was significantly increased in HH24 animals. This key gluconeogenic enzyme was also induced during chronic H in the grass shrimp, *Palaemonetes pugio*, and together with the results of the present study, emphasizes the conserved role for glycolytic production of ATP in the H response in shrimp (41). In addition to this, H24 lead to increased expression of GAPDH and fructose bisphosphate aldolase, both of which have been assigned as hypoxia response element (HRE)-containing genes in model mammalian systems (34, 45). H-induced mobilization of stored triglycerides has been demonstrated in the H-tolerant goby *Gillichthys mirabilis* (23). Similarly, triacylglyceride lipases were markedly induced in the current study at both H24 and HH24, suggesting a similar use of triglyceride mobilization for input into the glycolytic pathway. Furthermore, phosphoarginine stores are mobilized under hypoxic conditions in the kuruma prawn, *Marsupenaeus japonicus* (1), and in the current study, concerted upregulation of arginine kinase in response to H24 and HH24 exposure suggests a similar mechanism for ATP buffering occurring during anaerobiosis in *L. vannamei*.

Decapod crustaceans display a wide range of diversity in the structure, function, and properties of hemocyanin (17, 33). Hc in *L. vannamei*, like other penaeid shrimp, is freely dissolved in the hemolymph and is composed of small (HcS; 75 kDa) and large (HcL; 77 kDa) subunits that are arranged in groups of six, forming functional hexamers (1 × 6) and dodecamers (2 × 6). The NH₂-terminal domain is most variable between species and contains a conserved gate-keeper residue at Tyr68 that facilitates binding pocket accessibility. The M-domain is the most conserved domain, both between species and subunit types, and is located in the central portion of the subunit. Each subunit contains six conserved histidines in the M-domain that coordinate the reversible binding of one molecule of O₂ per Hc subunit. The COOH-terminal domain is important for maintaining quaternary structure but has also been shown to carry immune-like functions as well (31, 73). Hc is considered to be a very plastic molecule with evidence in other crustacean species that O₂-binding properties can differ between subunit types, and expression and assembly of higher affinity subunits into functional oligomers are mechanisms utilized to cope with long-term H exposure (18, 50, 51, 63).

Decapod crustacean Hc subunits have been classified into three different types referred to as α, β, and γ (52, 53). Phylogenetic analysis within the Malacostraca has identified the β-type to have diverged from the α/γ clade ~520 MYA (9, 27, 66), the α and γ-types separating ~400 MYA, with α- and γ-types more similar to each other than to the basal β-subunit type (27, 37, 52, 53, 55, 66). Until the present study, only Hc-γ type subunits have been identified in the Penaeoidea infraclass. Loss of at least one of the subunit types has been commonly observed in decapod crustaceans (53); however, all three subunit types were recently identified in the bamboo shrimp *Atyopsis moluccensis* (55). Interestingly, the protein encoding the β-type subunit in *A. moluccensis* could not be identified by mass spectrometry, suggesting that the β-type in this shrimp species does not constitute a major component of the higher order oligomer form (55), a finding that is contrary to other decapod crustaceans in which the β-type subunit is the main constituent (20, 52, 66). The current study identified the previously known *L. vannamei* γ-type HcS and HcL isoforms 1, 2, and 3 expression levels to represent ~16.5% of the hepatopancreas transcripts, with the newly identified β-type subunit expressed >2,700-fold less than the γ-types. Based on the three orders of magnitude expression difference, the question remains whether the protein encoded by the β-subunit is a constituent of the functional O₂-carrying oligomer in *L. vannamei*.

More broadly, the relevance of the transcriptional changes in the full and partial length sequences of Hc identified in the present study to the oligomeric configuration and O₂-transport functions of the respiratory pigment requires further study. To date, neither the subunit makeup, the O₂ affinities of the Hc oligomers nor possible shifts in oligomer composition in response to H or HH, have been reported for this species. The identification of the β-type Hc subunit in a penaeid shrimp with a differing expression signature than that of the γ-subunit types adds to our knowledge of the complexity underlying low dissolved O₂ sensing and tolerance in a decapod crustacean. The antagonistic profile of the γ-type subunits (HcS and HcL1-3) in HH24 shrimp may reflect a shift from requiring production of more Hc to offset O₂ delivery demand in H alone, which is suppressed by a CO₂-dependent increase in Hc O₂ affinity mediated by reduced hemolymph and tissue pH in the HH condition, and thus reducing the need for energetically costly Hc production. This reduction in Hc expression could also reflect a larger-scale metabolic reduction, a phenomenon observed for many crustacean species and recently demonstrated by a reduction in global protein synthesis rates during HH in *L. vannamei* (29).

Together, the distinct transcriptional changes observed after HH24 compared with H alone have led us to speculate about the role of elevated CO₂ in relation to O₂ sensing pathways in decapod crustaceans. The HIF-dependent pathway appears to be unresponsive to the addition of CO₂ under low O₂, as the transcriptional targets of this pathway are induced in both H and HH regimes. However, CO₂ appears to alter sensing mechanisms in the mTOR and UPR pathways and together provide an overall transcriptomic landscape promoting cell survival and growth. Since H induced the expression of the Hc...
γ-type subunits, but addition of CO₂ muted that response, we hypothesize that abundance of these transcripts may be regulated at least in part by mTOR and/or UPR response. The identification of the novel β-type subunit HcB not only brings to light the dramatic advantage of RNA-Seq for transcript discovery and quantification, but the synergistic effects of low O₂ and high CO₂ on its expression suggests the involvement of different regulatory mechanisms than the γ-type subunits. Thus, deciphering the involvement of HcB in O₂ delivery is imperative for enhanced understanding of the physiological response to low O₂ and elevated CO₂ in decapod crustaceans.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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