**Title(s)**: The transcriptomic response over a five-point time course exposure to and recovery from hypoxia in the copepod *Tigriopus californicus*

Time-course of gene expression during the onset of hypoxia and initiation of recovery in the intertidal copepod *Tigriopus californicus*

Patterns of gene expression over a time-course of hypoxia exposure and recovery characterizes the response of a hypoxia tolerant crustacean

Time-series transcriptomic profiles over the onset of hypoxia and initiation of recovery in a crustacean lacking HIF-α

Time-course analysis during hypoxia and recovery reveals expression patterns of genes required for hypoxia resistance in a crustacean lacking HIF-α

**Running head**: Time-series transcriptomics during hypoxia in *Tigriopus californicus*

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**Abstract**

The intertidal copepod *Tigriopus californicus* persists in supralittoral splash pools disconnected from the flow of the ocean. This copepod experiences daily bouts of hypoxia and anoxia that strike rapidly at the onset of nightfall and last until the sun rises. Despite the severity of this hypoxic exposure, *T. californicus* tolerates even anoxia with minimal mortality. However, this copepod species lacks genetic components found in the hypoxia response pathways of most metazoans, including the genes encoding the hypoxia inducible factor HIF-α and its repressor EGLN. Because *T. californicus* lacks these regulatory elements, it is still unclear which genes respond transcriptionally to hypoxia in this species and how the transcriptional response proceeds on a fine timescale. In this study, we captured gene expression during a time course of hypoxia that mimics that which the species experiences in the wild, sequencing mRNA during normoxia, mild hypoxia (~3.5 mg O2 l-1), at critical oxygen tension (Pcrit; ~0.5 mg O2 l-1), one hour into anoxia (0 mg O2 l-1), and two hours into recovery. We identified genes with altered expression over the time course and clustered these genes for downstream tests for enrichment of GO terms and transcription factor binding site motifs. We also examined genes grouped by biological function to describe how elements within these groupings responded during and after hypoxia exposure. We found that several groups of genes with known responses to hypoxia significantly responded, including genes with known interactions with HIF-α in other systems. We found evidence that further implicates regulation of genes related to sugar metabolism and exoskeletal modifications as potential adaptive responses to hypoxia by *T. californicus*.

**Introduction**

The intertidal zone in coastal habitats can be a volatile and stressful environment for the species which persist in pools. Environmental conditions in a tide pool are subject to cyclical changes based on the tides daily. This includes rapid swings in oxygen content of the water, where during the night when tides are low and fauna, such as micro and macro algae, deplete oxygen in the water when switching from photosynthesis to aerobic respiration. While pools close to the water line may be refilled with oxygen-rich seawater as the tides rise, splash pools above the water line may experience longer and more intense fluctuations in oxygen content. Any organism that inhabits these pools must be tolerant of extremely low oxygen content and reoxygenation when the sun rises.

The intertidal copepod *Tigriopus californicus* inhabits such supralittoral splash pools along the west coast of North America. Oxygen sensor data from *T. californicus* inhabited pools on the coast of Oregon, USA show that this species experiences extreme hypoxia and even anoxia on most nights in the summer months of July through September. These shifts occur after sunset when algae sharing the pools consume oxygen via aerobic respiration and instances of complete anoxia last on average for 5+ consecutive hours. Despite or perhaps due to these daily challenges, *T. californicus* is resistant to hypoxic stress and can survive long period of low oxygen up to 72 hours and even 24 hours of complete anoxia (CITE). However, while many species respond to hypoxic stress via the induction of the hypoxia inducible factor (HIF) pathway, *T. californicus* is among marine species recently discovered to lack the key transcription factor for this pathway, HIF-α, but not its dimer partner HIF-β/ARNT. Additionally, the oxygen sensing proline hydroxylase EGLN, which normally inhibits HIF-α activity, was also discovered to be absent in this copepod’s genome (CITE). Because *T. californicus* is tolerant to hypoxic stress despite the lack of the canonical transcription factor needed for hypoxia response across so many taxa, it is still not fully clear how this species responds transcriptionally to the onset of hypoxia, the complete lack of oxygen, and reoxygenation.

Previously, differential gene expression was captured in *T. californicus* at three time points: after 3 hours and 24 hours of hypoxia (~ 0.15-0.3 mg O2 l-1) exposure and after a full 24-hour recovery in normoxic water. This provided a good snapshot of 451 genes differentially expressed after 24 hours hypoxia exposure and 561 genes differentially expressed during recovery. Many of these were involved in a variety of key processes including glycolytic activity, mitochondrial function, and the metabolism of exoskeleton components were also identified as differentially expressed, highlighting the potential for interesting transcriptional response unique to the biology of *T. californicus*. This study also documented the prevalence of hypoxia response elements (HRE’s), the binding sites for HIF-α, upstream of genes in the *T. californicus* genome, but did not find that differentially expressed genes were associated with HRE’s more than expected by chance. Thus, the mechanism by which the transcriptional response in induced in this copepod remains unclear.

While pairwise comparisons between gene expression in normoxia and extended hypoxia are informative, we may gain a clearer picture of the transcriptomic response by *T. californicus* by quantifying gene expression on a time scale relative to the hypoxia exposure in copepod pools in the wild, where oxygen levels fall quickly and can be entirely depleted over a course of just a few hours (CITE). Moreover, the gene expression may be different on a scale of available oxygen, not just at different lengths of exposure to hypoxia. Indeed, the hypoxia response by *T. californicus* may initiate changes to gene expression before extreme levels of hypoxia are reached. Additionally, physiological breakpoints before oxygen is depleted, such as measured by the respiratory statistic *P*crit - the critical oxygen tension at which respiration rate can no longer be controlled by the organism independent of available oxygen in the water – may mark important transcriptomic changes. Because *T. californicus* has been shown to experience anoxia in their pools, measuring gene expression during exposure to oxygen-less environments could highlight mechanisms for persistence. Finally, the transcriptomic response to reoxygenation should need to be rapid to keep up with the flood of oxygen in the copepod pools once the sun rises; therefore, capturing gene expression during the initial phases of recovery will also be informative. Considering these elements of a hypoxia exposure event like that experienced by *T. californicus* in the wild will help uncover patterns that we predict will lead to mechanistic hypotheses about how this copepod achieves tolerance to drastic fluxes in oxygen.

Since the RNA-seq experiment was completed by Graham & Barreto (2019), we have made refinements to the process of exposing *T. californicus* to hypoxic stress that allow for tighter control over the time frame of hypoxia exposure and accurate induction of complete anoxia. This is made possible through closed chamber microplate respirometry, in which copepods experience hypoxia and eventually anoxia while we continuously monitor oxygen levels in their environment. To capture gene expression over a hypoxia exposure like that experience in the wild, we designed a time course experiment in which replicates of *T. californicus* copepods were sampled for RNA-seq at five time points (**Figure 1**). These were in order: 1) during normoxia, 2) during mild hypoxia (~3.5 mg O2 l-1) halfway through oxygen depletion, 3) at the species and population-specific *P*crit breakpoint (~0.5 mg O2 l-1), 4) after one hour exposure to complete anoxia (0.0 mg O2 l-1), and 5) 2 hours into recovery after reoxygenation in normoxic water.

We explored transcriptomic changes by identifying genes that significantly responded over the entire hypoxia time course. We then clustered these genes by their patterns of expression and implemented GO enrichment analyses to explore functional patterns within each cluster of genes. To supplement this data, we also compared expression between select (but not all) time course points in a pairwise manner such as typically done in a traditional RNA-seq experiment. Based on the previous RNA-seq results by Graham & Barreto (2019), the crustacean biology of *T. californicus*, and well-known hypoxia response pathways in other taxa, we also explored subsets of genes associated with specific process or pathways to see which genes in these groups responded significantly over the hypoxia course. These processes included genes involved with glycolysis and related pathways (pentose-phosphate, pyruvate, starch-sucrose, fructose-mannose, TCA), mitochondria-targeted genes, genes associated with antioxidants, chitin/cuticle/exoskeletal genes, and genes involved with carotenoid pigment metabolism.

**Methods**

**Copepod cultures**

Copepods used in this experiment were originally collected in San Diego, California (32.7333°N, 117.2500°W). Copepods were maintained in large, outbred lab cultures in 400-mL beakers for a minimum of three months (approx. three generations) prior to use in this study. The copepods we used in this study were newly hatched from these stock cultures. To create these experimental cultures, we removed developed egg sacs from gravid females in the laboratory stock cultures and placed them in new 400-mL beakers to hatch. Before sampling these copepods for this study, we waited until freshly hatched nauplii were fully mature at a minimum of 30 days old but no older than 44 days old so that all copepods sampled in this study were roughly age matched within 14 days of each other. The experimental cultures were maintained in the same conditions as stock cultures at 20°C in 35 ppt artificial saltwater (ASW) on a 12h:12h light:dark cycle in incubators. They were supplied fresh ad libitum mixtures of *Nannochloropsis* and *Isochrysis* microalgae and ground-up spirula wafers and Tetramin fish flakes (Tetra, Blacksburg, VA, USA).

**Hypoxia Time Course and RNA Extraction and Purification**

Copepods used in the hypoxia time course were isolated from the age-matched cultures 24 hours prior to experimentation and placed into clean ASW with no food. We isolated 125 males and 125 females to divide into five experimental groups with each group containing 25 copepods of each sex for a total of 50 copepods per group. On the day of the hypoxia time course, the groups of 50 copepods were added to clean, filtered ASW in 500 µL wells of a closed system microplate respirometer placed in a completely dark incubator at 20°C and sealed with air-tight film, a rubber gasket, and a weighted block as recommended by the respirometer manufacturer (Loligo Systems, Copehagen, Denmark). Because this respirometer is a closed system, copepods depleted oxygen in the water over approximately two hours eventually creating an anoxic environment. This mimics the onset of anoxic conditions and buildup of carbon dioxide that *T. californicus* experiences in the wild on a nightly basis (cite Powers et al. 2024 when published).

Copepods were randomly assigned to five exposures groups to be sampled along the course of hypoxia exposure: normoxia, mild hypoxia, severe hypoxia at *P*crit (critical oxygen tension), anoxia, and recovery (**Fig. 1**). Each group of copepods were sealed into the microplate at the same. This included copepod assigned to the normoxic time point to mimic handling stress experienced by the other groups. Normoxia group copepods were removed after just fifteen minutes in their well. For the mild hypoxia groups, we allowed them to deplete oxygen until DO levels were at 3.5 mg O2 l-1, which represents the halfway point of oxygen depletion in the closed system (**Fig. 1**), and then removed them from the well. For the severe hypoxia at critical oxygen tension group, we allowed the copepods to deplete DO to 0.5 mg O2 l-1 before removing them from the well, as this was determined to be the average point at which the San Diego copepods reach *P*crit (cite Powers et al. 2024 when published). The anoxia group depleted oxygen beyond *P*crit, all the way to 0 mg O2 l-1 and these copepods remained at anoxia for one hour before we removed them from the well. Copepods assigned to the recovery group were removed from their well at the same time as the anoxia group; however, the recovery group were added to clean, normoxic saltwater for two hours. We repeated this time course six times for a total of N = 6 replicates.

When each group was removed from their assigned conditions, they were immediately added to 350 µL of TRIzol® reagent (Invitrogen). Copepods were then homogenized with 1-mm zirconia-silica beads (Biospec) for 25 seconds at 3500 oscillations per minute to extract total RNA. Extracted RNA was stored at -80°C until RNA purification. Across the 6 replicates of 5 time-course exposures, we ended with 30 total RNA samples to be purified.

**RNA Library Preparation, Sequencing, and Data Processing**

Extracted RNA was purified using Direct-zol™ RNA MicroPrep Kit (Zymo Research, cat. no. R2062). Purified RNA was treated with TURBO DNase Inactivation Reagent then cleaned using RNA Clean & Concentrator™ -5 kit (Zymo Research, cat. no. R1016). RNA integrity was checked with Agilent 2100 bioanalyzer prior to library preparation.

Prior to library preparation, we randomly assigned samples into four groups to minimize the potential for batch effects. The four sets of libraries were prepared on separate days within a span of one week using the QuantSeq 3’ mRNA-Seq V2 Library Prep Kit FWD with UDI’s (Lexogen). Libraries were amplified using the PCR Add-on and Reamplification Kit V2 for Illumina (Lexogen). Prior to pooling samples, preparations were checked for quality using Agilent TapeStation High Sensitivity D5000 ScreenTape®. Samples were then pooled and sequenced as 100-bp single-end reads on a single lane of the Illumina NextSeq2000 (Oregon State University Center for Genome Research and Biocomputing). We obtained an average of 14,721,690 reads (min. 9,296,007; max. 17,740,487) before quality check and trimming (Table S1).

Following a quality check using FastQC v. 0.12.0 (CITE), reads were trimmed of adapter and UDI sequences using *bbduk* with a minimum read length ≥ 25 bp and average Phred score ≥ 20 and the following parameters: ftl = 10, k = 13, ktrim = r, usesshortkmers = t, mink = 5, qtrim = r, where ftl = 10 specifies a ‘forcetrimleft’ to remove the first 10 base pairs belonging to the Lexogen UDI tags. We retained an average of 13,377,440 reads per sample after trimming (min. 9,047,410; max. 15,882,022). RNAseq reads were mapped using STAR (CITE) against the *Tigriopus californicus* reference genome (CITE Barreto 2018), with the following parameters: outFilterMultimapNmax 10, alignIntronMax 10,000, alignMatesGapMax 10,000. We counted mRNA reads using FeatureCounts (CITE) with the following parameters: -t mRNA, -s 1, where -s 1 enables strand specific read counting. We obtained an average of 10,377,819 reads (min. 7,217,475, max. 11,886,378) per sample after mapping and counting. This represented an average read retention rate of 77.66% (min. 74.66%, max. 80.96%) after counting and mapping (Table S1).

**Identification of DEG’s**

Processing of count data and differential gene expression analyses were completed in R (CITE R core team). Sample counts were assembled into a count matrix using the ‘read\_featureCounts’ function from the hciR package v1.7 (CITE). Prior to normalization using the DESeq2 package v1.38.3 (CITE) with default parameters, the count matrix was filtered to remove counts of mitochondrial DNA and genes that did not have at least 10 counts in at least 6 samples based on recommendations when using DESeq2, where 6 represents the lowest number of replicates per timepoint in the experiment. After filtering, but before normalization with DESeq2, we also corrected the count matrix for potential batch effects from the replication of the hypoxia time course by using the ‘Combat\_seq’ function from the Combat-seq package integrated into the sva package v3.50.0 (CITE).

We identified genes that significantly changed their expression over the hypoxia time course using the maSigPro package v 1.74.0. This package creates a regression matrix to identify significant changes in expression per gene using the ‘p.vector’ function. We specified the model using with the following parameters: Q = 0.05, MT.adjust = “BH”, and counts = TRUE, where Q sets the threshold for identifying significant genes at p < 0.05, MT.adjust = “BH” specifies the Benjamin-Hochberg FDR adjustment, and counts = TRUE specifies the use of logistic regression using a negative-binomial link function with Θ =10 because our matrix was in the form of normalized count data. We specified the degree of the regression fit polynomial as ‘degree = 3’, which models the inclusion of a linear, quadratic, and cubic term. Statistically significant genes were filtered using an r-squared cutoff of rsq > 0.1 using the ‘get.siggenes’ function from maSigPro.

We also examined six key pairwise comparisons between time points in the time course using DESeq2. These included comparing gene expression at the normoxia time point to that at the 1) mild hypoxia, 2) *P*crit, 3) anoxia, and 4) recovery time points, 5) comparing gene expression between the anoxia and recovery time points, and 6) comparing expression between the mild hypoxia and anoxia time points. Differentially expressed genes were considered statistically significant using an FDR-adjusted threshold of α < 0.1. All code used in these analyses are provided in supplemental data and freely available at [INSERT GITHUB LINK].

**Cluster Analysis and Functional Enrichment**

Using the statistically significant genes identified using maSigPro, we performed a cluster analysis in R to group the genes based on their expression patterns over the hypoxia time course. We used the ‘see.genes’ function in maSigPro to cluster the significant genes using the “hclust” (hierarchical cluster analysis) method using Ward.D aggregation and the default k = 9 clusters.

We performed a Gene Ontology (GO) enrichment analysis in R using the topGO package v2.54.0 (CITE). We used a custom *T. californicus* GO annotation (available in the supplement). We probed the genes in each cluster defined by maSigPro as well as the significant differentially expressed genes identified via pairwise comparisons using DESeq2 to look for significantly enriched terms, focusing on the biological process ontology, using a FDR cutoff of α < 0.05. We grouped and visualized the GO enrichment results using the rrvgo package v1.14.2 (CITE). We specified the semantic similarity matrix and reduced similarity matrix in rrvgo using the “Wang” method (CITE) with a threshold of 0.8 for grouping GO terms.

Because GO term labels can be broad, even with reduction and grouping techniques such as offered by the rrvgo package, we also manually curated lists of genes in the *T. californicus* genome with roles in specific pathways or functions potentially related to hypoxia response in diverse taxa or general stress response in *T. californicus*. The first step to build these lists involved manual querying of the Kyoto Encyclopedia of Genes and Genomes (KEGG) for enzymes involved in the following pathways or orthology: oxidative phosphorylation (OXPHOS), glycolysis/gluconeogenesis, starch and sucrose metabolism, fructose metabolism, pentose phosphate pathway, pyruvate metabolism, citrate cycle (TCA), chitin metabolism, and carotenoid metabolism. If the enzyme was linked with a NCBI-gene ID from the published *T. californicus* genome, this was included in the list. If the enzyme was not linked to the *T. californicus* genome, the amino acid sequence for two other species were downloaded to build a custom blast database. We preferentially chose sequences belonging to invertebrates, including *Daphnia pulex* (Leydig, 1860, KEGG organism code: dpx) and *Drosophila melanogaster* (Meigen, 1830, KEGG organism code: dme). If sequences were not published for an invertebrate, we chose sequences belonging to *Homo sapiens* (Linnaeus, 1758, KEGG organism code: has) and *Mus musculus* (Schwarz and Schwarz, 1943, KEGG organism code: mmu). Using these sequences as a custom database, we performed a blastp search with the *T. californicus* genome as the query and max target sequences set to 1, retaining matches with an e-value of < 1e-20 and match length of > 60 amino acids. The final list of genes was rounded out by inclusion of genes based on literature searchers of relevant or important genes known to have roles in these processes in other taxa. Results of blastp searchers are provided in the supplement. The final total number of genes in each list is shown in **Table 1**.

**Enrichment of transcription factor binding site motifs**

We examined each of cluster of genes identified via maSigPro for enrichment of known transcription factor binding sites using the MEME suite of tools (CITE). We used the Analysis of Motif Enrichment (AME) tools to search the upstream regions of genes in each cluster (up to 1000 bp) for known motifs in the JASPAR database (2022\_core\_insect\_nonredundant). Motifs with significant enrichment were based on default parameters using the adjusted *P*-value from a Fisher’s exact text with Bonferroni correction.

**3. Results and Discussion**

**3.1 Significant genes identified by maSigPro and GO term enrichment within clusters**

Using maSigPro, we identified 1347 genes with expression that significantly responded to the hypoxia time course. Hierarchical cluster analysis using the maSigPro default k = 9 clusters classified these genes into groups with diverse expression patterns encompassing a range of changes over the exposure to and recovery from hypoxic stress (**Figure 2**). The largest of these clusters, cluster 2, contained 264 of the significant genes (**Figure 2B**) and the smallest cluster contained 56 genes (**Figure 2I**). Using DESeq2 to compare gene expression between two select time points at a time, we found a total of 1,630 statistically significant differentially expressed genes across the five pairwise comparisons we inspected (**Figure S1**). These included comparing expression under normoxic conditions to expression 1) in mild hypoxia, 2) at the critical oxygen tension (*P*crit) of *T. californicus*, 3) during anoxia, and 4) during recovery, as well as 5) comparing gene expression between the anoxia and recovery and 6) comparing expression between hypoxia and anoxia (**Figure S1**).

**3.2 Contents and expression patterns of significant gene clusters grouped using maSigPro**

Cluster 1 included 182 genes with expression that was variable through the start of hypoxia but most often increased during recovery for most genes in the cluster. Among the top 10 most statistically significant genes identified by maSigPro within this cluster, there are several genes predicted to aid in cell detoxification, antioxidant processes, and mitochondrial homeostasis following oxidative stress. These include the flavohemoprotein hmp, CHAC2, Mgst3, and SLC13A5 (**Table S2**). Significantly enriched GO terms for genes in this cluster involved innate immune response signaling, negative regulation of reactive oxygen species (ROS) metabolism, wound healing, regulation of glycogen and carbohydrate metabolism, estrogen and other hormonal regulation, protein stabilization, and the promotion of apoptosis (**Figure S2**).

Cluster 2 represents an interesting case where gene expression steadily increased then returned to pre-stress levels during recovery. GO enrichment of genes in cluster 2 revealed terms associated with a switch from aerobic respiration to anaerobic respiration via glycolysis/gluconeogenesis and metabolic pathways closely linked with this process (**Figure S3**) including those grouped under pentose metabolic process, intracellular glucose homeostasis, and positive regulation of phosphate metabolic process. The gene Gbs-76A, found in the top 10 most significant genes of this cluster, helps regulate the binding and biosynthesis of glycogen (**Table S2**). Also found in the top 10 statistically significant genes for cluster 2 was a cuticle-like protein used in exoskeleton modification (**Table S2**). This gene was the second most statistically significant across all gene clusters. The top 10 significant genes in cluster 2 also included important genes for cell detoxification, such as Tmem9 which reduces intracellular pH, arrd-17 which responds to osmotic stress, and DDO which is known to deaminate harmful D-amino acids. Notably, within the top 10 significant genes of this cluster resided Siah1, shown to be induced by the master hypoxia response regulator HIF-α despite previous work showing that HIF-α is missing in its canonical form in *T. californicus*. Cluster 2 was also enriched with genes grouped under broad stress response GO terms such as regulation of apoptotic processes, DNA damage response, and multicellular organismal response to stress (**Figure S3**) including the heat shock proteins Hsp67Bb, Hsp23, and hsp-16,48 and the gene pyx (also in the top 10 significant genes for this cluster), implicated in protection from heat stress in *Drosophila melanogaster* (CITE).

Genes in clusters 3 and 5 shared a similar pattern as cluster 2 with an increase in expression during the hypoxic time points followed by a sharp decrease during recovery. However, genes in cluster 3 showed had expression levels did not decrease back to that of normoxia and, conversely, expression levels during recovery in cluster 5 decreased below that of normoxia on average (**Figure 2**). Additionally, genes in cluster 5 showed the most marked increase in expression during anoxia, with little or slow induction through the timepoint at *P*crit. Though only comprising 65 genes, cluster 3 was enriched for genes associated with the GO terms involving phosphate metabolism (**Figure S4**). Cluster 5 was enriched with genes grouped under GO terms associated with hormonal transport and responses to hormone signals as well as genes involved with alterations to energy metabolism involving glucose transport (**Figure S6**). Additionally, there was an enrichment of genes associated with BMP signaling in this cluster (**Figure S6**). The BMP pathway has been shown to help mediate oxidative stress during reduced oxygen levels (CITE). Both groups of top 10 significant genes in clusters 3 and 5 also contained genes involved in hormone regulation. These were the cytochrome P450 Cyp49a1 in cluster 3 involved with hormone biosynthesis and nhr-14 in cluster 5 involved with the regulation of hormone binding activity (**Table S2**). The top 10 significant genes in cluster 3 included the genes UGP2, involved with anaerobic glycolysis, and Tret1, a trehalose transporter; trehalose is a key intermediate sugar in the starch-sucrose metabolic pathway and feeds products into glycolysis and chitin synthesis in invertebrates. The top 10 significant genes in cluster 3 also included CYP6J1 and Gsr which involved with the cell detoxification activities of the antioxidant glutathione (**Table S2**).

Genes grouped into cluster 4 show an inverse pattern of expression over the hypoxia time course compared to cluster 2, whereby expression in cluster 4 decreased through hypoxia exposure but then rapidly increased to return to normoxic levels (**Figure 2**). The 156 genes in this cluster were enriched for GO terms associated with steroid biosynthesis, regulation of apoptosis due to endoplasmic reticulum stress, regulation of immune response, regulation of muscle systems, and regulation of energy production including carbohydrate metabolism and glucose homeostasis (**Figure S6**). Among the top 10 most significant genes in this cluster were genes related to the reproductive process of spermatogenesis included Ptchd3 and Gmcl1. Also in this list are two genes involved in glucose metabolism, SIK2 and CPPED1. Like cluster 2, the top 10 significant genes in cluster 4 also contained an exoskeleton modifying gene, chitin deacetylase 5 (**Table S2**). A final notable gene in the top 10 significant genes for cluster 4 is METTL9 which has been demonstrated to methylate subunits of the electron transport system (ETS) in the mitochondria (CITE).

Genes in cluster 6 are characterized by expression levels that decreased rapidly at the onset of hypoxia, with less relative change across the remaining time course (**Figure 2**). Genes in this cluster were associated with enriched representation in GO terms related to stress response, including cellular response to mechanical stimulus and response to chemical stress (**Figure S7**). Enrichment for GO terms associated with cell detoxification and repair were also represented in this cluster, including positive regulation of DNA repair, response to unfolded protein, and nitrogen compound catabolism (**Figure S7**). Indeed, the top 10 most significant genes in this cluster included the antioxidant genes gst-6 and GstD1 that encode glutathione transferases. Tests for enrichment also revealed genes associated with protein localization to the mitochondria (**Figure S7**). Within the top 10 most significant genes for this cluster were three genes that may affect early protective or combative responses to hypoxia including slc46a1 which helps transport folate during stressful conditions (CITE), a luciferin 4-monooxyganase that promotes fatty-acyl-CoA biosynthesis for ATP production, a Tret1-2 homolog used in trehalose transport, and NDRG3 which is necessary for lactate signaling during hypoxic response (CITE).

Clusters 7 and 8 showed inverse patterns of expression. Cluster 7 housed 99 genes with expression levels that steadily increased through the hypoxia time course, while cluster 8 contained 236 genes with gradually decreasing expression (**Figure 2**). GO terms enriched in cluster 7 genes included cellular response to external stimulus, glutamate metabolism, and regulation of lipid homeostasis (**Figure S8**). Among the top 10 most significant genes in cluster 7 are several genes known to combat hypoxic stress in other systems including lhpp which inhibits aerobic, but not anaerobic, glycolysis (CITE), SQOR which detoxifies the mitochondria from sulfides, and slc22a7 which acts as a hub gene for excreting toxins during hypoxia (**Table S2**). Cluster 8, which contained twice as many genes as cluster 7 (**Figure 2**), was enriched with genes associated with several catabolic and metabolic processes (**Figure S9**). Genes of cluster 8 were also enriched with GO terms involving response to unfolded protein, response to oxygen-containing compounds, and response to cold (**Figure S9**). The top 10 most significant genes in cluster 8 contained the gene clpX (**Table S2**) which specifically helps with unfolded protein response in the mitochondria. Like cluster 6, there was also an enrichment of genes involved with regulation of nitric oxide (**Figures S7, S9**) and, like cluster 5, an enrichment of genes involved with hormone signaling (**Figures S6, S9**). Like clusters 2 and 4 that contained genes with a strong increase or decrease in expression, respectively, in their top 10 significant genes, clusters 7 and 8 also contained genes that encode products predicted to affect exoskeleton composition. These were laccase-2 in cluster 7 which is a key initiator the process of exoskeleton tanning (hardening and making insoluble) and agmo, an alkylglycerol monooxygenase that affects cuticular stability (**Table S2**).

Genes grouped into cluster 9 showed a general pattern of minimal or inconsistent changes in expression across the exposure to hypoxia but a drastic decrease in expression below normoxic levels during recovery (**Figure 2**). Despite being the smallest of the gene clusters grouped by maSigPro, cluster 9 genes were enriched for a variety of functions (**Figure S10**), including the regulation of insulin, ER stress response, fructose metabolism, and negative regulation of lipid storage. Enriched GO terms for genes in this cluster also involved stress response processes such as the response to acidic pH and toxic mercury buildup. This cluster also was enriched for genes associated with the maturation of pigment granules. This category included the most significant gene in this cluster, CD63, which is known to promote cell survival, cell adhesion, and sequestration of pigment (CITE) (**Table S2**). Within the top 10 significant genes of cluster 9 were mdh that is important for NADH supply to the mitochondria following the citric acid cycle, PNKP which associated with DNA damage repair following oxidative stress, and NA+/H+ exchanger beta that plays a role in insulin homeostasis (CITES).

**3.2 Transcription factor binding site enrichment in gene clusters**

Tests for enrichment of transcription factor binding site motifs in the promoter regions of genes in each cluster revealed 13 motifs across all 9 clusters (**Figure 3, Table S3**). The binding site for the transcription factor Clamp, involved in sex specific alternative splicing and the regulation of chromatin accessibility during exposure to abiotic stress, was significantly enriched in all 9 clusters. The next most common binding site belonged to Trithorax-like (Trl), present in all clusters except cluster 9. This factor also regulates chromatin accessibility and is connected to proper mitochondrial biogenesis whereby a reduction in Trl function can lead to increased ROS. Like Trl, there was significant enrichment for binding sites of Chorian factor 2 (Cf2) in clusters 1 through 8. This factor is associated with the regulation of muscle fiber genes at all stages of life; however, its enrichment could also be unrelated to the hypoxia time course and instead could be explained by its association with the circadian gene timeless (tim), which was in the top 10 most significant genes of cluster 1 thereby indicating at some changes to gene expression may be due to diel and rhythmic regulation. The binding motif for the zeste (z) transcription factor, shown to participate in transvection-based gene regulation, was also significantly enriched in several clusters included clusters 1,4, 6, and 7. The promoter region of genes in clusters 1 and 2 were enriched with motifs for ceramide synthase (CerS/Schlank) binding. This dual enzyme-transcription factor plays a critical role in lipid homeostasis during starvation conditions or when energy demands fluctuate (CITE).

Cluster 2 contained genes with expression that increased through anoxia but then returned to normoxic levels during recovery. The upstream promoter regions of these genes were significantly enriched with motifs for three binding sites not significantly enriched in any other cluster. These motifs matched that of knirps (kni), grainyhead (Grh), and caudal (cad) factor binding sites. Knirps-like factors play critical roles in the formation of eggs by invertebrates, mostly through a mechanism of transcriptional repression (CITE), caudal-like factors are known to affect gut homeostasis and increase in expression in response to oxidative stress (CITE), and grainyhead-like factors have been shown to influence the integrity and formation of the epithelium, cuticle, and exoskeleton in adult *Caenorhabditis elegans* (CITE). Grainyhead-like factors may also help influence exoskeleton modifications in *T. californicus*, considering previous results that suggest a role of exoskeletal modification to survive hypoxia shown previously (CITE Graham & Barreto) and the identification of several genes involved in chitin and cuticular modification identified in the present hypoxia time course (see sections 3.3.4 and 3.3.5).

**3.3 Changes in expression in genes grouped by select functions**

**3.3.1 Response to Oxygen and Mitochondria-targeted genes**

During and following a hypoxic event, many biological systems and hundreds of genes are engaged to combat cellular stress, prolong survival, and ensure recovery. The GO term “Response to Oxygen” includes 1,022 genes in the *T. californicus* genome that may participate in at least some small way in hypoxia and reoxygenation stress pathways. A total of 108 of these genes were identified by maSigPro to significantly change expression over the hypoxia time course, with 103 of these also identified by DESeq2 in pairwise contrasts of select time points. Pairwise comparisons between expression at normoxia and expression at each of the other four time points in the time course revealed that the number of genes in this GO term increased at each progressive stage of hypoxia and into recovery (**Figure 4A).**

When available oxygen is depleted, the production of ROS via mitochondrial ETS complex III occurs during the switch to anerobic metabolism. The production of these compounds continues during the initial phases of recovery when rapid reoxygenation floods the mitochondrial ETS with oxygen (CITE). Several genes that produce products targeted to or interacting with the mitochondria were found to be significantly differentially expressed over the hypoxia time course. Of the 600 genes with mitochondria-related products identified in the *T. californicus* genome, 50 of these were differentially expressed with 40 identified by maSigPro and an additional 10 uniquely identified by pairwise comparisons to normoxia using DESeq2. Like genes in the ‘Response to Oxygen’ GO term, the number of significant genes found to be differentially expressed from normoxia increased from the onset of hypoxia through anoxia and remained elevated during recovery (**Figure 4B**). The 40 genes identified and clustered by maSigPro were found in each cluster except 9 (**Table** ), with the largest number of these genes found in cluster 2 (10 genes) and cluster 8 (8 genes).

Cluster 3, which grouped genes with increased expression through anoxia but decreased during recovery, included the gene AOX1 which encodes an alternative mitochondrial oxidase. This enzyme acts as an alternate exit point for electrons in the ETS and is predicted to aid in mitochondrial stability during periods of oxidative stress. When the AOX enzyme is present, electron exit the system before they reach complex 3, dissipating the energy and producing heat (CITE). This eliminates activity of complex 4, cytochrome oxidase (COX), which normally catalyzes the transfer of electrons to oxygen. When oxygen is absent during anoxia, increased expression of AOX may allow complexes 1 and 2 to continue to utilize substrates and could avoid the overreduction of complexes 3 and 4 when electron supply is slow or when ROS compromise the inner mitochondrial membrane (CITES). This enzyme may be a unique adaptation in copepods that could have been acquired via lateral gene transfer (CITE).

There were no genes encoding subunits of the OXPHOS complexes in the ETS that were significantly affected by the hypoxia time course according to the maSigPro model. However, one gene, NDUFA6, which encodes a subunit of NADH dehydrogenase (ubiquinone) and complex 1 of the ETS, was identified by DESeq2 to have significantly higher expression solely during anoxia compared to normoxia. Complex 1 serves as one of the initial entry points for electrons to the mitochondrial membranes during the production of ATP and uses NADH from glycolysis and the TCA cycle and the NDUFA6 subunit may be important for complex 1 biogenesis. After an hour anoxia, aerobic respiration would be running low on substrates needed to produce ATP and anaerobic respiration would be the main producer of cellular energy. However, because *T. californicus* experience cyclical anoxic conditions in the wild, the biogenesis of complex 1 may indicate preparation for reoxygenation.

**3.3.2 Antioxidants**

The effects of ROS during hypoxia, anoxia, and reoxygenation represents a critical biotic challenge to survival. Of the 48 antioxidant-related genes identified in the *T. californicus* genome (CITE list in supplement), 12 of these were significantly differentially expressed during the hypoxia time course and were present in all gene clusters grouped by maSigPro except cluster 9 (**Table 1**). The number of these genes found to be significantly differentially expressed between normoxia and each of the subsequent time points by DESeq2 increased as hypoxia intensified and remained elevated during recovery (**Figure 4C**).

Many of these genes produce products involving the antioxidant molecule glutathione (GSH). The key antioxidant enzyme glutathione peroxidase (GPX2), the key cofactor of glutathione during the reduction of peroxides, was present in cluster 7 with genes that increased in expression through the time course. Several predicted glutathione S-transferase genes also responded significantly, including microsomal Mgst-like genes and Gst-like isoforms. glutathione S-transferases work with the reduced form of glutathione to detoxify cells of lipids damaged by ROS (CITE). Other important members of the glutathione metabolism pathway also significantly responded, including the mitochondria-targeted glutathione reductase Gsr that reduces glutathione disulfide to produce more GSH, the glutathione-specific glutamylcyclotransferase CHAC2 that degrades GSH, and the glutathione hydrolase Ggt1 that breaks down extracellular GSH. Altogether, these groups of genes make up the production, breakdown, and key enzymatic cofactors of glutathione and indicates that this antioxidant is critical for responding to hypoxic stress in *T. californicus*.

Why members of the superoxide dismutase (SOD) family, another group of powerful antioxidants, did not significantly respond to time course is unclear. Other antioxidants, such as catalase or γ-glutamylcysteine ligase, did not significantly respond to the hypoxia exposure either. However, the lack of a transcriptional response by these elements may not reflect an antioxidant response at the protein level, as post-translational modifications to antioxidant activity is a possible method for tuning responses to ROS. The expression of at least two other antioxidant genes besides GSH and SOD, did significantly respond to hypoxic stress. The glucose-6-phosphate 1-dehydrogenase (Zw), a member of the pentose phosphate pathway but also a predicted antioxidant, was found by DESeq2 to be significantly downregulated at anoxia compared to normoxia, but this could simply reflect changes to the pentose phosphate pathway during the switch to anaerobic respiration. The gene UGT8 gene which encodes a UDP-glucoronosyl transferase, another potential antioxidant (CITE), also significantly changed in expression over the time course and grouped in cluster 2 with genes with expression that increased during hypoxia but returned to normoxic levels during recovery.

**3.3.3 Carotenoid metabolism genes**

Carotenoid pigments are assumed to be both antioxidants and critical precursors to stress and immune responses in marine species (CITE), though the degree to which these pigments contribute to antioxidant activity in other taxa, particularly avian species, is still debated (CITE). Most animals, including *T. californicus*, cannot synthesize carotenoids de novo and must ingest dietary carotenoids, such as beta-carotene or lutein, to convert them into new forms for pigmentation or cleave them to produce precursors to vitamin A or various retinoids. The bright orange-red color of *T. californicus* is almost entirely derived from a modified carotenoids called astaxanthin (CITE). Out of 26 *T. californicus* genes putatively involved with the modification, transport, deposition, or cleavage of carotenoids, 9 were found to be significantly differentially expressed by maSigPro and grouped into clusters 1, 2, 6, 7, 8, and 9 (**Table 2**) and 1 additional gene was identified by DESeq2 to have increased expression in anoxia compared to normoxia. The number of these genes found differentially expressed between normoxia and subsequent time points increased through the time course (**Figure 4D**).

Four of the significant genes are predicted to encode carotenoid cleaving enzymes. These included three beta-carotenene oxygenases that nestled in cluster 8 with genes showing decreased expression through anoxia and into recovery and the fourth was ninaB, grouped in cluster 1 with genes that show a strong increase during recovery. Four cytochrome P450-encoding genes identified by Weaver et al. (2022) as candidate genes that may encode carotenoid modifying enzymes in *T. californicus* were found to significantly respond to hypoxia exposure. These included Cyp18a1 which grouped in cluster 1, mitochondrial Cyp301a1 which clustered with genes in cluster 2, and CYP3A24 and CYP15A1 which both grouped in cluster 6 with genes that decreased in expression at the start of hypoxic exposure.

Three genes involved with pigment storage, CD63-like genes, were also significant. Two of these, TCAL\_04752 and TCAL\_02439, grouped into clusters 7 and 9, respectively, while the third, TCAL\_09634, was identified using DESeq2 as upregulated between anoxia and normoxia. In other systems, CD63 encodes an antigen that promotes pigment storage in vesicles so the expression changes of these three genes may indicate an increase in pigment sequestration during hypoxic stress. Insofar, CD63 genes have been shown to primarily work with melanin pigments; however, they may also help store retinal pigments which can include carotenoids (CITE). Like many other crustaceans, *T. californicus* circulates carotenoids as lipid-like globules but also store them in their tissues in esterified forms (CITE). Carotenoids and CD63 antigens are predicted to participate in the immune and inflammatory response processes and therefore, it is possible these elements interact during *T. californicus*’ response to hypoxia.

At this time, it is unclear whether *T. californicus* relies on a single, multi-functional enzyme for carotenoid modification or if it requires multiple enzymes to convert dietary carotenoids to modified forms for pigmentation (CITE). However, the alterations to putative pigment modification genes, coupled with a decrease in beta-carotene oxygenase gene expression and an increase in pigment sequestration, may indicate that *T. californicus* was retaining carotenoids for antioxidant activity during anoxia or reoxygenation. The increase in the ninaB gene expression during recovery may indicate a switch to use some of these carotenoids to scavenge ROS during reoxygenation or to help aid homeostatic responses by the immune system. Carotenoids seem to be important in *T. californicus* for buffering against a variety of abiotic stressors (CITE) and, therefore, the accumulation of these pigments may be an important element that explains how this species is capable of tolerating extended exposure to hypoxia.

**3.3.4 Glycolysis related pathways and chitin metabolism**

When oxygen decreases and is eventually depleted, aerobic organisms often alter their metabolism to more heavily rely on the process of glycolysis instead of oxidative phosphorylation. This is possible because sugar substrates are still available during hypoxia even though oxygen, the terminal electron acceptor in the ETS, has become scarce (CITE). GO terms related to glycolysis and intertwined sugar metabolic processes including the pathways for fructose-mannose, starch-sucrose, pyruvate, pentose phosphate, and the TCA cycle were enriched in all clusters except clusters 6 and 7 (**Table 2, Figures S2-S10**). However, individual genes involved in these processes were found to be statistically significant and present across all nine clusters (**Table 1**). This emphasizes a switch by *T. californicus* to anaerobic metabolism as is seen in most aerobic species, a process which is usually regulated by the activity of HIFα (CITE). The transcriptomic response to hypoxia and reoxygenation by members of the metabolomic pathways is complicated and tissue specific (CITE). However, despite measuring gene expression in whole animal homogenates, we observed some patterns that stand out in our data despite the complexity of the transcriptional response among sugar metabolism pathways (**Figure S11**).

Glycolysis feeds into pyruvate metabolism through the multistep conversion of various glucose forms into pyruvate. Genes all along the glycolysis pathway responded to the hypoxic time course. Hexokinases are responsible for the initial conversion of α-D-glucose to glucose-6-phosphate at the start of this pathway (**Figure S11**). The hexokinase gene HKDC1, which encodes a whole-body hexokinase, grouped with cluster 2. Glucose may also be converted to β-D-Glucose, then to β-D-Glucose-6-phosphate, and then on to glucose-6-phosphate. It appears that genes encoding enzymes that participate in this alternative pathway significantly decrease in expression during hypoxia exposure with the aldose 1-epimerase GALM (TCAL\_12124) grouping in cluster 8 and a glucose-6-phophate 1-epimerase (TCAL\_04063) in cluster 4. It is possible that this alternative method for reaching glucose-6-phosphate may be energetically unfavorable given that it requires more steps that using a hexokinase alone, or that this is a possible mechanism for decreasing the activity of pathways that utilize β-D-glucose. This observation is also despite our observation that the gene PGM1, which encodes a phosphoglucomutase that also operates at the start of glycolysis to produce glucose-6-phosphate from the starch and sucrose metabolic pathway, had increased expression starting at the initial hypoxia time point and became significantly higher during anoxia compared to normoxia according to DESeq2.

In the middle of the glycolysis pathway, the genes FBP1 which encodes a fructose-1,6-bisphosphotase (also active in the pentose phosphate pathway), Pfkfb4 which encodes a 6-phosphofructokinase (also active in the fructose and mannose pathway), Pgk which encodes a phosphoglycerate kinase, and PGAM2 that encodes a phosphoglycerate mutase all increased in expression. Pfkfb4 grouped in cluster 9 with genes that showed an increase in expression as hypoxia worsened with a sudden dip in expression at *P*crit and again in recovery. FBP1 grouped with cluster 1 genes that generally shared a pattern of highest expression in recovery (**Figure S11**). Pgk and PGAM2 were identified by DESeq2 to have significantly higher expression in anoxia compared to recovery and normoxia, respectively. These expression patterns are consistent with the functions of the known roles of these genes’ products, whereby FBP1 is a repressor of HIF activity (thus explaining its heightened expression post-hypxia in our results), and Pfkfb4, Pgk, and PGAM2 encode enzymes needed to complete glycolysis.

The significant induction of many genes in the glycolysis and related pathways (**Figure 4E**) indicates that anaerobic metabolism is an important and rapid response to decreasing oxygen in *T. californicus*. Indeed, a key regulatory gene that normally inhibits glycolysis called TIGAR, which encodes a fructose-2,6-bisphosphatase, was grouped in cluster 4 with genes that decreased in expression during through anoxia but returned to normoxic expression levels during recovery (**Figure S11**). Like the gene Siah1, TIGAR has been shown to be a target of HIF-α (CITE). Siah1 and TIGAR were members of clusters 2 and 4, respectively, and these two clusters had inverse patterns of expression. Siah1 has been shown to promote the degradation of the proline hydroxylases that destroy HIF-α, thereby further stabilizing the activity of HIF-α during the hypoxia response. Moreover, the glycolysis genes mentioned above, FBP1, Pfkfb4, and Pgk have each been shown to possess hypoxia response elements that interact with HIF-α as well. The observations that Siah1, TIGAR, FBP1, Pfkfb4, and Pgk expression respond as they normally would in systems where HIF-α is known to be present is notable because HIF-α has been shown to be missing from the *T. californicus* genome. It is possible that there is another key regulatory element of the hypoxia response in *T. californicus* that is performing the same role, or that HIF-α exists in the genome but not in its canonical form.

The gene Pepck, which encodes a phosphoenolpyruvate carboxykinase that exerts strong control over the rate of gluconeogenesis, sits at the interface of glycolysis, the TCA cycle, and the pyruvate pathway. This gene was another member of cluster 2, showing increased expression through anoxia then decreasing during recovery (**Figure S11**). Another key gene in the pyruvate pathway that was upregulated in hypoxia and into recovery was PC, which encodes a pyruvate carboxylase needed to convert pyruvate into oxaloacetate which then feeds into the TCA cycle during the process of energy production. This gene grouped with cluster 1 genes that had the highest expression during recovery; however, its expression was elevated even at the start of hypoxia exposure (**Figure S11**). While shown to be suppressed during hypoxia in cancer cells (CITE), this genes elevated activity during the hypoxia time course in *T. californicus* may be reflective of this species’ extremely low *P*crit and their ability to regulate normal respiration even down to very low levels of oxygen (CITE our paper).

We also observed some interesting patterns with genes involved with the byproducts of glycolysis and the pyruvate pathway, ethanol and lactate. The gene ldhA encoding a lactate-dehydrogenase clus was upregulated though hypoxia and into recovery (**Figure S11**). Four alcohol dehydrogenase genes also significantly responded to the hypoxia time course (**Figure S11**). However, unlike the lactate dehydrogenase, these Akr1a1-like genes clustered mostly in cluster 8 with genes that decreased in expression during the time course (**Figure S11**). This may be explained via the activity of the the acetyl-CoA synthetase Acss1 in cluster 7 and the aldehyde dehydrogenase aldh4a1 in cluster 5 and that showed a marked increase in expression especially starting at the *P*crit time point. These three genes can reduce the production of ethanol by converting acetaldehyde and acetate into forms used in other pathways, such as butanoate or propanoate metabolism.

Many of the sugars, byproducts, and enzymes related to the glycolysis, pyruvate, fructose mannose, pentose phosphate, and TCA cycle pathways (such as hexokinases, for example) are also present in the starch-sucrose and the chitin metabolic pathways. The involvement of chitin metabolism in the hypoxia response of *T. californicus* has been proposed previously based on a strong transcriptional response of chitin, cuticle, and exoskeletal genes (CITE Graham & Barreto 2019). Our results recapitulate this result, with altered expression for many genes related to these processes (**Table 1, Figure S11, Figure 4F**).

One of the first substrates required to produce chitin is glucose (CITE). However, glucose used to produce chitin can also come from the disaccharide trehalose. Two genes that generate trehalose in the starch-sucrose pathway were upregulated. These were tpsA which encodes an alpha-trehalose-phosphate synthase and tpsp which encodes a bifunctional trehalose-6-phosphate synthase (**Figure S11**). Tpsp groued in cluster 2 with genes that increased during hypoxia and returned to normoxic expression levels in recovery and tpsA was identified by DESeq2 to be more highly expressed in recovery compared to normoxia. However, genes encoding trehalases, which are responsible for converting trehalose to β-D-glucose needed for chitin synthesis did not significantly respond to the hypoxic stress. Instead, we observed that ten different predicted trehalose transport genes (Tret1 or Tret1-2 like) did significantly respond (**Figure S11**). Seven of these decreased expression during hypoxia exposure grouping with clusters 4, 6, and 8. The other three increased in expression, with one clustering in cluster 1 (up drastically in recovery), another in cluster 3 (up during through anoxia but falling in recovery), and the third being identified via DESeq2 to be differentially expressed at *P*crit compared to normoxia. Trehalose phosphate synthases, like tpsA and tpsp, and trehalose itself are both implicated in buffering against hypoxic stress in multiple taxa through a proposed mechanism of protein stabilization. It is possible that *T. californicus* takes advantage of this mechanism as well, regulating its transport of trehalose toward stabilizing proteins.

*Tigriopus californicus* may also need to transport some trehalose toward chitin metabolism. We observed a significant transcriptional response of genes in the chitin synthesis pathway (**Figure S11**). These included UGP2 which encodes a glucose-1-phosphate uridylytransferase, three glutamine/fructose-6-phosphate transaminase genes (Gfpt1, GFPT1, and GFPT2-like), NAGK that encodes an N-acetyl-D-glucosamine kinase, PGM3 that encodes a phosphoacetylglucosamine mutase, and Slc35a3 which encodes a UDP-N-acetylglucosamine transporter. This list also may include the hexokinases like HKDC1 and Hex-t2, as these enzymes catalyze conversion of glucose to glucose-6-phophate at the start of this pathway. HKDC1 and UGP2, both of which help make glucose-6-phosphate, were both upregulated during hypoxia belonging to clusters 2 and 3, respectively. Likewise, the transaminase encoding GFPT2 which is active in the process of cell adhesion, was also upregulated in hypoxia and belonged to cluster 5 (**Figure S11**). Conversely, the GFPT1-like transaminases were downregulated and grouped in cluster 4 (**Figure S11**). This contradicts previous results that show hypoxia induces Gfpt1 expression during hypoxia in mice, but could be explained by an apparent role of utilizing GFPT2 for cell survival when glutamine uptake is high, as would be the case during hypoxia.

Chitin catabolism can proceed to two ways. Chitin can be broken down by chitinases to produce N-acetyl-glucosamine or chitin can be deacetylated to produce chitosan and N-glucosamine. We observed inverse patterns between these two processes (**Figure S11**), where chitinases were decreased in expression as hypoxia progressed and remained depressed into recovery, with two of these grouping in cluster 8 by maSigPro. Conversely, three of four chitin deacetylase genes (TCAL\_13698, TCAL\_05770, and TCAL\_13801) identified by maSigPro increased in expression becoming most highly expressed into recovery (cluster 1). The fourth chitin deacetylase gene (TCAL\_13736) grouped in cluster 4 with genes that decreased during hypoxia but increased in recovery. These two sets of results with chitinases and deacetylases, combined with the increased expression of genes in the chitin synthesis pathway, could indicate that *T. californicus* increases the production of chitin hypoxia exposure. Chitinase expression is decreased at the same time, so that any chitin breakdown that occurs is in favor of the production of chitosan, especially during recovery. This may be due to a possible role in the response to ROS by chitosan, possibly through interactions with antioxidants.

**3.3.5 Other cuticle and exoskeleton genes**

Increased expression of genes involved in chitin synthesis and decreased expression of chitinase genes could also be explained through the lens of exoskeletal modification. Invertebrate exoskeletal structure is also determined by cuticle composition, and we observed several cuticle-modifying genes that responded significantly to the hypoxia course. In fact, the most significantly differentially expressed gene in cluster 2 encodes a cuticle-like protein (TCAL\_01551, **Table S2**). In total, five other cuticle-like genes also significantly responded to hypoxia. Of these, two identified via maSigPro belonged to clusters 4 and 8. The remaining three were identified by DESeq2 and two of these showed increased expression during recovery compared to normoxia while the last showed increased expression during anoxia compared to normoxia. Four other genes predicted to encode cuticle modifying proteins also significantly responded during the time course. These included three genes predicted to encode obst-E proteins, which play a role in chitin binding during cuticle formation (CITE), and the another predicted to encode a pro-resilin gene, which helps make the elastic components of the cuticle. Two of the obst-E genes were upregulated during hypoxia exposure with one of these two grouping in cluster 2 and the other identified by DESeq2 to be upregulated in anoxia and recovery compared to normoxia. The third obst-E gene was grouped in cluster 8. The pro-resilin gene was identified by DESeq2 to be upregulated at the *P*crit time point compared to normoxia.

The processes of exoskeletal modification through changes to chitin and cuticle genes is complicated and the specifics of how mechanistically *T. californicus* may be altering its exoskeleton over the exposure to and recovery from hypoxia is unclear. However, it is possible that the exoskeleton becomes denser and less permeable during hypoxia. Previously, Graham & Barreto (2019) described a general pattern of expression after of 24 hours of hypoxia that showed chitin degradation genes being downregulated and chitin synthesis genes being upregulated (CITE). The patterns of expression by chitin genes over the hypoxia time course in this study seem to be consistent with that conclusion. Although it the expression patterns of cuticle-modifying genes, such as the cuticle-like or obst-E protein encoding genes, do not offer a clear picture of how they might be affecting the exoskeleton of *T. californicus*, we hypothesize that rearrangement of the cuticle to make the exoskeleton denser and less permeable is the overall effect. Indeed, within the top 10 most significant genes across all the clusters lies the gene lcc2 (Laccase-2, **Table S2**), a gene that has been implicated in beetles as a critical component of cuticle tanning (CITE). The process tanning in invertebrates involves the sclerotization and pigmentation of the cuticle, making it insoluble, hard, and pigmented. Across the hypoxia time course, lcc2 expression increased in response to hypoxia, grouping with cluster 7 genes.

Based on transcriptional changes to the chitin synthesis pathway, the significant responses of cuticle modifying genes, and the involvement of a known cuticle tanning gene in lcc2, our results corroborate observations that exoskeletal changes occur in *T. californicus* during hypoxia exposure (CITE Graham & Barreto 2019). Chitin synthesis does appear to be tied to hypoxia induced transcriptional changes, albeit with the expression of the canonical HIFα gene that is lacking in *T. californicus*. Additionally, thickening of the cuticular lining of the trachea in response to hypoxia has been documented previously in nematodes. It is possible that *T. californicus* modifies its exoskeletal composition as an adaptive response to survive hypoxia. This species lacks a circulatory system, respiratory pigments, or gills and passively diffuses oxygen across its cuticle to respire. Rearrangement and tanning of the cuticle and the build-up of chitin (to reinforce the exoskeleton or produce chitosan in response to oxidative stress) may then allow *T. californicus* to sequester what little oxygen remains when dissolved oxygen in the environment becomes scarce. This strategy would contrast with that of plants, which increase cuticle permeability to facilitate greater gas exchange during hypoxia. Regardless, an adaptive role of cuticle and chitin modification in response to hypoxia remains speculative and correlational at this stage and further experimentation that simultaneously documents physiological changes via microscopy may help validate physiological changes that correspond to altered gene expression during hypoxia.

**4. Conclusion**

* The RNA-seq data in this study indicate a multi-faceted response on both short- and long-term time scales to hypoxia stress by the copepod *Tigriopus californicus*.
* Canonical responses to hypoxia, including changes in expression of genes involved in abatement of oxidative stress due to reactive oxygen or nitrogen species were also observed, as was changes in expression to genes with products that operate in the mitochondria itself. This includes the gene AOX, which encodes an alternative oxidase in the mitochondrial ETS and a gene that encodes a subunit of complex 1.
* Enrichment of genes found in unfolded protein response was seen in gene clusters involved with decreased expression during hypoxia or an increase during recovery.
* The involvement of carotenoid processing genes, including those involved in their breakdown like beta-carotene oxygenase genes, reinforces previous studies that advocate for carotenoid pigments as important buffers to environmental and internal stress in *T. californicus*.
* Changes in the expression of genes involved in anaerobic metabolism were observed in clusters of genes that responded early and transiently. These included increased expression in genes involved in glycolysis and the metabolism and transport of sugars. We observed differential expression of many genes associated with the transport of trehalose, a dissacharide shown to reduce oxidative stress and display antioxidant activity in other systems.
* Genes involved in chitin synthesis, chitin degradation, cuticle modification, and cuticle tanning are differentially expressed during the hypoxic time course. Genes in cluster 2 with transiently increased expression during hypoxia are enriched with binding site motifs for grainyhead-like factors, known to affect cuticle formation in other systems. This corroborates previous RNA-seq data suggesting exoskeletal modifications by *T. californicus* may be taking place during hypoxia. This includes
* Many of the genes found to significantly respond to the hypoxic course involve systems normally regulated by the activity of HIFα. Additionally, key genes that are known to interact directly with HIFα via hypoxia response elements significantly responded, including Siah1, TIGAR, FBP1, Pfkfb4, and Pgk. This raises the question as to whether a closely related hypoxia inducible factor with a non-homologous gene sequence to HIFα initiates the hypoxic response in this species. The canonical downstream response to HIF is clearly intact, plus transcriptional responses in systems more special to *T. californicus* like carotenoid or exoskeletal modifying genes.

**References**

**…**

**Tables**

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| --- | --- | --- | --- |
| **Table 1**: Total number of *T. californicus* genes curated under biological functions of interest. | | | |
| **Function or pathway** | **Total** | **# Significant (maSigPro)** | **Clusters** |
| Glycolysis | 58 genes | 15 genes | 2, 3, 4, 5, 6, 7, 8 |
| Fructose and mannose metabolism | 22 genes | 7 genes | 2, 4, 6, 8, 9 |
| Starch and sucrose metabolism | 54 genes | 20 genes | 2, 3, 8 |
| Pentose phosphate pathway | 22 genes | 8 genes | 1, 2, 3, 6, 8 |
| Pyruvate metabolism | 40 genes | 9 genes | 1, 2, 3, 5, 6, 7, 8 |
| TCA cycle | 26 genes | 2 genes | 1, 2 |
| Response to oxygen (GO Term) | 1022 genes | 108 genes | 1, 2, 3, 4, 5, 6, 7, 8, 9 |
| Antioxidants | 48 genes | 12 genes | 1, 2, 3, 4, 5, 6, 7, 8 |
| Mitochondria-targeted | 600 genes | 40 genes | 1, 2, 3, 4, 5, 6, 7, 8 |
| Carotenoid and pigment genes | 26 genes | 10 genes | 1, 2, 6, 7, 8 |
| Chitin, cuticle, and exoskeleton | 224 genes | 52 genes | 1, 2, 3, 4, 5, 6, 7, 8 |
| The totals for glycolysis and related pathways (fructose, starch/sucrose, pentose phosphate, pyruvate, and TCA) also include genes that are known to be present in two or more of these pathways. Likewise, there is probable overlap in the genes in the antioxidant, mitochondria targeted, response to oxygen, and carotenoid pathways. | | | |

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Table 2:** A summary of select categories taken from the visualization of enriched GO terms created by the ‘rrvgo’ package across all 9 maSigPro clusters of genes significantly affected by the hypoxia time course. | | | | | | | | | |
| **Broad GO Term Processes** | **Clusters enriched for term** | | | | | | | | |
| **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** |
| **Immune response** | **X** |  |  | **X** | **X** | **X** |  |  |  |
| **Hormone, steroid, or estrogen regulation** | **X** |  |  | **X** | **X** |  |  | **X** |  |
| **Oxidative stress response** | **X** |  |  |  |  |  |  | **X** |  |
| **Cell detoxification or nitrogen-compound metabolism** |  |  | **X** |  |  | **X** |  | **X** | **X** |
| **\*Glucose homeostasis, glycolysis, and related pathways** | **X** | **X** | **X** | **X** | **X** |  |  | **X** | **X** |
| **Mitochondrial-specific targeting and OXPHOS** | **X** |  |  |  | **X** | **X** |  |  |  |
| **Apoptosis** | **X** | **X** |  | **X** |  | **X** |  |  |  |
| **DNA damage repair** |  | **X** |  |  |  | **X** |  |  |  |
| **Unfolded protein response** | **X** |  |  | **X** |  | **X** |  | **X** | **X** |
| **Response to external stimuli or stress** | **X** | **X** |  | **X** |  | **X** | **X** |  |  |
| **Heat or cold response** | **X** |  |  |  |  |  |  | **X** |  |
| **Nervous system regulation and retinal response** | **X** | **X** |  | **X** | **X** |  | **X** | **X** |  |
| **Circadian rhythm or clock regulation** | **X** |  |  |  |  |  |  |  |  |
| **Pigment storage** |  |  |  |  |  |  |  |  | **X** |
| **Regulation of sperm or egg development** | **X** |  |  |  |  |  |  | **X** |  |
| **Iron ion, calcium ion, or other metabolite homeostasis** | **X** | **X** |  | **X** | **X** | **X** |  |  |  |
| **Lipid or fatty-acid homeostasis** | **X** | **X** |  | **X** |  | **X** | **X** | **X** | **X** |
| **BMP signaling pathway** |  |  |  |  | **X** |  |  |  |  |
| **\*Includes fructose, pentose-phosphate, sucrose and carbohydrate, TCA, and pyruvate pathways** | | | | | | | | | |

**Figures**

**A screen shot of a video game

Description automatically generated**

**Fig. 1** Experimental setup of the hypoxia course.

A group of graphs showing the number of clusters

Description automatically generated

Figure 2: The expression patterns of genes identified to significantly respond over the hypoxia course by maSigPro. Genes were clustered into groups using the default “hclust” (hierarchical cluster analysis) method using Ward.D aggregation and the default k = 9 clusters by maSigPro. Black dots are the mean expression at each time point and error bars represent the standard error of the mean.

A collage of multiple colored text

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Figure 3: A subset of significantly enriched transcription factor binding site motifs across clusters. A full list can be found in Table S3.

A graph of numbers and lines

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Figure 4: The number of significant differentially expressed genes detected using DESeq2 when comparing in a pairwise fashion the expression at normoxia to each of the time points during hypoxia exposure and during recovery.

**Do we want to refer to any of these figures below in the text?**

A diagram of a graph

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AI-generated content may be incorrect.A diagram of a network

AI-generated content may be incorrect.A graph of different lines and dots

AI-generated content may be incorrect.A diagram of a graph

AI-generated content may be incorrect.