

Loss of the HIF pathway in a widely distributed intertidal crustacean, the copepod *Tigriopus californicus*

Allie M. Graham^{a,1} and Felipe S. Barreto^a

^aDepartment of Integrative Biology, Oregon State University, Corvallis, OR 97330

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Hypoxia is a major physiological constraint for which multicellular eukaryotes have evolved robust cellular mechanisms capable of addressing dynamic changes in O₂ availability. In animals, oxygen sensing and regulation is primarily performed by the hypoxia-inducible factor (HIF) pathway, and the key components of this pathway are thought to be highly conserved across metazoans. Marine intertidal habitats are dynamic environments, and their inhabitants are known to tolerate wide fluctuations in salinity, temperature, pH, and oxygen. In this study, we show that an abundant intertidal crustacean, the copepod *Tigriopus californicus*, has lost major genetic components of the HIF pathway, but still shows robust survivorship and transcriptional response to hypoxia. Mining of protein domains across the genome, followed by phylogenetic analyses of gene families, did not identify two key regulatory elements of the metazoan hypoxia response, namely the transcription factor HIF- α and its oxygen-sensing prolyl hydroxylase repressor, EGLN. Despite this loss, phenotypic assays revealed that this species is tolerant to extremely low levels of available O₂ for at least 24 h at both larval and adult stages. RNA-sequencing (seq) of copepods exposed to nearly anoxic conditions showed differential expression of over 400 genes, with evidence for induction of glycolytic metabolism without a depression of oxidative phosphorylation. Moreover, genes involved in chitin metabolism and cuticle reorganization show categorically a consistent pattern of change during anoxia, highlighting this pathway as a potential solution to low oxygen availability in this small animal with no respiratory structures or pigment.

hypoxia | transcription factor | gene expression

Multicellular eukaryotes have evolved specialized cellular mechanisms to enhance O₂ uptake and distribution, resulting in dynamic respiratory and circulatory systems capable of responding to changes in O₂ availability. These responses are mediated in part through the induction of hypoxia-inducible factors (HIF), the regulatory components that are highly conserved both in form and function across phyla (1, 2). The main transcription factors associated with the HIF pathway are HIF- α (HIF-1 α , HIF-2 α /EPAS1 in vertebrates; HIF-1 α in invertebrates) and HIF- β /ARNT, which form a dimer and move into the nucleus. The activity of the HIF- α subunit is governed posttranslationally via a repression machinery (EGLN, or PHD: prolyl hydroxylase); during normoxic conditions, HIF- α is hydroxylated by EGLN and subsequently destroyed before dimerization with HIF- β /ARNT. However, during hypoxic conditions, EGLN activity is inhibited and the HIF heterodimer is allowed to form in the nucleus. HIF recognizes and attaches to DNA regions called HREs (hypoxia response elements) located upstream of target genes, thus manipulating their expression patterns (3). As “master regulators” of oxygen sensitivity, the regulatory machinery and downstream genes are frequent targets of selection for populations living in low oxygen environments in a variety of organisms (4–6).

Hypoxia in ocean waters has been rapidly increasing in distribution, frequency, and severity (7), and certain coastal ecosystems even reach levels of anoxia seasonally (8). This adversely

affects a wide range of organisms, with fishes and crustaceans, in general, showing lower levels of tolerance (7). From an evolutionary perspective, the ability of marine species to adjust to stressful levels of abiotic factors, such as temperature, pH, and oxygen, is a major determinant of their distributions at small and large scales.

The copepod *Tigriopus californicus* has become an excellent model for studies of physiological adaptations in the marine environment. It is an abundant resident of supralittoral rock pools along the west coast of North America and shows a pattern of strong genetic differentiation among populations (9). Because supralittoral pools are refreshed primarily by wave splash instead of high tides, their inhabitants are under extreme environmental stress in the form of salinity, temperature, pH, and oxygen fluctuations. In such pools, oxygen becomes more limited at night, when respiration by fauna and flora can make dissolved oxygen (DO) levels drop to ~0 mg/L (10). Multiple studies have quantified *T. californicus* physiological tolerance and gene expression under high temperatures (11–13) and high and low salinities (14, 15). Patterns and mechanisms of hypoxia tolerance, however, have been largely overlooked. A single prior work showed that *T. californicus* is tolerant to anoxic conditions for at least 18 h (16), with no further investigation. Species of planktonic

Significance

Oxygen availability is essential for development, growth, and viability of aerobic organisms. The genes in the hypoxia-inducible factor (HIF) pathway are considered master regulators of oxygen sensitivity and distribution inside cells, and they are hence highly conserved across animal groups. These genes are frequent targets of natural selection in organisms living in low-oxygen environments, such as high-altitude humans and birds. Here, we show that the abundant tidepool copepod *Tigriopus californicus* can withstand prolonged exposure to extreme oxygen deprivation, despite having secondarily lost key HIF-pathway members. Our results suggest the existence of alternative mechanisms of response to hypoxic stress in animals, and we show that genes involved in cuticle reorganization and ion transport may play a major role.

Author contributions: A.M.G. and F.S.B. designed research, performed research, analyzed data, and wrote the paper.

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Data deposition: RNA-seq data were deposited in the NCBI Sequence Read Archive database, <https://www.ncbi.nlm.nih.gov/sra>, both for the hypoxia experiment (BioProject PRJNA503487, accessions SRR8146267–SRR8146290) and for the assembly of additional copepod transcriptomes (BioProject PRJNA504307, accession nos. SRR8168003 and SRR8168004).

¹To whom correspondence may be addressed. Email: grahaall@oregonstate.edu.

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copepods have been shown to tolerate moderate hypoxic conditions, with exposures ranging from 2.4 to 1.0 mg O₂/L for 24 h before significant mortality occurs (17–19). However, little is known about the genetic mechanisms of hypoxia response in any copepod or intertidal invertebrates at large. Here, we examine phenotypic and transcriptional patterns of hypoxia response in *T. californicus* and show that *T. californicus* has secondarily lost key members of the HIF pathway, as well as suffered a reduction in the fidelity of HREs across their genome.

Results

HIF Pathway Genes. Examination of the original annotation of the *T. californicus* genome (20) did not reveal the presence of HIF-1 α or EGLN1, but such absence may be the result of an incomplete assembly or annotation of the draft genome sequence. We thus performed targeted computational analyses, independent of homology BLAST methods, to search for them. Besides the genome-derived protein sequences, we included transcriptome assemblies that were generated de novo from five different populations. We reasoned that these would provide complementary probabilities that these genes were sequenced and assembled in any of the *T. californicus* assemblies.

HMM searches of protein domains and phylogenetic analyses showed no evidence for the presence of either HIF- α or EGLN1 genes, suggesting that *T. californicus* has lost the canonical HIF pathway. A phylogenetic tree including sequences with *e* value < 0.01 yielded no *T. californicus* sequence within the invertebrate/vertebrate HIF- α clade (Fig. 1). We did, however, clearly detect representatives from the other bHLH-PAS-containing protein groups (AhR/AHRR, ARNT/ARNTL, CLOCK, NCOA1/2/3, NPAS1/2/3/4, SIM1/2, MET). A sequence previously identified as “HIF- α -like” protein (accession no. Cin4277) from the tunicate *Ciona intestinalis* rooted within a group of *Tigriopus* sequences near NCOA1-3, distantly placed from the well-supported HIF- α clade (Fig. 1). We scrutinized the five *T. californicus* genome sequences in this group by assessing their domain architectures via InterProScan (21) and found that they represent a variety of genes that contain PAS domains but do not have bHLH or other HIF-like domains. One of these proteins is PERIOD

(circadian-like, IPR02278), three belong to a group with voltage-dependent channel domain (IPR027359) and cAMP-binding domains (IPR018490), and one is a member of a protein kinase-like domain superfamily (IPR011009). The fact that these *T. californicus* genes are not nested within those of other taxa is an artifact of our methods, since we did not necessarily include all PAS-containing genes from other species. In addition, the location of the *C. intestinalis* sequence has low bootstrap support, and is likely due to long-branch attraction, frequently associated with the tunicate genome (22). Therefore, we are confident none of the *T. californicus* sequences in this group represent a form of HIF- α gene.

Because the HIF pathway canonically comprises the presence of both a HIF- α and an EGLN, they are often examined together to confirm the existence of this oxygen-sensing mechanism (1, 23, 24). Similar to the bHLH-PAS tree, the phylogenetic tree of all P4HC-containing proteins did not show any *T. californicus* representatives grouping within the EGLN clade but did show representatives of other P4HC proteins (*SI Appendix, Fig. S1*). Although this tree had lower levels of bootstrap support across internal nodes compared with the bHLH-PAS tree, the locations of highly supported nodes suggest that no *T. californicus* are EGLN-like. In addition, original BLAST annotation of the *T. californicus* genome did not include any EGLN-like sequences.

Anoxia Tolerance Assays. To assess levels of tolerance to anoxia in *T. californicus*, we quantified growth rate and viability of copepods immersed in water containing nearly no dissolved oxygen (<0.5 mg of O₂/L). Copepod larvae (1 d after hatching) exposed to anoxia for 24 h showed a significant delay in initial and full onset of metamorphosis compared with their full siblings subjected to normoxic conditions (*n* = 36 clutches, paired Wilcoxon test, initial: *V* = 63, *P* = 0.0138; full: *V* = 58.5, *P* = 0.0134). However, there was no significant difference in onset of first adult male (*V* = 161, *P* = 0.33), nor in survival to day 20 after treatment (*V* = 269, *P* = 0.136) (Table 1 and *Dataset S14*). In addition, exposing the 20-d-old adults to anoxic environments for 24 h resulted in no mortality (*n* = 48 clutches), regardless of which treatment they received as hatchlings (*Dataset S14*). A

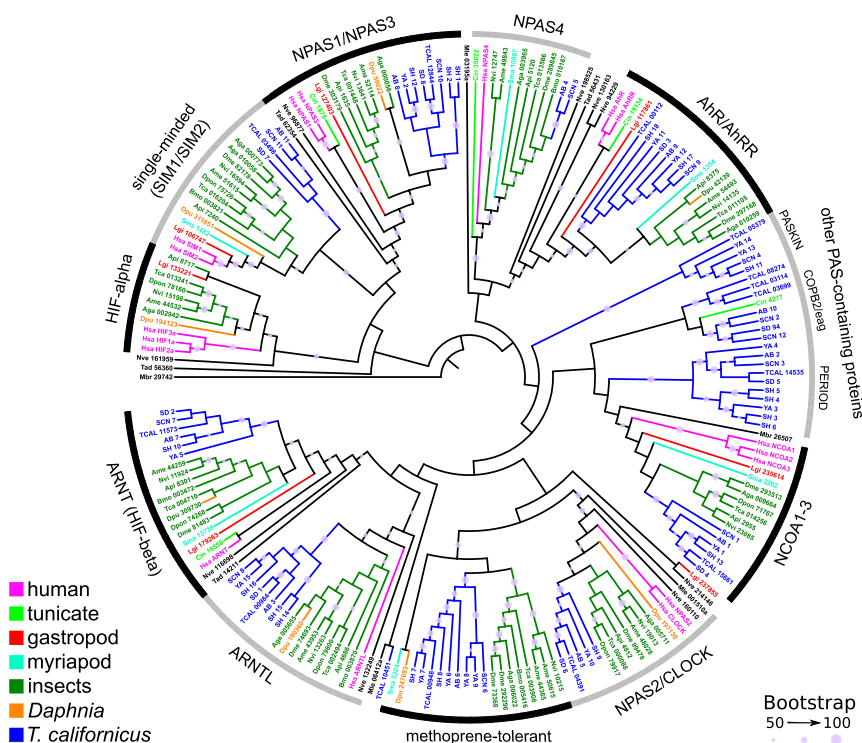


Fig. 1. Maximum likelihood tree of bHLH-PAS proteins across metazoans. Accessions for *T. californicus* include protein models from the reference genome (TCAL), as well as from transcriptomes from five populations (AB: Abalone Cove, SCN: Santa Cruz, SD: San Diego, SH: Strawberry Hill, YA: Yachats). Taxa abbreviations: Aga, *Anopheles gambiae*; Ame, *Apis mellifera*; Api, *Acyrtosiphon pisum*; Bmo, *Bombyx mori*; Cin, *Ciona intestinalis*; Dme, *Drosophila melanogaster*; Dpon, *Dendroctonus ponderosae*; Dpu, *Daphnia pulex*; Hsa, *Homo sapiens*; Lgi, *Lottia gigantea*; Mbr, *Monosiga brevicollis*; Mle, *Mnemiopsis leidyi*; Nve, *Nematostella vectensis*; Nvi, *Nasonia vitripennis*; Sma, *Strigamia maritima*; Tad, *Trichoplax adhaerens*.

of anoxia, such genes were largely down-regulated (Fig. 3B). Conversely, cuticle/proresilin structural proteins, as well as chitin synthesis genes, returned to lower levels or even became down-regulated (Fig. 3A and B). The majority of MTP genes that were up-regulated during anoxia returned to normal levels, but three genes significantly increased their expression during recovery (Fig. 3D). Overall, the recovery treatment was characterized by GO categories involved in muscle development and activity (e.g., “muscle structure development,” “actin-myosin filament sliding”), glycolysis/respiration (6-phosphofructo-2-kinase, fructose-2,6-bisphosphate 2-phosphatase), as well as “structural constituent of cuticle” (SI Appendix, Table S3).

We independently validated our RNA-seq results by repeating the experiment using new individuals and quantifying expression using reverse-transcription qPCR (RT-qPCR) of 16 genes of interest across multiple functional categories (SI Appendix, Table S4). RT-qPCR data were fully supportive of the RNA-seq results, with very similar pattern of expression levels and variation (SI Appendix, Fig. S2) and with strong correlation across genes (Spearman correlation; 24-h time point: $r = 0.838$, $P = 5 \times 10^{-5}$; recovery time point: $r = 0.821$, $P = 0.000131$).

Distribution of HREs. HIF transcription factor dimers attach to HREs located in the promoter regions of target genes, typically in dense clusters within 1,000 bp upstream of transcription start sites (25). We detected HREs in the promoter of 5,161 protein-coding genes across the *T. californicus* genome (Dataset S3) and tested the association of HRE presence with putative gene function (based on GO term annotation) and hypoxia response. Among genes annotated to participate in oxygen homeostasis, 36.7% had HRE in their promoter region. This proportion was not significantly greater than the fraction of all other genes containing HREs (35.9%; Table 2; G test, $G = 0.276$, $P = 0.599$), indicating no HRE enrichment in hypoxia-associated genes according to GO categorization.

Independent of GO annotation, we examined the distribution of HREs among genes based on whether they responded to 24-h exposure to anoxia. We found no evidence that genes that were significantly differentially expressed during anoxia had HREs overrepresented in their promoter regions. The proportion of DE genes with HREs (26.2%) was actually lower than that of nonchanging genes that contained HREs (31.0%; Table 3; $G = 4.80$, $P = 0.028$). This was true also when only up-regulated genes were considered (27.7% up-regulated vs. 30.8% down-regulated or nonchanging genes; $G = 1.69$, $P = 0.193$).

Transcription Factor Binding Site Motif Discovery and Enrichment. We performed computational analyses of transcription factor binding site (TFBS) motif discovery and enrichment to identify other classes

of transcription factors potentially involved in response to hypoxia. These searches were performed on promoter sequences of the 451 DE genes in the 24-h treatment. The motif associated with the transcription factor hunchback (hb) was found in both types of analyses (SI Appendix, Figs. S3 and S4), with motifs associated with Trithorax-like (Trl), Chorion factor 2 (CF2), zeste (z), and odd skipped (odd) found in the AME analysis (SI Appendix, Fig. S4). Among the five transcription factors, four contain C2H2 Zinc Fingers, while two are considered Polycomb-group proteins (SI Appendix, Table S5).

Discussion

The HIF pathway is highly conserved among animals, and its primary components were shown to have appeared early in Metazoan evolution, being absent from sponges and ctenophores but already present in placozoans and cnidarians (23, 24). Within crustaceans, HIF was examined and found in Branchiopoda (*Daphnia* spp.; ref. 26) and Malacostraca (27, 28). In *T. californicus*, we readily identified homologs for all gene families that contain bHLH-PAS and P4HC, including HIF- β /ARNT, partner of HIF- α , which is known to be constitutively active and to dimerize with other members of bHLH-PAS gene families across multiple pathways. We did not, however, find any copies of HIF- α or EGLN. Altogether, our analyses suggest that *T. californicus* lost the functionally linked HIF- α /EGLN gene pair, and, hence, cannot produce the HIF- α /HIF- β heterodimer. Consistent with this loss is the apparent deterioration of HRE binding domain specificity in genes involved in hypoxia response, likely as a result of relaxation of selection for maintenance of these noncoding sites. To our knowledge, a putative secondary loss of HIF- α and EGLN has been documented in only one other species, a tardigrade, although the authors were not explicitly examining this loss (29).

Despite the loss of HIF, *T. californicus* can survive in water with near zero O_2 for at least 24 h. The genus *Tigriopus* is known to have a high surface area-to-volume ratio, no gills, and no respiratory pigment, suggesting that they rely on cutaneous diffusion of gaseous exchange (30). These aspects of their physiology may have facilitated the loss of such an otherwise crucial pathway, and additional mechanisms were possibly coopted to modulate oxygen homeostasis.

Our study also showed that transcriptional response to hypoxia in *T. californicus* is dynamic and robust. Genes responding to prolonged hypoxia (24 h) in *T. californicus* provide interesting comparisons to similar studies in other systems. Under hypoxic conditions, a switch from oxidative phosphorylation to glycolysis is expected (31), with a predicted increase in expression of genes mediating this switch. Consistent with this prediction, we observed a significant increase in pyruvate dehydrogenase kinase

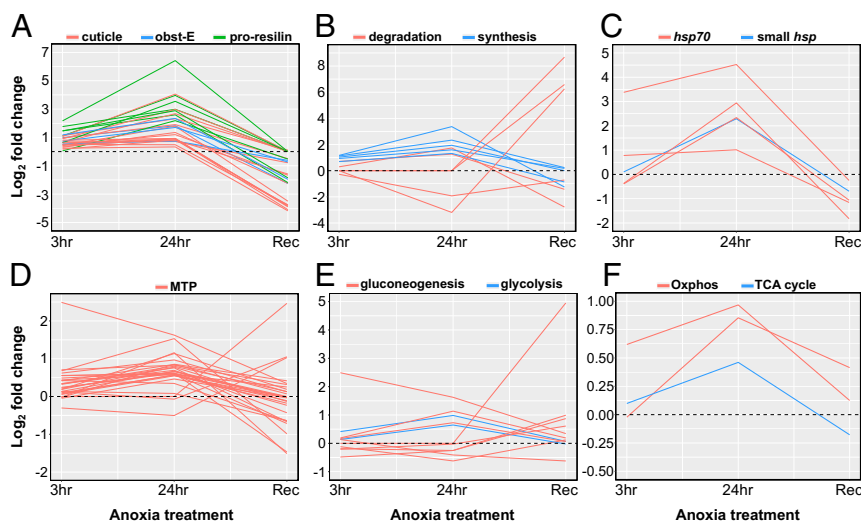


Fig. 3. Temporal patterns of differential gene expression during anoxia. Shown are gene functional categories that showed consistent patterns or belong to metabolic pathways. Each line represents a gene. Fold-change levels plotted are the mean values across replicates and represent the expression level during anoxia relative to the control (normoxia) treatment at each time point. Plotted are only genes that showed significant differential expression in at least one time point. Gene groups: structural cuticular proteins (A), chitin metabolism (B), heat-shock proteins (C), mitochondrially targeted proteins (D), gluconeogenesis and glycolysis (E), oxidative phosphorylation and tricarboxylic acid cycle (F).

Table 2. Association between GO annotation and HREs

	GO term		
Presence of HRE in promoter	Oxygen homeostasis*	Other	Totals
With HRE	444	3,422	3,866
Without HRE	765	6,096	6,861
Totals	1,209	9,518	10,727
Proportion with HRE	0.367	0.359	0.360

Shown are the number of *T. californicus* genes with GO annotations, sorted by the presence or absence in their respective promoter region (*G* test, $G = 0.276$, $P = 0.599$).

*This combines multiple GO terms associated with oxygen homeostasis: "heme-", "hemato-", "angio-", "oxygen", "hypoxia."

(*Pdk*) mRNA levels, an enzyme that is known to catalyze the step inhibiting pyruvate from entering and fueling the tricarboxylic acid (TCA) cycle (32, 33). In mammals, however, *Pdk* is a direct target of HIF-1 and, hence, contains HREs in its promoter region; in *T. californicus*, we did not detect HREs within a 1-kb promoter of the *Pdk* start codon. Therefore, identifying the *trans*-acting factor involved in *Pdk* transcription may be key in understanding hypoxia response in *T. californicus*.

Hypoxia response in other systems is also characterized by widespread transcriptional changes in subunits of oxidative phosphorylation (OXPHOS) complexes (34–37). We observed no change in expression of mtDNA-encoded subunits, and only in two nuclear-encoded OXPHOS genes (of 59 annotated). These two genes are associated with the cytochrome *c* oxidase complex (complex IV); one is involved in complex assembly (*COX15*), while the other is a known to help maintain mitochondrial function during hypoxia (homolog of human *HIG1*) (38). Up-regulation of certain complex IV subunits during hypoxia provides a possible submechanism of tolerance. Under low-oxygen conditions, HIF has been shown to regulate the transcriptional switch between two isoforms of certain COX subunits [from *COX4-1* to *COX4-2* in mammals (39), and from *COX5a* to *COX5b* in yeast (40)], with the alternative isoforms increasing efficiency of OXPHOS. Induction of *COX* genes in *T. californicus* hence warrants further targeted examination.

Perhaps the most unexpected pattern in our RNA-seq study was the transcription of several genes involved in cuticle and chitin metabolism. Structural components of the arthropod exoskeleton, like chitin and resilins, are crucial in forming a stable network with a high degree of flexibility and mobility (41, 42). After 24 h of anoxia, *T. californicus* showed increased transcription in chitin synthases and associated hormones (*EH* and *JH*) in parallel with up-regulation of 16 structural cuticular protein genes. Many other copies of cuticular genes ($n = 56$) did not reach statistical thresholds but had patterns of expression that were highly concordant with the ones above (*SI Appendix, Fig. S5*). During recovery in normoxia, these genes returned to lower transcription levels, likely driven by the high induction (~70–400-fold up-regulation) of genes involved in chitin biodegradation, such as chitin deacetylases. These results provide strong evidence for the potential involvement of chitin metabolism in hypoxia tolerance in

this species. Unlike terrestrial members of the Pancrustacea, copepods do not have trachea; instead they are thought to rely on cutaneous diffusion and possibly use integumental windows and segmental podocytes as their respiratory structures (43). We hypothesize that a highly plastic transcriptional response of cuticular genes during low-oxygen conditions permits the exoskeleton to undergo changes in structure, potentially through a modification of permeability via cuticle reorganization. Ultimately, microscopy techniques could be utilized to track physical changes in the exoskeleton before and during hypoxia, to ascertain how these transcriptional differences manifest themselves on the exoskeleton. Finally, current approaches for estimating coexpression networks should prove useful in this pursuit, but this will require an effort with large sample sizes (*SI Appendix*).

Conclusions

Hypoxia is a major physiological constraint for organisms who depend on aerobic respiration. Typically, the resulting physiological response is governed by the conserved HIF pathway, led by its regulatory machinery comprising *HIF- α* , *HIF- β* , and *EGLN*. We documented the loss of *HIF- α* and *EGLN* in an abundant crustacean that inhabits a highly variable environment, suggesting alternative molecular mechanisms of response to low oxygen availability may be more common than previously assumed. Our results suggest a strong role of chitin metabolism during hypoxia, which may work to maximize residual oxygen uptake in this species that lacks respiratory structures and pigments. This system will provide unique opportunities to examine the evolution of other oxygen-sensing gene families and how different cellular stress response mechanisms may evolve dual roles. While these key HIF pathway members are well described in the Crustacea (26–28), it is still unknown whether loss of these genes is idiosyncratic of *Tigriopus* or occurred earlier or in other lineages of the Copepoda.

Materials and Methods

Anoxia Tolerance Experiments. Copepods were collected from intertidal rocky pools in Ocean Beach, San Diego, California (SD: 32.7333°N, 117.2500°W), and maintained in large, outbred laboratory cultures for a minimum of three generations. Egg sacs ($n = 36$) were hatched in individual plates, and 1-d-old nauplii were used to perform split-brood treatments of anoxia (<0.50 mg O₂/L) or normoxia (≥ 6.5 mg O₂/L) in 20-mL sealed glass vials for 24 h. Larvae were

Table 3. Association between differential expression and HREs

	Anoxia vs. Normoxia		
Presence of HRE in promoter	Differentially expressed	No change	Totals
With HRE	118	3,539	3,657
Without HRE	333	7,895	8,228
Totals	451	11,434	11,885
Proportion with HRE	0.262	0.310	0.308

Shown are the number of *T. californicus* genes examined for differential expression during 24 h of anoxia relative to normoxia controls, sorted by the presence or absence in their respective promoter region (G test, $G = 4.80$, $P = 0.028$).

then returned to plates and monitored for metamorphosis rate, time to maturity, and survival to age 20 d. Adults surviving to age 20 d were then subjected to a 24-h anoxia treatment, and survivorship was measured after 3 d. Using a new batch of individuals at age 20 d, we exposed sets of 10 copepods to anoxia or normoxia for four time periods (24, 48, 72, 96 h) and quantified survivorship for 3 d after removal from experimental exposures. This time series experiment was also performed with a new batch of 1-d nauplii.

Search for HIF Machinery. We queried the protein sequences from the *T. californicus* reference genome (20), as well as translated sequences from five independently assembled de novo transcriptomes from multiple populations (SI Appendix). We used the HMMER 3.0 program to identify two sets of proteins, those that contained a PAS domain (PF00989.24, present in HIF- α or HIF- β) or that contained a P4HC domain (PF13640.5, present in EGLN). Alignment and phylogenetic methods followed those from a recent study of these gene families (2).

Transcriptional Response to Hypoxia. Clutches ($n = 24$) were hatched and raised separately in normal culture conditions until age 20 d. Broods were assigned randomly to normoxia or anoxia replicates and experimentally treated as above, and four replicates from each condition were terminated at each of three time points: 3 h (3 h), 24 h (24 h), and 24 h in normoxia after the 24-h treatment (recovery). The recovery treatment involved transferring copepods from anoxia vials into culture plates with normoxic water. RNA isolation, mRNA library preparation, sequencing, and data processing were

performed as described previously (44) (SI Appendix). Differential gene expression at each time point was quantified with edgeR by comparing anoxia samples to their respective normoxia control group and assessed with a false discovery rate of 0.10. Scripts are available at <https://github.com/amgraham07>.

Identification and Enrichment of HREs and Other TFBS. Promoter regions (1 kb upstream) of every gene in the *T. californicus* genome were extracted and searched for the presence of an HRE, based on its motif: (A/G)CGTG(A/C/G)(A/G)(A/G) (25). Detailed scripts are available on GitHub (<https://github.com/amgraham07>). These were then used to test for overrepresentation among genes with relevant GO terms or with hypoxia-induced expression. Alternative TFBS motifs were searched along the promoter regions of genes that were differentially expressed in the 24-h anoxia treatment. The MEME suite was used to search for known or “novel” potential TFBS motifs; known motifs were then identified by comparisons to the JASPAR database, while “novel” motifs were assigned a potential identity via the tool Tomtom in the MEME suite (45).

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