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

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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.

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.

The authors declare no conflict of interest.

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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 PRJNA504307, accession nos. SRR8168003 and SRR8168004) and for the assembly of additional copepod transcriptomes (BioProject PRJNA504307, accession nos. SRR8168003 and SRR8168004).

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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-1α 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/ARHR, 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 SI.1). 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 SI.1). A
new assessment for 24-h anoxia again showed no significant difference in survival compared with the normoxic controls (two-sample Wilcoxon test, $W = 18.5, P = 0.194$, Fig. 2A). Exposure to anoxia for longer periods, however, resulted in significant yet not complete mortality relative to normoxia controls, with mean survivorship of 60.7% after 48 h ($W = 22.5, P = 0.025$), 10.7% after 72 h ($W = 25, P = 0.011$), and 6% after 96 h ($W = 25, P = 0.0086$) (Fig. 2A and Dataset S1B). The median lethal time $LT_{50}$ ($±SE$) was 51.4 h ($±2.71$).

Juvenile copepods (1 d old) exposed to anoxia for 24 h also showed nearly total survivorship 3 d after treatment (Fig. 2B, $W = 17.5, P = 0.465$), but they were significantly more sensitive than adults after longer exposures, with only 4% survival after 48 h ($W = 30, P = 0.0050$) and 0% survival at 72–96 h ($P = 0.0022$; Fig. 2B and Dataset S1C). The median lethal time $LT_{50}$ for nauplii was 38.2 h ($±1.05$).

**Differential Gene Expression.** We examined transcriptional response to anoxia (<0.5 mg/L DO) by performing RNA-sequencing (seq) analyses of 20-d-old copepods after culturing for 3 h or 24 h in anoxic water, as well as of copepods transferred to normoxic water for 24 h after a 24-h anoxia treatment (“recovery” treatment). Each time point treatment was compared with its own control group of copepods in normoxic water. The three time points differed substantially with respect to number and function of gene differentially expressed (DE) between anoxia and normoxia treatments. After 3 h, there were relatively few significantly DE genes (26 total), with all but one being significantly up-regulated (Dataset S2B). Among these, we detected four members of solute carrier families, one of which is associated with protection or tolerance from high temperature stress (psk), a heat shock protein gene ($Hsp67B$), and a mitochondrial metabolic gene ($Pepck$). The single down-regulated gene at the 3-h time point is a gene which modulates the repression activities of genes involved in apoptosis ($SPOP$). These genes showed a significant enrichment of Gene Ontology (GO) terms associated with various ion transport and absorption ($SI$ **Appendix, Table S1**).

After 24 h of exposure to anoxia, the repertoire of genes changed substantially (Dataset S2C). Of the 451 DE genes, 368 were up-regulated and had GO terms associated with redox reactions (“oxidation-reduction”), as well as various metabolic processes (aminoglycan, chitin, polysaccharide metabolism) and ion transport/absorption ($SI$ **Appendix, Table S2**). Notably, a number of genes associated with chitin/exoskeleton (proresilins, cuticle proteins, ost-1, obst-E, $CUA2B$, $SgAbd-2$; $n = 17$), chitin synthesis ($CHS$; $n = 1$), keratinization ($SPRR3$; $n = 2$), and hormones associated with eclosion/metamorphosis ($ALAS1$, $IF$; $n = 2$) were significantly up-regulated (Fig. 3A and B and Dataset S2C). Cuticle proteins, including proresilins, consistently showed the highest magnitude of up-regulation, with four of these among the overall top 15 genes and with fold change (log$_2$) ranging from 2.98 to 6.42 (Fig. 3A). Conversely, genes involved in chitin degradation (chitinase and chitin deacetylase) were significantly down-regulated (Fig. 3B). Four $Hsp$ chaperones became significantly up-regulated, with three from the $Hsp70$ family and one small $Hsp$ (Fig. 3C). In addition, 20 nuclear-encoded mitochondrially localized protein (MTP) genes are significantly up-regulated (Fig. 3D); these genes span a range of mitochondrial metabolic processes, including glucose metabolism ($PC$, $Pepck$, $Pdk$; Fig. 3E), heme binding/biosynthesis (COX15, $ALASI$, $CYP301a1$, $SUOX$), tricarboxylic acid cycle ($DHTKD1$; Fig. 3F), cytochrome c oxidase complex ($HGI1$, COX15; Fig. 3F), and fatty acid synthesis/acetyl-CoA ($ACS3$, $ACSI$).

At the recovery time point, 561 genes were DE and involved a higher proportion of down-regulated genes (355 down-regulated versus 205 up-regulated; Dataset S2D), which contrasts with the general response to 24-h anoxia. Multiple chitin disaccharide deacetylase ($deuA$) genes were highly up-regulated, while at 24 h

**Table 1. Developmental rate and viability of copepods exposed to different dissolved oxygen conditions**

<table>
<thead>
<tr>
<th>Trait</th>
<th>Normoxia</th>
<th>Anoxia</th>
<th>Difference</th>
<th>Paired Wilcoxon test</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial metamorphosis</td>
<td>6.28 (0.88)</td>
<td>6.69 (0.92)</td>
<td>0.42 (0.93)</td>
<td>$V = 63, P = 0.0138$</td>
<td></td>
</tr>
<tr>
<td>Full metamorphosis</td>
<td>7.47 (0.94)</td>
<td>8.06 (1.31)</td>
<td>0.58 (1.36)</td>
<td>$V = 58.5, P = 0.0134$</td>
<td></td>
</tr>
<tr>
<td>First adult male</td>
<td>13.78 (1.29)</td>
<td>14.11 (1.21)</td>
<td>0.33 (1.77)</td>
<td>$V = 161, P = 0.338$</td>
<td></td>
</tr>
<tr>
<td>Survivorship at day 20</td>
<td>86.13%</td>
<td>80.07%</td>
<td>$-6.06% (0.18)$</td>
<td>$V = 269, P = 0.136$</td>
<td></td>
</tr>
</tbody>
</table>

One-day-old hatched larvae were exposed to anoxia (<0.5 mg of O$_2$/L) or normoxia ($≥6.5$ mg of O$_2$/L) for 24 h. For developmental traits, data shown are mean ($±SD$) number of days since hatching. $n$ = 36 clutches.
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 downregulated (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-photophofructo-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: \( r = 0.838, P = 5 \times 10^{-8} \); 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) 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 (Tri), Chorion factor 2 (C2), 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-\( \beta \)/ARNT, partner of HIF-\( \alpha \), 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-\( \alpha \) or EGLN. Altogether, our analyses suggest that *T. californicus* lost the functionally linked HIF-\( \alpha \)/EGLN gene pair, and, hence, cannot produce the HIF-\( \alpha \)/HIF-\( \beta \) 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-\( \alpha \) 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 \( \text{O}_2 \) 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 critical 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
(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 mRNA-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-I 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 resilin, 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 2. Association between GO annotation and HREs |
|-------------------------------|-------------------|-------------------|
| Presence of HRE in promoter | Oxygen homeostasis* | Other |
| With HRE                     | 444               | 3,422             |
| Without HRE                  | 765               | 6,096             |
| Totals                       | 1,209             | 9,518             |
| Proportion with HRE          | 0.367             | 0.359             |

Table 2. Association between GO annotation and HREs

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-”, “oxy-”, “gen”, “hypoxia.”

**Table 3. Association between differential expression and HREs**

<table>
<thead>
<tr>
<th>Anoxia vs. Normoxia</th>
<th>Differentially expressed</th>
<th>No change</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>With HRE</td>
<td>118</td>
<td>3,539</td>
<td>3,657</td>
</tr>
<tr>
<td>Without HRE</td>
<td>333</td>
<td>7,895</td>
<td>8,228</td>
</tr>
<tr>
<td>Totals</td>
<td>451</td>
<td>11,434</td>
<td>11,885</td>
</tr>
<tr>
<td>Proportion with HRE</td>
<td>0.262</td>
<td>0.310</td>
<td>0.308</td>
</tr>
</tbody>
</table>

Table 3. Association between differential expression and HREs

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 survivability was measured afterward. 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 survivability 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 HMMPR2 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 PHD 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/amrahamo7.

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) upstream) of every gene in the T. californicus genome. Nucleic Acids Res. 38, 408–415 (2010).

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