The hypoxia response pathway in the Antarctic fish *Notothenia coriiceps* is functional despite a poly Q/E insertion mutation in HIF-1α

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ABSTRACT

Antarctic notothenioid fishes, inhabiting the oxygen-rich Southern Ocean, possess a polyglutamine and glutamic acid (poly Q/E) insertion mutation in the master transcriptional regulator of oxygen homeostasis, hypoxia- inducible factor-1α (HIF-1α). To determine if this mutation impairs the ability of HIF-1 to regulate gene expression in response to hypoxia, we exposed *Notothenia coriiceps*, with a poly Q/E insertion mutation in HIF-1α that is 9 amino acids long, to hypoxia (2.3 mg L^{-1} O_2) or normoxia (10 mg L^{-1} O_2) for 12 hours. Heart ventricles, brain, liver, and gill tissue were harvested and changes in gene expression quantified using RNA sequencing. Levels of glycogen and lactate were also quantified to determine if anaerobic metabolism increases in response to hypoxia. Exposure to hypoxia resulted in 818 unique differentially expressed genes (DEGs) in liver tissue of *N. coriiceps*. Many hypoxic genes were induced, including ones involved in the MAP kinase and FoxO pathways, glycolytic metabolism, and vascular remodeling. In contrast, there were fewer than 104 unique DEGs in each of the other tissues sampled. Lactate levels significantly increased in liver in response to hypoxia, indicating that anaerobic metabolism increases in response to hypoxia in this tissue. Overall, our results indicate that the hypoxia response pathway is functional in *N. coriiceps* despite a poly Q/E mutation in HIF-1α, and for the first time, establish that Antarctic fishes are capable of altering gene expression in response to hypoxia.

Keywords: hypoxia, Antarctic fish, RNA-seq

ABBREVIATIONS

differentially expressed genes, DEGs; dissolved oxygen content, DO; dual specificity phosphatase, DUSP1; extracellular signal-regulated kinases, ERK; Forkhead Box O, FoxO; gene ontology, GO; heat shock element, HSE; hemoglobin, Hb; hypoxia- inducible factor-1, HIF-1; hypoxia response element, HRE; lactate dehydrogenase A, c-jun N-terminal kinase, JNK; lactate

dehydrogenase A, LDHA; mitogen activated protein kinase, MAPK; Nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB; Per-Arnt-Sim, PAS; placenta growth factor B, PGFB; principal component analysis, PCA; reactive oxygen species, (ROS); transactivation domains, TAD; vascular endothelial growth factor A, VEGFA.

INTRODUCTION

Similar to all other regions on earth, climate change is rapidly altering the Southern Ocean ecosystem with increases in ocean temperature, acidification, and deoxygenation threatening marine biodiversity (Chown et al., 2015). Climate change is especially pronounced in the western Antarctic Peninsula region where air temperatures have increased by nearly 3°C and summer sea surface temperatures by more than 1°C since 1951 (Clarke et al., 2007; King, 1994; Meredith and King, 2005; Vaughan et al., 2003). Coincident with warming, the oxygen content in the world's oceans has declined by approximately 2% since 1960 (Schmidtko et al., 2017), driven by increases in temperature that reduce oxygen solubility, and increases in precipitation and/or glacier melt that reduce mixing (ventilation) (Keeling et al., 2010). As a result of ocean deoxygenation, suitable habitats capable of sustaining the aerobic metabolic demands of marine organisms are predicted to severely decline during the next century (Deutsch et al., 2015).

Antarctic notothenioids are the dominant members of the fish fauna in the Southern Ocean, and among the most vulnerable groups of fishes to climate change (Bilyk and Devries, 2011; Eastman, 2005). Their long evolution in the cold, oxygen-rich waters of the Southern Ocean has reduced selective pressure to maintain some traits, resulting in the loss of phenotypic plasticity (Bilyk et al., 2018; Place et al., 2004). For example, the low thermal tolerance of Antarctic fishes is associated with an inability to regulate the expression of molecular chaperones and genes in the unfolded protein response pathway (Bilyk et al., 2018; Place et al., 2004).

Although the stenothermic nature of Antarctic fishes has been well characterized (Beers and Sidell, 2011; Bilyk and Devries, 2011; Somero and DeVries, 1967), much less is known about their hypoxia tolerance. Few studies have investigated the hypoxia tolerance of Antarctic notothenioids (Holeton, 1970; Wells et al., 1989), and available evidence suggests they have a limited capacity to adjust metabolism and gene expression to withstand hypoxia, although no

large-scale RNA sequencing studies on their hypoxia response have been conducted (O'Brien et al., 2020).

Fish are vulnerable to hypoxia that accompanies warming because as temperature increases, the dissolved oxygen content (DO) in water declines and metabolic rate increases, impinging on an organism's ability to produce ATP via aerobic metabolic pathways. Fish employ multiple behavioral, physiological, and biochemical strategies to withstand hypoxia (Farrell and Brauner, 2009). Rates of oxygen uptake may increase by increasing ventilation frequency and amplitude; oxygen delivery may increase through an increase in cardiac output, hematocrit and tissue vascularization; and oxygen demand may decline by switching from aerobic to anaerobic metabolic pathways for synthesizing ATP (Farrell and Brauner, 2009). Consistent with this, hypoxia tolerance is often positively correlated with liver and brain glycogen stores that can be mobilized during hypoxia to support anaerobic metabolism (Speers-Roesch et al., 2013) and by high maximal activities of glycolytic enzymes in some tissues (Mandic et al., 2013). At the molecular level, many of the genes induced in response to hypoxia are regulated by the master transcriptional regulator of oxygen homeostasis in metazoans, hypoxia-inducible factor-1 (HIF-1) (Nikinmaa and Rees, 2005).

HIF-1 is a heterodimer composed of HIF-1α and HIF-1β subunits (Wang and Semenza, 1995). HIF-1β, also known as the aryl hydrocarbon receptor nuclear translocator protein (ARNT), is constitutively expressed and the protein is stable under normoxic conditions (Jiang et al., 1996). In contrast, HIF-1α is transcribed and translated under normoxia but is hydroxylated at two proline residues in an oxygen-dependent reaction catalyzed by prolyl hydroxylase (PHD), which targets the protein for polyubiquitination and degradation by the 26S proteasome (reviewed by Semenza, 2007). Under hypoxic conditions, HIF-1α accumulates and translocates into the nucleus, dimerizes with HIF-1β, and binds to hypoxia responsive elements (HREs), transactivating the expression of a myriad of genes that mediate angiogenesis, glycolysis, cell growth, and erythropoiesis (Semenza, 1998).

The HIF-1α protein contains several functional domains that are conserved between fish and mammals. These include a basic helix loop helix (bHLH) domain for DNA binding, two Per-Arnt-Sim (PAS) domains for heterodimerization and DNA binding, two transactivation domains (TAD), and two proline residues that regulate its sensitivity to oxygen (Hon et al., 2002; Lando

et al., 2002; Semenza et al., 1997). Although the functional domains of HIF-1α are highly conserved among notothenioids, other teleosts, and humans, in notothenioids, the protein contains a polyglutamine (Q) and glutamic acid (E) insertion mutation between the second PAS domain and the first conserved proline residue that may alter its function (Rix et al., 2017). The length of the poly Q/E region varies between 9 and 34 amino acids in notothenioids and is longest in members of the Channichthyidae (icefishes) family that lack the oxygen-binding protein hemoglobin (Hb) (Rix et al., 2017). In contrast, rainbow trout and the sub-Antarctic notothenioid *Eleginops maclovinus* possess a poly E insertion only four amino acids in length, and humans lack E and Q residues within this region (Rix et al., 2017).

We hypothesized that the long evolution of Antarctic notothenioids in an oxygen-rich environment may have reduced selective pressure to maintain an intact HIF-1α, resulting in the poly Q/E insertion mutation that may impair the ability of HIF-1 to induce the expression of hypoxic genes. To test this hypothesis, we exposed *Notothenia coriiceps* to normoxia (10 mg L^{-1} O_2) or hypoxia (2.3 mg L^{-1} O_2) for 12 hours and analyzed changes in gene expression in liver, brain, heart ventricle, and gill using RNA sequencing (RNA-seq). This level of hypoxia has previously been shown to result in an increase in HIF-1α protein levels in nuclei isolated from cardiomyocytes of *N. coriiceps* (O'Brien et al., 2020). Levels of lactate and glycogen were also quantified to determine if hypoxia induces an increase in anaerobic metabolism.

MATERIALS AND METHODS

Animals

Notothenia coriiceps (Richardson 1844; 1202 ± 504 g) were collected in 2017 using baited pots and otter trawls deployed from the ARSV *Laurence M. Gould* off the southwestern shore of Low Island (63°30′S, 62°42′W) and in Dallmann Bay (64°08′S, 62°40′W). Animals were held in flow-through seawater tanks and transported to the U.S. Antarctic research station, Palmer Station, where they were held in covered circulating seawater tanks at 0 ± 1 °C and fed chopped fish every 2–3 days. Animals were allowed to recover from the stress of handling and transport for at least 1 week before experiments began.

N. coriiceps were held at normoxia (10 mg L^{-1} O_2, n = 6) or exposed to hypoxia (2.3 mg L^{-1} O_2, n = 6) for 12 hours. Two animals at a time were transferred to a 56-gallon tank with flow through sea water where they were allowed to recover from the stress of handling for two hours. Tanks were covered with plastic to minimize oxygen diffusion from air to water, seawater flow was reduced, and for the hypoxia exposures, oxygen levels were decreased at a rate of 4 mg L^{-1} per hour to 2.3 mg L^{-1} O_2 by bubbling nitrogen gas into the tank. Dissolved oxygen (DO) levels were monitored and regulated using a galvanic oxygen probe connected to a DO process controller (Hanna Instruments) that controlled the flow of nitrogen gas into the tank. The oxygen probe was calibrated daily with oxygen-saturated sea water. Control animals were handled in the same way but oxygen levels were maintained at 10 mg L^{-1} O_2 with air diffusers. At the end of the experimental period, animals were euthanized by a sharp blow to the head followed by spinal cord transection. Tissues were harvested, flash frozen and stored at -80°C for later use. All procedures were approved by the University of Alaska, Fairbanks Institutional Animal Care Committee (570217-9).

RNA isolation

Total RNA was extracted from tissues (n = 4 per treatment and tissue with two males and females in each treatment group) using Qiagen RNeasy Plus Universal mini kit following manufacturer's instructions (Qiagen, Hilden, Germany). RNA samples were quantified using Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and RNA integrity (RIN) was evaluated using an Agilent TapeStation 4200 (Agilent Technologies, Palo Alto, CA, USA). The TapeStation uses a microfluidics-based method for separating RNA by molecular mass and an algorithm to assess RNA quality based on the electropherogram (Schroeder et al., 2006).

Library preparation

RNA sequencing libraries were prepared using the NEBNext Ultra RNA Library Prep Kit for Illumina and following manufacturer's instructions (NEB, Ipswich, MA, USA). Briefly, mRNAs were initially enriched with Oligo(dT) beads. Enriched mRNAs were fragmented for 15 minutes at 94 °C. First strand and second strand cDNA were subsequently synthesized. The cDNA fragments were end repaired, adenylated at 3'ends, and universal adapters were ligated to

cDNA fragments, followed by index addition and library enrichment by PCR with limited cycles. The sequencing library was validated with an Agilent TapeStation (Agilent Technologies, Palo Alto, CA, USA), and quantified using the Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA), as well as by quantitative PCR (KAPA Biosystems, Wilmington, MA, USA). The sequencing libraries were clustered on a flow cell. After clustering, the flow cell was loaded on the Illumina instrument (HiSeq4000 or equivalent) according to manufacturer's instructions. The samples were sequenced using a 2x150bp Paired End (PE) configuration. Image analysis and base calling were conducted by the Control software. Raw sequence data (.bcl files) generated by the sequencer were converted into fastq files and de-multiplexed using Illumina's bcl2fastq 2.17 software. One mismatch was allowed for index sequence identification.

Identification and Characterization of Differentially Expressed Genes

Phase 1: Mapping to the N. coriiceps genome

To generate alignments, the paired-end fastq files were mapped to the NC01 *N. coriiceps* assembly (ReqSeq accession: GCF_000735185.1) with HISAT2 v2.1.0. Count estimation was performed with featureCounts v1.6.4 (Liao et al., 2014) and the resultant expression data analyzed with DESeq2 v1.26.0 (Love et al., 2014) to determine differentially expressed genes (DEGs). Every position of all fastq files had an average Phred-scaled quality score of 32 or higher. The criterion used to identify a DEG was a Benjamini-Hochberg adjusted *p*-value less than or equal to 0.05. OrthoFinder v2.3.12 (Emms and Kelly, 2015) was used to assign these to orthologous genes of model organisms: *Danio rerio* (ReqSeq accession: GCF_000002035.6) and various stickleback fish (via "stickleback" search term from the UniProtKB database on uniprot.org). For the latter, a custom reference set was provided by the ClueGO developer to enable stickleback gene ontology (GO) with *N. coriiceps* input. DEG lists for each of the 4 tissues for both sets were input into the network visualization software Cytoscape v3.8.0 (Shannon et al., 2003). The ClueGO v2.5.7 (Bindea et al., 2009) and CluePedia v1.5.5 plug-ins (Bindea et al., 2013) were employed for pathway enrichment analysis. The *p*-value threshold was set to 0.05 and the GO Term Fusion option was enabled for each run to eliminate redundant GO terms; all other settings remained at default.

Phase 2 Mapping to the N. rossii genome

Because the *N. rossii* genome assembly is more complete than that of *N. coriiceps* (82.7% vs 75.9% as estimated by BUSCO v4.0.1 (Simao et al., 2015)), the paired-end fastq files were also mapped to the *N. rossii* genome. The *N. rossi* genome assembly was provided by the Wellcome Sanger Institute because it was not yet publicly available when we conducted the analysis (GCA_943590865.1; Bista et al., 2023). Additionally, Sanger provided RNA-seq reads, also not yet publicly available, so that we could annotate the *N. rossi* genome. A reference-guided transcriptome assembly of the RNA-seq reads was generated with Trinity v2.9.1 (Grabherr et al., 2011), and evaluated by BUSCO to be 87.7% complete. RepeatModeler v2.0.1 (Flynn et al., 2020) was used to make a repeat library from the *N. rossii* genome. The library, the transcriptome, and the reads were subsequently input into MAKER v2.31.10 (Cantarel et al., 2008) to generate a *.gff* annotation file and a protein fasta file. The remainder of the analysis pipeline was performed as before, starting with alignments of the *N. coriiceps* RNA-seq reads to the *N. rossii* genome using HISAT2 v2.1.0. The MAKER-produced *N. rossii* annotation file was required input for abundancy estimation with featureCounts (Liao et al., 2014). The protein fasta file was necessary to find orthologs (once again to *D. rerio* and sticklebacks) via OrthoFinder (Emms and Kelly, 2015) to enable downstream pathway enrichment analysis with ClueGO/CluePedia/Cytoscape (Bindea et al., 2013; Bindea et al., 2009; Shannon et al., 2003).

Quantification and analysis of lactate and glycogen levels

Tissues were homogenized using a Tekmar Tissumizer in ice cold 0.5 M Tris buffer (pH 8.3) for quantifying lactate levels and in MiliQ water for quantifying glycogen levels. For measuring lactate, homogenates were centrifuged at 16,100 *g* at 4°C for 15 minutes and the resulting supernatant was filtered with Amicon Ultra 10K filtration units. For glycogen measurements, homogenates were heated to 95 ºC for 10 minutes and then centrifuged for 10 minutes at 16,100 *g* at 4°C. Filtrates and homogenates were stored frozen at -80°C until use. Lactate levels were quantified as described previously (Devor et al., 2016). Glycogen was quantified using a glycogen assay kit (Sigma-Aldrich, MAK016). Absorbances at 440 nm

(lactate) or 570 nm (glycogen) were measured using a SpectraMax Plus plate reader with the software SoftMax Pro 6.3 (Molecular Devices, San Jose, CA, USA). All samples and standards were measured in triplicate.

Data analyses

Significant differences in lactate and glycogen levels between normoxic and hypoxic individuals within each tissue type were analyzed using GraphPad Prism version 9.3.0 (GraphPad Software, San Diego, California USA) with significance set at $p < 0.05$. Outliers were identified using a Grubb's test and removed from subsequent analyses. Normality was determined using a Shapiro-Wilke's test, and equal variance was evaluated using an F-test. A two-tailed unpaired Student's t-test was used to identify significant differences in glycogen and lactate levels within each tissue when data were normally distributed and had equal variance. A Mann-Whitney test was used to identify significant differences when data were not normally distributed. For data sets lacking equal variance, a Student's t-test with Welch's correction was used.

RESULTS

RNA-seq

The Relative Integrity Number (RIN) for all RNA samples was ≥ 8.3 and the mean RIN was 9.3 ± 0.44 (std. dev.). The average number of raw reads per tissue was 41,746,014 ± 837,102 (mean ± standard error). The average alignment rate of reads to the *N. rossii* genome assembly (66%) was marginally better than that of the reads to the *N. coriiceps* genome (63%).

Overall trends in differential gene expression

A Principal Component Analysis (PCA) indicates that gene expression differed more among tissues than between treatment groups (Fig. 1). PC1 separates liver from the other three tissue types and explains 47.93% of the variation in gene expression. PC2 separates brain from liver, gill, and heart ventricle and explains an additional 22.03% of the variation in gene

expression among the four tissues. There was no clear differentiation in gene expression between the hypoxia and normoxia treatment groups within any tissue .

There were significantly more DEGs in liver compared with brain, gill, and heart ventricle using either the *N. coriiceps* or *N. rossii* reference genome (Table 1, Fig. 2). Combining the alignments to both the *N. coriiceps* and *N. rossii* genomes, there were 818 unique DEGS in liver, 104 in brain, 57 in heart and 35 in gill (Table 1, Fig. 2). Although liver displayed the greatest number of DEGs, gill and heart tissues displayed a large range in log2-fold change of gene expression (Fig. 2). For example, in the heart ventricle, there was one downregulated gene and three upregulated genes with a greater than 20 log2-fold change (considering both analyses, Supplemental Tables). The most strongly induced gene in the heart ventricle was one with ubiquitin-protein transferase activity (LOC104952699; Supplementary Tables).

Most of the identified DEGs were unique to each tissue (Fig. 3), although we recognize this may be incomplete, as not all genes were identified. Only two genes were upregulated in all four tissues, one of which was unidentified. There were no down-regulated genes identified in common among all tissues.

Pathway analysis

Because of the limited number of DEGs in tissues other than liver, pathway analysis only yielded results for upregulated genes in the liver. Gene ontology (GO) analysis indicates that the largest number of upregulated genes are involved in the immune response (22 unique identified genes). The second most abundant group of upregulated genes were ones involved in the MAP kinase pathway (13 unique identified genes), and several were involved in the response to hypoxia (11 unique identified genes), including the well-known HIF-1 induced genes: lactate dehydrogenase A (LDHA), vascular endothelial growth factor A (VEGFA), and placenta growth factor B (PGFB) (Table 2). KEGG pathway analysis also identified several pathways known to be involved in the hypoxia response in liver, including Forkhead Box O (FoxO) and VEGF signaling, and pyruvate metabolism (Table 2).

Lactate and glycogen levels

Lactate levels were 5.5-fold higher in livers of *N. coriiceps* exposed to hypoxia compared with *N. coriiceps* at normoxia ($P = 0.015$; t =3.54; Table 3). Lactate levels were equivalent between normoxic and hypoxic hearts, brains and gills of *N. coriiceps*, and glycogen levels were equivalent between normoxic and hypoxic *N. coriiceps* in all tissues analyzed.

DISCUSSION

Our results indicate that many genes in the hypoxia response pathway are upregulated in the liver of the Antarctic fish *N. coriiceps* in response to hypoxia, suggesting that HIF-1 is active despite an insertion mutation in HIF-1α. Although the number of DEGs in response to hypoxia is small in *N. coriiceps* compared with temperate fishes, canonical hypoxic genes, such as the glycolytic enzyme, LDHA, and angiogenic regulator, VEGFA, are induced in liver. These results show for the first time that Antarctic fishes are capable of regulating gene expression in response to hypoxia despite their long evolution in an oxygen-rich environment.

The hypoxia response varies among tissues

Similar to other metazoans, including fish, the molecular response to hypoxia is tissue specific in *N. coriiceps* (Gracey et al., 2001; Lau et al., 2019). Most of the identified DEGs are unique to each tissue (Fig. 3). Only two genes, one of which was unidentified, were upregulated in all of the four tissues. The other, dual specificity phosphatase (DUSP1), displayed more than a 2 log2-fold increase in all tissues (Supp. Tables 2,4,6,8). DUSP1 is a member the mitogen-activated protein kinase (MAPK) family of phosphatases known to be induced by hypoxia and other stressors (Seta et al., 2001). DUSP1 inactivates several MAPKs, including JNK, p38, and ERK, by dephosphorylating threonine and tyrosine residues (Hirsch and Stork, 1997). DUSP1 may serve a protective mechanism by minimizing hypoxia-induced apoptosis, cell proliferation and/or inflammation driven by MAPK (Kim and Choi, 2010).

Liver

The liver displayed the most robust response to hypoxia with 651 genes upregulated and 167 genes downregulated (Table 1). Genes upregulated by hypoxia in *N. coriiceps* include ones that are known to be regulated by hypoxia in other organisms, such as LDHA, VEGFA, and PGFB, a member of the VEGF family (Rossi et al., 2016). The increase in mRNA levels of LDHA suggests a shift towards anaerobic metabolism that is also reflected in an increase in lactate levels in liver.

The immune response pathway comprised the largest number of genes upregulated in liver in response to hypoxia with 22 unique genes induced, including ones that regulate the inflammatory response (GO:0006954), chemokine signaling (GO:0070098), T cell differentiation (GO:0030217), and lymphocyte (GO:0072676) and leukocyte (GO:0050900, GO:0030595, GO:0002521, GO:0002688) activities (Table 2). There is extensive crosstalk between the hypoxia and immune response pathways in part because the increase in energetic demands of immune cells increases oxygen demand, leading to hypoxia (Taylor and Colgan, 2017). However, there are also regulatory factors that stimulate both pathways (Taylor and Colgan, 2017). For example, chronic hypoxia increases the production of reactive oxygen species (ROS) by mitochondria, which activate nuclear factor-κB (NF-κB), a family of pro-inflammatory transcription factors (D'Ignazio et al., 2016; Morgan and Liu, 2011), and stabilize HIF-1α levels by inhibiting PHD (Lee et al., 2016). The increase in NF-κB amplifies the hypoxia response because it transactivates HIF-1α expression (van Uden et al., 2008). HIF-1, in turn, regulates a variety of immune cell functions, such as the survival, differentiation and proliferation of T-cells in mammals (Taylor et al., 2016) and fish (He et al., 2022; Shi et al., 2023). An upregulation of the immune response pathway may be amplified in livers of *N. coriiceps* in response to hypoxia because the liver harbors a high density of nematode parasites (Palm et al., 1998; personal observation). Moreover, the presence of nematodes consuming oxygen may lower tissue PO_2 and exacerbate hypoxic conditions in the liver.

Several genes involved in regulating kinase activity, especially in the MAP kinase pathway, were induced in liver in response to hypoxia (GO:0043410, GO:0071900, GO:0043405, GO:0032147, GO:0000187, GO:0004709, GO:0070374). For example, the growth arrest and DNA damage-inducible 45 (GADD45) family of proteins, which arrest the cell cycle and activate apoptosis and the MAPK pathway, were induced (Tamura et al., 2012; Yang et al.,

2009). MAPKs regulate a variety of cellular processes, including inflammation, proliferation and apoptosis (Kim and Choi, 2010). The MAP kinases ERK1/2 also regulate the expression of HIF-1α by phosphorylating and increasing the activity of p300, the co-transcriptional activator of HIF-1 (Sang et al., 2003), and thus, may play a role in strengthening the response to hypoxia.

The AGE-RAGE pathway is induced in response to hypoxia in the liver of *N. coriiceps*, similar to the temperate fish species, *Sillago sihama* (silver sillago), (Tian et al., 2020). Genes in this pathway promote oxidative stress and apoptosis and are activated by advanced glycation end products (AGEs) under hyperglycemic conditions that may occur during hypoxia as glycogen is hydrolyzed to glucose to provide substrates for glycolysis (Waghela et al., 2021).

FoxO proteins are transcription factors that mediate a variety of cellular processes including metabolism, oxidative stress and mitophagy, and are important for maintaining homeostasis (Gui and Burgering, 2022). Genes encoding proteins of the FoxO pathway, including AKT, which phosphorylates and increases the activity of FoxO (Tzivion et al., 2011), were induced in response to hypoxia in *N. coriiceps* liver. In response to hypoxia in mammalian cells, FoxO proteins reduce mitochondrial mass and the production of ROS and thus protect against oxidative damage (Jensen et al., 2011). In a study investigating the role of miRNA in regulating the cellular stress response in the gills of the Antarctic notothenioid, *Trematomus bernacchii*, 8 of the 9 differentially expressed miRNA upregulated after 7 days exposure to an acute heat stress targeted mRNAs involved in the FoxO signaling pathway, indicating this pathway is of central importance in regulating the response of notothenioids to stress (Vasadia et al., 2019).

Considered together, the pattern of DEGs in liver suggests that in response to hypoxia, glucose metabolism increases, stress may be mitigated by an induction of the immune system and antioxidants defenses, and damaged cells may be replaced by cell proliferation driven by the MAPK pathway. Many of the same genes that are induced in response to hypoxia in the liver of *N. coriiceps*, such as ones in the MAPK pathway and those involved in immunity and inflammation, are upregulated in notothenioids in response to a variety of stressors, including heat (Bilyk and Cheng, 2014; Bilyk et al., 2018; Huth and Place, 2013) and heat in combination with elevated pCO_2 (Huth and Place, 2016), suggesting that the response to hypoxia may be a part of a general stress response in notothenioid fishes.

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344 *Heart and Gill*

345 Results from the principal component analysis indicate that the heart and the gill are most
346 similar with respect to DEG expression. Although the number of DEGs are modest in gill (35)
347 and heart (57), the magnitude of change in gene expression is large (Fig. 2). For example, in the
348 heart ventricle, there was one unidentified gene downregulated and three genes upregulated with
349 a greater than 20 log2-fold change (Supplemental Table 6). The gene with the largest increase in
350 expression in the heart (and the only one of the three most upregulated genes identified) has
351 ubiquitin-protein transferase activity, which may be required for cardiac remodeling in response
352 to hypoxia. In mammals, hypoxia induces the expression of fetal genes in cardiac muscle,
353 including glycolytic genes, energy efficient isoforms of myosin (Razeghi et al., 2003), and genes
354 that stimulate right ventricle hypertrophy (Smith et al., 2020). The membrane protein, seizure
355 protein 6 homolog (SEZ6B) displayed a 9.3 log2-fold change in the heart and was the fourth
356 most highly upregulated gene. In mammals, SEZ6B stimulates the cell cycle and is upregulated
357 in several cancers (Chen et al., 2022). Cardiac hypertrophy occurs in response to hypoxia in
358 some fishes, such as rainbow trout (Simonot and Farrell, 2007), zebrafish, and cichlids (Marques
359 et al., 2008) and may be important for increasing cardiac output and oxygen delivery. SEZ6B
360 was also highly induced by hypoxia in the gill (7.8 log2-fold change) and liver (6.2 log2-fold
361 change) but not the brain of *N. coriiceps*.

362 The gills of some temperate fishes undergo extensive remodeling to increase oxygen
363 uptake in response to hypoxia (Gilmour and Perry, 2018). The gill surface area for oxygen
364 diffusion may increase by shedding interlamellar cell masses (ILCM), although this has the
365 potential to increase energetic costs for osmoregulation (Gilmour and Perry, 2018). In some
366 species, there are also changes in the size and/or morphology of mitochondria-rich cells,
367 pavement cells and/or mucous cells that may prevent ion loss and lower energy demand (Matey
368 et al., 2011; Matey et al., 2008). It is unknown if gill remodeling occurs in Antarctic fishes in
369 response to hypoxia but an increase in the expression of GADD45A in the gills of *N. coriiceps*,
370 suggests that it may. GADD45A stimulates apoptosis in response to hypoxia in fish (Fang et al.,
371 2018) and it was strongly induced in gills of *N. coriiceps* in response to hypoxia with a 2.77
372 log2-fold change, suggesting that apoptosis of ILCM and remodeling may occur.

Brain

The brain displayed relatively few DEGs (104), which was somewhat surprising given its sensitivity to hypoxia (Erecinska and Silver, 2001). In mammals, vascularization increases in the brain to a greater extent than other organs, such as the liver (Sapkota et al., 2023) and yet VEGF signaling was not induced in *N. coriiceps* brain. However, there is some evidence that fish, similar to mammals, can redistribute blood flow in response to hypoxia to protect vital organs such as the brain (Kuwahira et al., 1993; Li et al., 2023). Consistent with this, there were only four DEGs in brains of *Gillichthys mirabilis* exposed to 10% normoxia for up to 6 days (Gracey et al., 2001). In brains of *N. coriiceps*, there was a 4 log2-fold increase in expression of CLDN7, which encodes a claudin involved in forming tight junctions in epithelial cells (Sapkota et al., 2023), suggesting that with a longer hypoxia exposure, an increase in vascularization may occur. Interestingly, the brain was the only tissue sampled in which there were a larger number of downregulated genes (58) than upregulated ones (46). Some of the downregulated genes included members of the solute carrier family of proteins (SLCs). Given the energetic cost of membrane transport, downregulating the expression of these genes may be an effective strategy for conserving energy and oxygen demand (Pizzagalli et al., 2021). One limitation of our analysis is that we analyzed the entire brain, rather than specific regions, which may respond differently to hypoxia (Zhou et al., 2008).

A diminished hypoxia response in N. coriiceps compared with temperate fish species

Overall, there were fewer DEGs in *N. coriiceps* compared with temperate fish species in response to hypoxia (e.g.; Akbarzadeh et al., 2020; Sun et al., 2020; Xia et al., 2018). There are several possible explanations for this observation. First, it's conceivable that evolution in an oxygen-rich environment has resulted in the loss of HREs in the DNA where HIF-1α binds to transactivate gene expression. There is precedence for this conjecture: Antarctic notothenioids have fewer heat shock elements (HSEs) in the DNA where the heat shock transcription factor binds to induce the expression of genes in response to heat stress (Bogan and Place, 2019). As a result, fewer genes are transcribed in response to heat stress compared with the related cold-

temperate notothenioid, *E. maclovinus* (Bilyk et al., 2018). A similar loss of HREs may minimize the hypoxia response in Antarctic notothenioids. Alternatively, it's possible that a more robust response to hypoxia would be elicited with a longer and/or lower DO hypoxia exposure. Little is known about the hypoxia tolerance of Antarctic fishes. One prior study determined that the minimum DO required to maintain standard metabolic rate (P_{crit}) (Somo et al., 2022) of *N. coriiceps* was 20% of air saturation (~ 2.0 mg O_2 L^{-1}) but it was measured in one or few individuals using closed-chamber respirometry (Heise and Abele, 2008). In another study, *N. coriiceps* survived DO levels as low as 1.5 - 3.0 mg O_2 L^{-1} (Hemmingsen et al., 1969). If the level of DO used in our study was above P_{crit}, increases in oxygen delivery may have been sufficient to maintain PO_2 levels in most tissues above levels needed to induce a hypoxia response. The lack of an increase in lactate levels in tissues other than liver, and a lack of a decrease in glycogen in all tissues of *N. coriiceps* in response to hypoxia suggest this may be the case. Although we did not quantify Hb or hematocrit in *N. coriiceps*, there is evidence from other studies that Antarctic notothenioids can increase oxygen delivery in response to hypoxia. Exposure of the Antarctic notothenioid, *Pagothenia borchgrevinki* to ~ 7.9 mg O_2 L^{-1} for 11-14 days resulted in an increase in hematocrit and Hb levels, as well as Hb oxygen-binding affinity but not plasma lactate levels, indicating that Antarctic fishes are able to improve oxygen delivery similar to temperate fishes to minimize anaerobic metabolism (Wells et al., 1989). Using a targeted approach (quantitative PCR), it was determined that globin genes (ie; neuroglobin, cytoglobin and myoglobin) are induced in brain, gill and retina of the Antarctic notothenioids *Trematomus bernacchii* and *Chionodraco hamatus* in response to a longer, mild hypoxia exposure of 8 mg L^{-1} O_2 for 5 days (Giordano et al., 2021). Previous studies have shown that changes in gene expression in response to hypoxia are transient and often increase with time (e.g.; Gracey et al., 2001; Zhang et al., 2021). Thus, a longer exposure to hypoxia may elicit an increase in the number of DEGs. Lastly, there is some evidence that fish, similar to mammals, can redistribute blood flow in response to hypoxia (Kuwahira et al., 1993; Li et al., 2023), which, if activated in *N. coriiceps* might also contribute to a muted molecular response to hypoxia in some tissues. Future studies quantifying the level of HIF-1α in all tissues would provide insight to differences in tissue PO_2 levels.

Conclusions

Our results suggest that the poly Q/E insertion in HIF-1α of *N. coriiceps* does not disrupt its function given that some hypoxic genes were induced, especially in liver. It is conceivable that rather than being deleterious, this insertion mutation may be advantageous in the cold. Poly Q regions in proteins typically form disordered, flexible regions that facilitate protein-protein interactions (Schaefer et al., 2012), and may be important for maintaining protein flexibility and function at low temperature. However, extended poly Q regions may cause protein aggregation and disrupt function (Adegbuyiro et al., 2017). Current studies in our lab are aimed at determining whether the longer poly Q/E regions in HIF-1α of members of the hemoglobinless Channicthyidae family alter HIF-1 function. Overall, our results suggest that Antarctic notothenioids maintain the capacity to mount a molecular response to hypoxia as the Southern Ocean warms and becomes deoxygenated.

DATA AVAILABILITY

RNA-seq data will be available upon publication at http://www.ncbi.nlm.nih.gov/bioproject/1036368.

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FIGURE LEGENDS

Figure 1. Principal component analysis of differentially expressed genes in four tissues of *N. coriiceps* at normoxia and hypoxia.

Figure 2. Volcano plots illustrating the number of differentially expressed genes in four tissues of *N. coriiceps* exposed to hypoxia. The horizontal grey line on each plot represents the line of significance (adjusted $p = 0.05$). Each dot represents a gene and its color, the adjusted *p*-value (Padj). DEGs include only ones identified using the *N. coriiceps* genome for mapping.

Figure 3. Venn diagram illustrating the number of shared and unique differentially expressed genes in tissues of *N. coriiceps* in response to hypoxia. The numbers of upregulated genes (A) and down regulated genes (B) include only ones identified using the *N. coriiceps* genome for mapping.

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Table 1. Number of differentially expressed genes in 4 tissues of *N. coriiceps* in response to hypoxia.

	Brain	Gill	Liver	Heart
Phase 1				
Upregulated	40	17	546	22
Down regulated	51	6	118	21
TOTAL	91	23	664	43
Phase 2				
Upregulated	22 (6)	13 (9)	225 (105)	10 (7)
Down regulated	17 (7)	4 (3)	65 (49)	8 (7)
TOTAL	39	17	290	18

Genes included are those that displayed a significant log 2-fold change ($P < 0.05$). Numbers in parenthesis for phase 2 analysis indicate the number of unique genes identified that do not overlap with ones identified in phase 1 of the analysis.

774 **Table 2. Pathway analysis of upregulated genes in liver tissue of *N. coriiceps* exposed to**
775 **hypoxia.**

Biological Processes			
GOID	**GO Term**	**P-value (corrected)**	**# genes**
GO:0045937	positive regulation of phosphate metabolic process	0.0000	14
GO:0050900	leukocyte migration	0.0002	8
GO:0051480	regulation of cytosolic calcium ion concentration	0.0005	9
GO:0060326	cell chemotaxis	0.0010	8
GO:0030198	extracellular matrix organization	0.0011	8
GO:0043410	positive regulation of MAP cascade	0.0016	8
GO:0071900	regulation of protein serine/threonine kinase activity	0.0025	8
GO:0016616	oxidoreductase activity, acting on the CH-OH group donors, NAD or NADP as acceptor	0.0025	6
GO:0050930	induction of positive chemotaxis	0.0026	3
GO:0072554	blood vessel lumenization	0.0026	3
GO:0030595	leukocyte chemotaxis	0.0026	6
GO:0043405	regulation of MAP kinase activity	0.0048	6
GO:0002521	leukocyte differentiation	0.0052	6
GO:0009617	response to bacterium	0.0052	8
GO:0030217	T cell differentiation	0.0053	4
GO:0060348	bone development	0.0063	5
GO:0050920	regulation of chemotaxis	0.0088	5
GO:0050679	positive regulation of epithelial cell proliferation	0.0089	3
GO:0002688	regulation of leukocyte chemotaxis	0.0102	3
GO:0006954	inflammatory response	0.0107	7
GO:0032147	activation of protein kinase activity	0.0172	6
GO:0001666	response to hypoxia	0.0176	3
GO:0071902	positive regulation of protein serine/threonine kinase activity	0.0193	5
GO:0097529	myeloid leukocyte migration	0.0193	5
GO:0000187	activation of MAPK activity	0.0228	4
GO:0032496	response to lipopolysaccharide	0.0241	4

GO:0032103	positive regulation of response to external stimulus	0.0248	4
GO:0004709	MAP kinase kinase kinase activity	0.0264	3
GO:0030593	neutrophil chemotaxis	0.0326	3
GO:0005539	glycosaminoglycan binding	0.0330	4
GO:0099565	chemical synaptic transmission, postsynaptic	0.0335	4
GO:0050727	regulation of inflammatory response	0.0366	3
GO:0072676	lymphocyte migration	0.0366	3
GO:0070098	chemokine-mediated signaling pathway	0.0385	4
GO:0070374	positive regulation of ERK1 and ERK2 cascade	0.0433	3
GO:0002573	myeloid leukocyte differentiation	0.0442	3
GO:003333	fin morphogenesis	0.0508	3
GO:0008009	chemokine activity	0.0554	3
KEGG:04510	focal adhesion	0.0000	13
KEGG:04810	regulation of actin cytoskeleton	0.0005	11
KEGG:04933	AGE-RAGE signaling pathway in diabetic complications	0.0026	7
KEGG:04068	FoxO signaling pathway	0.0089	5
KEGG:04512	ECM-receptor interaction	0.0102	5
KEGG:04672	intestinal immune network for IgA production	0.0366	3
KEGG:00620	pyruvate metabolism	0.0409	3
KEGG:00330	arginine and proline metabolism	0.0508	3
Molecular Function			
GO:0004620	phospholipase activity	0.0401	4

Table 3. Major groupings of GO terms from pathway analysis of genes upregulated in liver *of N. coriiceps* in response to hypoxia

GO term	GO or KEGG ID	Associated genes	Known HIF-1 targets*
HYPOXIA RESPONSE (11 unique genes)		**ldha, pgfb, vegfaa, cyp17a1, me2, me3, phgdh, si:dkey-180p18.9, msna, rasa3, sh3kbp1**	**LDHA, VEGFA**
response to hypoxia	GO:0001666	ldha, pgfb, vegfaa	
oxidoreductase activity, acting on the CH-OH group of donors	GO:0016616	cyp17a1, ldha, me2, me3, phgdh, si:dkey-180p18.9	
blood vessel lumenization	GO:0072554	msna, rasa3, sh3kbp1	
IMMUNE RESPONSE (22 unique genes)		**csf3r, fam49al, gpr183a, irf7, rag1, slc7a7, cxcr4b, pgfb, vegfaa, c5ar1, ccl44, cxcl19, mmp9, ptger2a, tnfaip6, ccl25a, jund, nos1, zswim6, cxcr2, hist1h2a11, ripk2**	**FAM107B, GPR25,IRF1, RRAGD, SLC2A1, SLC25A36, SLCO5A1, SLC38A3, CXCR4, VEGFA, VEGFB, TNFAIP3, JUNB**
leukocyte differentiation	GO:0002521	csf3r, fam49al, gpr183a, irf7, rag1, slc7a7	
myeloid leukocyte differentiation	GO:0002573	csf3r, gpr183a, slc7a7	
regulation of leukocyte chemotaxis	GO:0002688	cxcr4b, pgfb, vegfaa	
inflammatory response	GO:0006954	c5ar1, ccl44, cxcl19, cxcr4b, mmp9, ptger2a, tnfaip6	
chemokine activity	GO:0008009	ccl25a, ccl44, cxcl19	
T cell differentiation	GO:0030217	fam49al, gpr183a, irf7, rag1	
neutrophil chemotaxis	GO:0030593	ccl44, cxcl19, cxcr4b	
leukocyte chemotaxis	GO:0030595	ccl44, cxcl19, cxcr4b, gpr183a, pgfb, vegfaa	
response to lipopolysaccharide	GO:0032496	cxcl19, cxcr4b, jund, nos1	
regulation of inflammatory response	GO:0050727	cxcr4b, mmp9, tnfaip6	
leukocyte migration	GO:0050900	ccl25a, ccl44, cxcl19, cxcr4b, gpr183a, mmp9, pgfb, vegfaa	
regulation of chemotaxis	GO:0050920	cxcr4b, gpr183a, pgfb, vegfaa, zswim6	
induction of positive chemotaxis	GO:0050930	cxcr4b, pgfb, vegfaa	
cell chemotaxis	GO:0060326	ccl25a, ccl44, cxcl19, cxcr2, cxcr4b, gpr183a, pgfb, vegfaa	
chemokine-mediated signaling pathway	GO:0070098	ccl44, cxcl19, cxcr2, cxcr4b	
myeloid leukocyte migration	GO:0097529	ccl44, cxcl19, cxcr4b, pgfb, vegfaa	
lymphocyte migration	GO:0072676	ccl25a, ccl44, gpr183a	
response to bacterium	GO:0009617	cxcl19, cxcr2, cxcr4b, hist1h2a11, jund, mmp9, nos1, ripk2	
KINASE ACTIVITY (13 unique genes)		**gadd45aa, paqr7b, ripk2, smarca4a, c5ar1, ccl44, cd27, gpr183a, dusp1, dusp2, dbf4, stk39, ccnd2a**	**GADD45b, GPR25, DUSP1**
activation of MAPK activity	GO:0000187	gadd45aa, paqr7b, ripk2, smarca4a	
MAP kinase kinase kinase activity	GO:0004709	gadd45aa, ripk2, smarca4a	
positive regulation of MAPK cascade	GO:0043410	c5ar1, ccl44, cd27, gadd45aa, gpr183a, paqr7b, ripk2, smarca4a	
regulation of MAP kinase activity	GO:0043405	dusp1, dusp2, gadd45aa, paqr7b, ripk2, smarca4a	
activation of protein kinase activity	GO:0032147	dbf4, gadd45aa, paqr7b, ripk2, smarca4a, stk39	
positive regulation of ERK1 and ERK2 cascade	GO:0070374	c5ar1, ccl44, gpr183a	
regulation of protein serine/threonine kinase activity	GO:0071900	ccnd2a, dbf4, dusp1, dusp2, gadd45aa, paqr7b, ripk2, smarca4a	
positive regulation of protein serine/threonine kinase activity	GO:0071902	dbf4, gadd45aa, paqr7b, ripk2, smarca4a	
MISCELLANEOUS			**PLCH2, TNFAIP3, EMID2, CXCR4, PPP1R9A**
phospholipase activity	GO:0004620	anxa2a, plcl2, smpdl3b, vegfaa	
glycosaminoglycan binding	GO:0005539	ccn5, pcolcea, tnfaip6, vegfaa	
extracellular matrix organization	GO:0030198	col18a1a, col1a2, col5a2a, col5a3b, emid1, hmcn2, kirrel3b, mmp9	
positive regulation of response to external stimulus	GO:0032103	cxcr4b, mmp9, pgfb, vegfaa	
fin morphogenesis	GO:0033334	frem1a, hmcn2, mmp9	
positive regulation of phosphate metabolic process	GO:0045937	c5ar1, ccl44, cd27, dbf4, gadd45aa, gpr183a, nos1, paqr7b, pgfb, ppp1r15b, ripk2, smarca4a, stk39, vegfaa	
positive regulation of epithelial cell proliferation	GO:0050679	c5ar1, pgfb, vegfaa	
regulation of cytosolic calcium ion concentration	GO:0051480	c5ar1, clic2, cxcr2, cxcr4b, grm1a, grm1b, lpar5b, ptger2a, wnt5a	
bone development	GO:0060348	col6a2, rarga, spp1, wls, wnt5a	
chemical synaptic transmission, postsynaptic	GO:0099565	chrna10a, grm1a, grm1b, p2rx5	
KEGG PATHWAY			
Arginine and proline metabolism	KEGG:00330	ckbb, nos1, p4ha1b	
Pyruvate metabolism	KEGG:00620	ldha, me2, me3	
FoxO signaling pathway	KEGG:04068	akt3a, ccnd2a, gadd45aa, grm1a,grm1b, hrasb, rag1	
VEGF signaling pathway	KEGG:04370	akt3a, hrasb, pxna, src, vegfaa	
Focal adhesion	KEGG:04510	LOC100535278, akt3a, ccnd2a,col1a2, col6a2, col6a4a, flna,hrasb, itga11a, pgfb, pxna, src,vegfaa	
ECM-receptor interaction	KEGG:04512	LOC100535278, col1a2, col6a2,col6a4a, itga11a	
Intestinal immune network for IgA production	KEGG:04672	ccl25a, cxcr4b, pigr	
Regulation of actin cytoskeleton	KEGG:04810	apc2, cxcr4b, fgd, hrasb, iqgap1,itga11a, msna, pip5k1bb, pxna,rras, src	
AGE-RAGE signaling pathway in diabetic complications	KEGG:04933	akt3a, col1a2, hrasb, nox4, pim1,serpine1, vegfaa, serpine1, vegfaa	

Multiple GO terms were combined into 4 categories (hypoxia response, immune response, kinase activity and miscellaneous). Unique genes within each category (except miscellaneous and KEGG pathway genes) are listed in bold font in the column of associated genes. Within each category, genes were identified as known HIF targets from Ortiz-Barahona et al. (2010).

Table 4. Lactate and glycogen levels in tissues of *N. coriiceps* in normoxia and hypoxia.

	NORMOXIA	HYPOXIA
Heart		
Lactate (μmol g^{-1})	26.33 ± 2.42 (6)	22.04 ± 2.49 (6)
Glycogen (mg g^{-1})	11.03 ± 2.06 (4)	6.67 ± 2.12 (6)
Liver		
Lactate (μmol g^{-1})	0.17 ± 0.04 (6)	0.94 ± 0.21 (6)*
Glycogen (mg g^{-1})	39.20 ± 5.22 (5)	26.27 ± 12.10 (6)
Brain		
Lactate (μmol g^{-1})	6.21 ± 0.64 (6)	6.79 ± 1.00 (6)
Glycogen (mg g^{-1}).	0.41 ± 0.10 (6)	0.56 ± 0.09 (6)
Gill		
Lactate (μmol g^{-1})	0.10 ± 0.01 (5)	0.31 ± 0.12 (5)
Glycogen (mg g^{-1})	0.49 ± 0.02 (6)	0.43 ± 0.06 (6)

The asterisk indicates a significant difference in levels between *N. coriiceps* held at normoxia and hypoxia as determined by an unpaired t-test or Mann-Whitney U test ($P < 0.5$). The number of individuals (*n*) is indicated in parentheses.