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Severe Hypoxia Up-regulates Gluconeogenesis in Daphnia

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Severe Hypoxia

Up-regulates Gluconeogenesis in *Daphnia*

by

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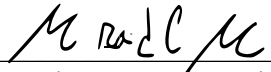
East Tennessee State University



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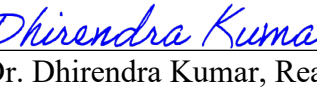

Dr. Dhirendra Kumar, Reader

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Abstract

Hypoxia is a significant low oxygen state that has complex and diverse impacts on organisms. In aerobes, various adaptive responses to hypoxia are observed that vary depending on the level of oxygen depletion and previous adaptation, hence the continued attention to hypoxia as an important abiotic stressor. Adaptive responses to hypoxia are primarily governed by the hypoxia-inducible factors (HIFs), which activate downstream genetic pathways responsible for oxygen transport and metabolic plasticity. In aquatic habitats, oxygen availability can vary greatly over time and space. Therefore, aquatic organisms' adaptation to hypoxia is likely pervasive, especially in genotypes originating from waterbodies prone to hypoxia. Here we report the transcriptional response to severe hypoxia in the freshwater crustacean *Daphnia magna*. We observe improved survival in media containing elevated calcium ion (Ca2+) concentrations. Additionally, we observe changes in lactate and pyruvate concentrations within tissues. To elucidate the transcriptome basis of these effects, we examine transcripts with known gene ontologies indicating roles in Ca2+ homeostasis and signaling, and in pyruvate metabolism, including gluconeogenesis (GNG). We observe the up-regulation of numerous transcripts encoding GNG pathway enzymes, including the rate-limiting enzyme phosphoenolpyruvate carboxykinase (PEPCK-C) and fructose-1,6-bisphosphatase (FBP). In contrast, no transcripts involved in Ca2+ homeostasis or signaling showed any significant differential expression. Some GNG transcripts are more up-regulated in clones from permanent waterbodies not prone to hypoxia, inconsistent with the hypothesis about its protective effects. One exception is the FBP transcript, which has been identified to be up-regulated in some hypoxia-tolerant aquatic organisms.

Contents

Introduction

Hypoxia as an Ecological Constraint

In metazoan life, oxygen is fundamental. As part of oxidative phosphorylation, oxygen (O_2) serves as the final electron acceptor in the electron transport chain. Below normoxic levels, there is a gradient in which organisms experience and react to stress. A decrease from normoxia to moderate hypoxia generally does not cause changes in how energy is produced, with ATP and O_2 fluxes remaining constant. Severe hypoxia occurs when there is a significant lack of O_2, leading to a critical imbalance in mitochondrial oxidative phosphorylation and the insufficient reoxidation of NADH, leading to an overall reduction in adenosine triphosphate (ATP) production. Anoxia occurs when there is a complete loss of O_2, resulting in the cessation of aerobic respiration. In both severe hypoxia and anoxia, ATP generation in invertebrates comes from inefficient substrate-level anaerobic phosphorylation (Grieshaber et al., 1993; Sokolova et al., 2019).

In aquatic ecosystems, severe hypoxia is defined as dissolved oxygen levels of less than 2 mL O2/L (equivalent to 2.8 mg O2/L) (Wu, 2002). Due to low O_2 solubility in water, its availability to aquatic organisms is poorer than terrestrial ones and exhibits considerably larger fluctuations in aquatic habitats than on land. The often-unpredictable O_2 dynamics in estuaries, coastal zones, meromictic lakes, and vertical stratification caused by haloclines and thermoclines are significant ecological constraints on aquatic inhabitants (Sokolova et al., 2019; Wu, 2002). Many smaller aquatic environments, such as tidepools, experience vast fluctuations in O_2 regularly (Richards, 2011). In addition, O_2 fluctuations can range from up to 300–400% of saturation level during high algal photosynthetic events to near-complete anoxia within a short period (Richards, 2011). Pollution and the introduction of anthropogenic nutrients and organic matter into bodies of water, in addition to changes in ocean temperature caused by climate change and aquaculture farming, can all result in hypoxic zones developing within ecosystems (Wu, 2002). Overall, O2 availability is a limiting factor for aquatic organisms' survival and growth; hence, research into how hypoxia impacts aquatic life is prevalent (Zheng et al., 2021).

As a result of this factor, aquatic organisms have adapted to such an unpredictable O_2 environment much more so than terrestrial organisms, where O_2 levels only become relevant at high altitudes or in underground burrows (Sokolova et al., 2019). General behavioral changes, avoidance and escape from hypoxic areas, reduced feeding rates, and increased water filtration are

common adaptations to hypoxia (Zheng et al., 2021). Moreover, several invertebrates and crustaceans alike have been identified as having much greater survivability in hypoxia, including the ability to survive for prolonged periods in anoxia (Hand & Menze, 2008; Zheng et al., 2021). Much interest has been taken in better elucidating these mechanisms of hypoxia tolerance from adaptive traits through an evolutionary and ecological perspective (Richards, 2011; Brennan et al., 2018; Andersen et al., 2020; Borowiec & Scott, 2020; Crispo et al., 2020). Among invertebrates, such studies are rarer (Sandoval-Castillo et al., 2018). Therefore, investigations into invertebrate hypoxia tolerance through the lenses of evolution and metabolism could offer better insights into how tolerance evolved and reveal novel mechanisms for hypoxia survival.

Hypoxia-Inducible Factors

Hypoxia-inducible factors (HIFs) are a class of proteins that serve as the master regulators of transcriptional responses to hypoxia in metazoans (Semenza, 2012). HIFs are comprised of two major isoform subtypes: (HIFα), which is oxygen-sensitive, and (HIFβ), which is oxygen-insensitive (Prabhakar & Semenza, 2012; Hankinson, 2008). HIFβ is also termed an aryl hydrocarbon receptor nuclear translocator (ARNT) (Yeo, 2019). HIFα and HIFβ subunits are all constitutively expressed and function as α–β heterodimers to bind to the hypoxia response element (HRE) at the transcriptional enhancer, transactivating target genes downstream (Ravenna et al., 2015; Webb et al., 2009). The oxygen-sensitive HIFα isoforms contain the oxygen-dependent degradation (ODD) domain, which facilitates HIFα stability and abundance by oxygen-sensing proteins' prolyl hydroxylase domains (PHD, also known as EGLN) (Ravenna et al., 2015). A representative list of HIF-1 target gene products can be found in Table 1 in Yeo, 2019.

In an adequate supply of oxygen or normoxia, HIFα chains are rapidly tagged and then degraded (Webb et al., 2009). Using oxygen as a substrate, HIF PHD hydroxylates two prolyl residues in the ODD domain of HIFα chains, which in turn promotes the interaction with von Hippel–Lindau protein (pVHL) and other proteins, forming pVHL-Elongin B/C-Cullin 2 (VEC), which finally directs ubiquitin-mediated proteasomal degradation (Koh et al., 2008; Yeo, 2019). During hypoxia, the loss of substrate O_2 prevents PHD activation, which results in HIF1α and HIF2α stabilization. HIFs are translocated into the nucleus where they dimerize with the HIFβ subunits and the coactivators P300/CBP are phosphorylated by ERK1/2, finally promoting the binding to the HRE in the promoter or enhancer regions of targeted genes, thus modulating their

expression (Ratcliffe, 2007; Webb et al., 2009; Yeo, 2019). Additionally, HIF1A is significantly up-regulated during hypoxia and numerous factors have been shown to regulate HIFα expression and activity at the transcriptional, translational, and post-translational levels in both an oxygen-dependent and oxygen-independent manner, including NF-κB, OS-9, SSAT2, etc. (Koh et al., 2008; Yeo, 2019). Overall, in metazoans, HIFα and HIFβ are always expressed, with HIF1α acting as the variable response to O_2 levels that are also regulated by an array of other factors (Table 2; Yeo, 2019).

Metabolic Responses in Hypoxia

The transition from moderate to severe hypoxia is a significant one and leads to a series of cascading metabolic changes. In general, hypoxia-mediated responses would typically manifest as compensatory morphological and physiological changes, including increased production of oxygen-transporting molecules such as hemoglobins (Sokolova et al., 2019). Severe hypoxia will increase the conversion of glucose to lactate, termed the Pasteur effect, by way of the activation of anaerobic glycolysis via HIFs while also causing metabolic rate depression (Kim et al., 2006). Crustaceans act the same, with the mobilization of glycogen for anaerobic metabolism producing lactic acid (Zheng et al., 2021). Overall, hypoxic activation of HIF-1 increases the conversion of pyruvate to lactate by up-regulating pyruvate kinase and lactate dehydrogenase. This produces ATP, but in an inefficient manner that may be inadequate due to lower amounts made with the creation of reactive oxygen species (ROS) in the mitochondria owing to the leakage of electrons from the respiratory chain (Kim et al., 2006). Therefore, ROS production must be mitigated to prevent its toxic buildup and mutagenic effects. HIF-1 will up-regulate antioxidants to eliminate ROS such as superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), and glutathione S-transferase (GST) (Zheng et al., 2021). Numerous metabolic and harm mitigation adjustments occur as survival in severe hypoxia is extremely stressful for the mitochondria, including after reoxygenation (Sokolova et al., 2019). The influx of lactate produced during anaerobic metabolism is toxic and will drop cellular pH if cells are not continuously perfused or if the buildup of toxic products is not reduced. Moreover, sudden oxygen reperfusion to ischemic cells will result in mitochondrial dysfunction and cardiac cell damage, with the most serious injury being a result of reperfusion rather than anoxia (Solaini & Harris, 2005).

In crustaceans, mitochondrial enzyme complexes like cytochrome C oxidase, succinate dehydrogenase, and ATP synthase have all been found to be down-regulated in response to hypoxia. Furthermore, antioxidant activity is broadly up-regulated. However, SOD, GPX, CAT, and GST activities can be expressed differently, at least in decapod crustaceans, depending on their hypoxia tolerance strategies (Table 2, Zheng et al., 2021). AMPK is an AMP-activated protein kinase that is up-regulated when the AMP to ATP ratio increases and may function as a sensor that regulates metabolism under hypoxia in crustaceans, regulating enzymes that promote an increase in ATP production while decreasing its consumption (Sheng et al., 2012; Zhang et al., 2017). Within *Daphnia*, hemoglobin (Hb) is up-regulated to deliver more oxygen in response to hypoxia (this is visually obvious with redder daphnids in hypoxia) and several Hb subunits in *Daphnia* can be differentially expressed at various conditions (Lee et al., 2022; Weber & Vinogradov, 2001). In hypoxia-acclimated *D. pulex,* only the glycolytic enzyme enolase involved in anaerobic metabolism was up-regulated slightly, and there was a strong induction of cellobiohydrolases, which may break down structural polysaccharides (Zeis et al., 2009). Overall, apart from hemoglobin, not much is known about the transcriptional response of metabolic enzymes to hypoxia, hence such studies can lend better insights into possible adaptive responses.

Gluconeogenesis

Gluconeogenesis (GNG), in general, can be considered the opposite of glycolysis, with distinct rate-limiting enzymes different from glycolysis enzymes, accomplishing several key steps. While in glycolysis, glucose is catabolized into pyruvate via glycolytic enzymes, producing NADH from NAD+ to fuel oxidative phosphorylation. In GNG, non-carbohydrate substrates like lactate, amino acids, and glycerol are converted into glucose. First, lactate and alanine are converted into pyruvate, then in the mitochondria, they are carboxylated to oxaloacetate (OAA) by pyruvate carboxylase (PC). OAA is reduced to malate, which is shuttled to the cytoplasm to again be converted to OAA. OAA is converted to phosphoenolpyruvate (PEP) by cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C). OAA may also be converted to PEP directly by mitochondrial PEPCK (Stark et al., 2014). PEP will then enter several reverse glycolysis steps via the gluconeogenic cycle, yielding fructose 1,6-bisphosphate (F1,6BP). F1,6BP is then dephosphorylated by fructose 1,6-bisphosphatase (FBPase), forming fructose 6-phosphate, which is then converted to glucose-6-phosphate (G6P) by phosphoglucoisomerase. In the final step of

GNG, G6P is converted to glucose via dephosphorylation by glucose-6-phosphatase (G6Pase). Overall, there are four unique gluconeogenic enzymes in GNG: PC, PEPCK, FBPase, and G6Pase (Figure 4) (Zhang et al., 2019).

In mammals, gluconeogenesis is up-regulated by HIF-1 during fasting, mostly in the liver and kidneys, to maintain glucose homeostasis (Zhang et al., 2019). Recent studies have revealed the up-regulation of GNG-specific enzymes in response to hypoxia in aquatic organisms. Vora et al. (2021) found that HIF-1 activation directly promotes GNG and the glyoxylate cycle in C. elegans. GNG, via PEPCK, provides carbon needed to produce NADPH and the antioxidant glutathione, which reduces oxidative stress. They also found that PEPCK is needed for hypoxia survival. The glyoxylate cycle allows the mobilization of lipids into acetyl-CoA for use in GNG and/or lactic acid production and may also produce glucose. In many aquatic organisms, fructose 1,6-bisphosphatase has also been found to be up-regulated in hypoxia and is regulated by HIF-1 activation (Cota-Ruiz et al., 2016; Crocker et al., 2013).

Calcium in Hypoxia Tolerance and Mitochondrial Homeostasis

In addition to oxygen, calcium (Ca2+) is one of the critical abiotic factors for eukaryotic survival. On the cellular level, the role of Ca2+ is ambivalent and is involved in intracellular signaling, myosin contraction, secretion, gene transcription, enzyme control, and apoptosis; and on the organismal scale, Ca2+ is required for mineralized tissues like bones and cuticles (Carafoli et al., 2001; Wheatly et al., 2002). In general, Ca2+ can enter the cell passively via cation exchangers Na^{+}/Ca^{2+} (or NCX) and a verapamil-sensitive channel or actively via a plasma membrane Ca^{2+} ATPase (PMCA) channel (Ahearn et al., 2004). Voltage-gated calcium channels (VGCCs) also channel Ca2+ in excitable cells such as muscles and neurons (Yamakage & Namiki, 2002). Ca2+ is stored by many organelles into calcium-storage proteins, like calsequestrin, calreticulin, chromogranin, etc., to increase its uptake into the cell while preventing any negative effects from high concentrations of Ca2+. Likewise, storage proteins are also important for Ca2+-dependent cellular processes and overall calcium homeostasis (Carafoli et al., 2001).

In mitochondria, Ca2+ uptake occurs primarily by the mitochondrial calcium uniporter (MCU), which takes up Ca2+ from the cytosol or by microdomains of stored Ca2+ from the ER into the mitochondrial matrix. Ca2+ is exported from the mitochondria by antiporter exchangers using the Ca^{2+}/H^{+} antiporter in most cells or Na^{+}/Ca^{2+} in excitable cells (Marchi & Pinton, 2014).

Overall, mitochondrial Ca2+ uptake controls intracellular Ca2+ signaling, cell metabolism, cell survival, and the regulation of mitochondrial effectors (Rizzuto et al., 2012). A list of components of the mitochondrial Ca2+ uniporter complex and their functions can be found in Table 1 Marchi & Pinton, 2014.

Cellular apoptosis and mitochondrial homeostasis are fully linked. One of the fundamental steps of programmed cell death is the opening of the mitochondrial permeability transition pore and the release of pro-apoptotic factors like cytochrome c (cyt-c). At least two of the factors that can trigger this mitochondrial-driven apoptosis (at least in mammals) are limited energy states (such as hypoxia) and elevated Ca2+ levels. However, organisms that are likely to experience hypoxia, like aquatic crustaceans, must be able to prevent mitochondrial-driven apoptosis to survive in such energy-limited states for extended periods. Therefore, it is becoming evident that there are functional trade-offs between the regulation of cell death and major fluctuations in energy states and Ca2+ content within hypoxia-tolerant organisms. For example, the brine shrimp, *Artemia franciscana*, experiences a complete loss of metabolism during very long periods of anoxia and shows differences in their mitochondria from those of mammals. Such differences are in the mammalian mitochondrial permeability transition pore (MPTP), capacity for calcium uptake, and their protease enzymes, showing different responses to calcium to blunt apoptosis (Hand & Menze, 2008). Additionally, it is important to note that *Daphnia* abundance is positively correlated with environmental calcium concentrations. This is likely due to higher energetic costs in the intake of Ca in poor-Ca media, leading to poorer growth and inefficiencies in storing Ca, especially after molting (Tan & Wang, 2009). For *D. magna*, the Ca survival threshold was found to be between 0.1 and 0.5 mg Ca L^{-1} (Hessen et al., 2000).

Methodology

Maintenance of Daphnia Clones

The four laboratory clones used in this study (IL, FI, GB, and HU) were obtained from the *Daphnia* Stock Collection at Basel University, Switzerland (Dieter Ebert), and were kept in COMBO water (Kilham et al. 1998) at 20 °C under a 16:8 D:L cycle and fed a diet of *Scenedesmus acutus*. The geographic origin of the four clones is listed in Appendix 2. The following procedure was used to establish all experimental cohorts: Five randomly selected females (grandmothers) from each clone were maintained from birth in COMBO water at a density of 1 individual per 20 mL, with the water changed every four days, and fed a *Scenedesmus* concentration of 10^5 cells/mL of COMBO daily. The offspring from the grandmothers' second and third clutches were used to establish the (mother) generation. The mothers were maintained in the same conditions until enough neonates from their second or consecutive clutches could be collected to form the experimental cohorts. Neonates were maintained in groups of 20 in 200 mL jars of COMBO for the first 6 days of their lives until transferred to the corresponding experimental tanks.

Chronic Mild Intermittent Hypoxia Treatment and Control

The *Daphnia* cohorts were kept in four 5L aquarium tanks, each containing eight transparent plastic containers, each with an open top for airflow and a 1mm nylon mesh bottom to keep cohorts separated in the same tank while allowing for free water exchange and the removal of neonates during water changes every three days. 10^5 *Scenedesmus* cells were added per mL twice daily, with the water volume and daily food ratios adjusted every 4 days to maintain 20 mL of water per individual in the tank. The chronic mild intermittent hypoxia treatment (CMIH) was performed by bubbling N_2 through the experimental tanks with continuous monitoring of the oxygen concentration by the Extech DO210 probe (Nashua, NH, USA) twice daily until the O_2 concentration was lowered to 4 mg/L. The oxygen concentration was typically elevated naturally to 6.5 mg/L between these daily procedures. During the same time, the control tanks were aerated with ambient air to 8 mg/L. The control tanks typically experienced a drop in oxygen concentration between aeration treatments of 7 –7.5 mg/L. Of the four tanks, two were CMIH and the other two were controls. Each tank was set up with 120 individuals, 30 per clone, each split into two containers per tank.

Severe Hypoxia Experiment and RNA-Seq Sample Collection

For the first acute severe hypoxia (ASH) tolerance measurements, daphnids were sampled from each of the four tanks at 25 days old (18 days at either normoxic or CMIH tank conditions) and moved into 70 mL cell culture flasks filled with COMBO water. The concentration of oxygen was bubbled at or below 1 mg/L using N_2 gas, sealed without air bubbles, and kept at 20˚C. 7 Daphnia per flask, 5 replicate flasks per clone per treatment. The acute hypoxia experiment was set up at 9:00 p.m. and the mortality was recorded 12 hours later and every hour thereafter, by observing the loss of movement after agitation by shaking and inverting the flask. For the RNA sampling, two individuals per clone from the CMIH and control tanks were sampled and frozen at the beginning of the ASH experiment. These constitute the ASH controls. Two individuals per clone were sampled after 12 hours of exposure before any mortality occurred from the ASH treatment. And the water was re-topped off with 1 mg O2/L of water and sealed again to maintain the survival experiment.

Ca2+ Severe Hypoxia Experiments

For the Ca2+ acute severe hypoxia experiments, the COMBO medium (Kilham et al., 1998) was compared to the alternative commonly used *Daphnia* medium, ADaM (Klüttgen et al., 1994), which contains a significantly higher Ca2+ concentration (Appendix 3). ADaM and COMBO, with or without the addition of Ca2+, were also compared. The four media types were prepared (Appendix 3), and the same procedure was performed as described in (Daphnia Clones and Maintenance) to generate neonates for each of the four media cohorts. Beginning on days 20-25 after placement into their respective media at random and thereafter, daphnids from each of the four clones and treatments were removed and placed individually into small 5 mL vials filled with 1 mg/L O_2 media of their corresponding media type. A total of 112 individuals were used, split between 2 blocks. Because late embryos in the brood chamber consume a significant amount of oxygen, only daphnids with no eggs in the brood chamber or with eggs younger than 48h were selected for this experiment. Ovary/egg status was recorded in the following categories: no clutch and no visible ovaries; no clutch and visible ovaries with fully developed oocytes; clutch <12 old in the brood chamber; clutch between 12h and 24h old in the brood chamber; and, finally, occasional individuals (4 total out of 112) with a clutch of 24 to 48h old in the brood chamber.

This ovary/clutch status was used as a covariable in the proportional hazards analysis of acute hypoxia survival time, as was the individuals' body length. Mortality was recorded similarly (as described in Severe Hypoxia Experiment and RNA-Seq Sample Collection). Daphnids not selected for the first experiment were consolidated and removed individuals were replaced with new neonates for additional experimental runs. In all, 2 blocks of measurements were conducted.

RNA Sequencing

During the acute hypoxia experiment, two individuals per flask from each of the four clones (IL, FI, GB, and HU) and each of the hypoxia treatments were sampled and frozen (see Severe Hypoxia Experiment and RNA-Seq Sample Collection). The four treatments were: daphnids reared at normoxia, daphnids reared at normoxia and exposed to acute hypoxia for 12 hours, daphnids reared in CMIH, and daphnids reared in CMIH and exposed to acute hypoxia for 12 hours. Daphnids were thawed and RNA was extracted using the Qiagen RNAeasy kit (Cat. ID: 74134) and quantified using a Qubit (ThermoFisher) fluorometer.

Following the extraction, RNA was reverse transcribed, and the sequencing libraries were constructed from the cDNAs as prescribed by the Oxford Nanopore Technology (Oxford, UK) PCR-cDNA Barcoding kit protocol (SQK-PCB109), with 3 biological replicates per clone per treatment, each replicate consisting of RNA extracted from two individuals. Each of the barcoded samples from the 4 treatments within each clone was pooled together into 3 replicate libraries, purified separately, and pooled together immediately before adding the sequencing adapter. Finally, the libraries were sequenced using Oxford Nanopore MinION for 24-48 hours per sequencing run, obtaining 2-4 Gb of reads in each run.

RNA Sequencing Data Analysis

Base-calling and reads filtering, demultiplexing, trimming, and mapping were performed using ONT Guppy software (ver. 3.6). *Daphnia magna* reference transcriptome 3.0 (D.Ebert and P.Fields, personal communication) was used as a reference. The mapping reference only contained the longest isoform for each gene, for a total of 33,957 transcripts, of which at least one read mapped to 22,445 transcripts. Transcripts were then filtered to retain only those that contained at least 72 reads across all samples, resulting in a set of 6050 transcripts retained for further analysis.

As each library and each sequencing run consisted of 3 biological replicates of each of 4 combinations of CMIH and ASH treatments from a single clone, clones were fully confounded with replicate library preparation and sequencing runs. The advantage of this design is that each library preparation contains a balanced set of all 4 treatments with a clone-library replicate combination that can either be used as a random block effect in a 3-way ANOVA or pulled with biological replicates for Likelihood Ratio tests (see below). The disadvantage of this design is the lack of the ability to test for the difference among clones, untangling the variance among clones from random variance among library preparation and sequencing runs.

Differential expression was analyzed using Likelihood Ratio tests (LRT) in DESeq2 (Love et al. 2014) and by 3-way ANOVA with RPKM as the response variable and CMIH treatment, ASH treatment, and habitat of origin as fixed effects and clones as a random block effect, implemented in JMP (ver. 16, SAS Institute 2021). In DESeq2 analysis, log fold change noise was shrunk by the apeglm algorithm for the Wald test (Zhu et al. 2019). DESeq2 analysis was conducted separately for the full data, for the two habitats of origin, and for the analysis of the CNIH factor for the subset of samples not exposed to ASH treatment (ASH = Control). For LRT analysis, the reduced model consisted of all factors except the factor being tested and all its interactions. Wald and LRTs yielded similar results, with only LRT results reported. An arbitrarily adjusted p-value of $padj<0.1$ was chosen as the cut-off for reporting differential gene expression in any given transcript. For 3-way ANOVAs, a false discovery rate (FDR) procedure was implemented for the multiple test correction, again with the cut-off of $FDR<0.1$.

Enrichment analysis was conducted separately for lists of transcripts with a possible significant effect for each of the main treatments (CMYH, ASH, habitat type) and interactions. CMYH was not reported. Two sources of gene lists and two types of annotation data were used. Gene lists were obtained by selecting transcripts with an uncorrected $p<0.01$ separately in the DESeq2 LRT analysis or in 3-way ANOVAs. The results of these analyses were similar, and only the LRT enrichment results will be reported, with one exception. The two approaches to the annotation data were: a closed, non-overlapping list of transcripts with functions a priori known to be hypoxia-related; and an open, overlapping list of GO categories. Firstly, we constructed a non-overlapping list of annotation terms that characterized pathways and functions a priori known to play a role in hypoxia response. The only deviations from the non-overlapping principle were the fused genes containing vitellogenin and superoxide dismutase domains (Kato et al. 2004), which

appeared both in the "vitellogenins" and "antioxidant pathway" gene lists. The source of annotation was a combination of descriptions obtained from the *D. magna* genome annotation available at (http://arthropods.eugenes.org/genepage/daphniamagna/Dapma7bEVm000001t1; Gilbert 2002) and annotations were obtained for the *D. magna* 3.0 transcriptome by blast2GO (Götz et al. 2008) and PANTHER (Mi et al. 2013) software. Secondly, we used all GO annotations generated by blast2GO and PANTHER to construct an open list of all GOs; this list was filtered to include only GOs represented in the reference transcriptome by at least 10 transcripts. In both cases, the Fisher exact test was used to test for enrichment, testing the hypothesis that a given pathway, function, or GO has a higher than random chance of occurring in the list of candidate genes (left-handed FET).

Lactate and Pyruvate Measurements

Lactate and pyruvate assays were conducted using CellBiolab kits on 3 daphnids from the acute hypoxic treatment per clone (see above) and 5 daphnids as controls. Each daphnid was homogenized in 100 µL ice-cold PBS with a pestle, and the homogenates were centrifuged at 4˚C. 25 µL of supernatant were pipetted into each lactate and pyruvate assay plate using the manufacturer's protocol (CellBiolab catalog #s 101820174 & 82320181). Additionally, two replicate aliquots of 15 µL of the supernatant each was used to quantify soluble proteins by the Bradford assay, with 185 µl of Bradford colorimetric reagent added to each well. All assay well plates were analyzed using a BIOTEK plate reader (Agilent, Santa Clara, CA, USA). Lactate and pyruvate fluorescence assay sensitivity was set to 35, and the Bradford assay absorption was measured at 595 nm.

Results

Daphnia Survival in Severe Hypoxia

The acute hypoxia experiment that was used to generate RNAs for the RNA-seq experiment showed that *Daphnia* reared in mild hypoxia had, unexpectedly, lower survival times in acute hypoxia, eliminating any hypotheses about possible hormetic effects of mild hypoxia (Fig. 1, Table 1). Importantly, this experiment also demonstrated that *Daphnia* from intermittent hypoxia-prone habitats showed higher survival times than their counterparts from permanent habitats unlikely to experience frequent hypoxia episodes (Fig. 1, Table 1,). No significant interactions were detected either.

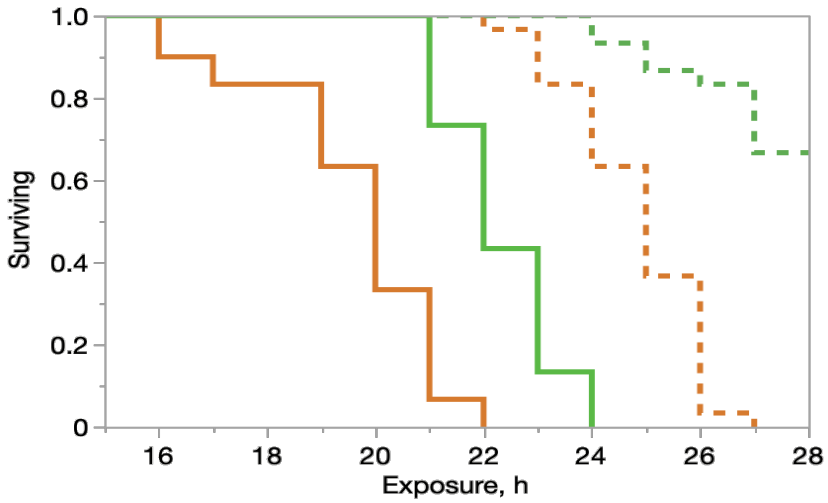


Figure. 1: Survival of *Daphnia* in severe acute hypoxia (1 mg O_2) for 28 hours from intermittent habitats (dotted lines) and permanent habitats (solid lines) exposed to normoxia (green) or chronic mild intermittent hypoxia (4 mg O_2 /L twice daily, orange). See Table 1 for survival analysis.

Table 1: Likelihood Ratio proportional hazards tests of the effects of habitat type, clones nested within habitats, chronic hypoxia, and their interactions on *Daphnia* acute hypoxia survival (Fig. 2).

Source	DF	Log Ratio χ^2	P-value
habitat	1	149.5	<0.0001
clone[habitat]	2	5.69	0.058
CMIH	1	66.7	<0.0001
CMIH *habitat	1	0.199	0.6553
CMIH *clone[habitat]	2	5.39	0.0674

Effects of Medium Ca2+ Content on Severe Hypoxia Survival

We tested the hypothesis that two commonly used Daphnia media, COMBO and ADaM affect Daphnia survival in acute hypoxia and that this difference is caused by different Ca2+ ions content (See Appendix Table 3). Indeed, *Daphnia* reared and tested in the COMBO medium (blue line) showed a significantly shorter survival time than those reared in either ADaM medium, or ADaM with lowered Ca2+ concentration, or in COMBO supplemented with Ca2+ (Fig. 2, Table 2). Ovaries/clutch status was a significant covariable with *Daphnia* carrying the youngest clutch showing the longest survival times and the ones with no clutch but fully developed ovaries showing the lowest survival times, along with the few individuals with the oldest clutches. There was also a significant difference among clones (Table 2), with the IL clone showing the longest survival times, as well as a slightly significant clone-by-medium interaction. However, the clone effect disappeared when body length was added as a covariable to the proportional hazards analysis (Appendix Table 4), making it impossible to untangle innate differences among clones from the direct effect of body size.

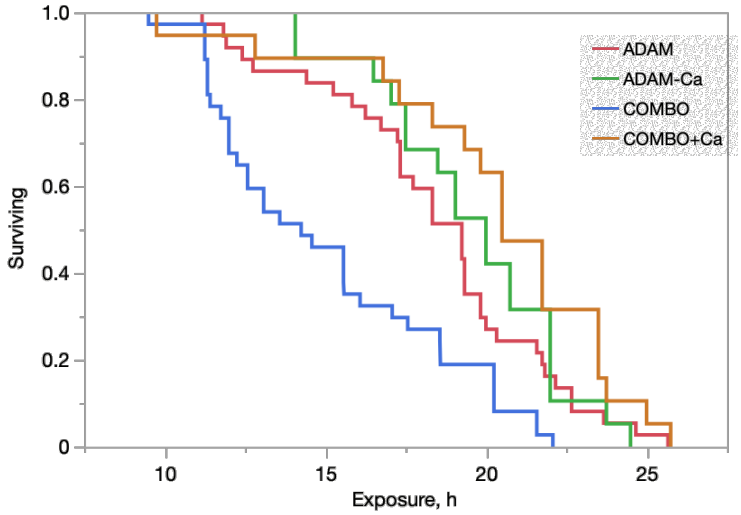


Figure 2: Acute hypoxia hourly survival of *Daphnia* in four mediums: ADaM (red), ADaM minus calcium (green), COMBO (blue), and COMBO with addition of Ca2+ (orange). See Table 2 for statistical details.

Table 2. Likelihood Ratio Tests (Proportional Hazards) of the effects of water medium with or without Ca2+ addition, clones, and ovaries status as a covariable on survival in acute hypoxia (Fig. 2). Only COMBO with no Ca2+ added was statistically different from the other mediums in the proportional hazards model (P<0.0001).

Source	DF	L-R Chi-square	Prob>ChiSq
Clone	3	11.451	0.0095
Medium	3	17.217	0.0006
Clone*Medium	9	17.355	0.045
ovaries cycle phase	4	15.105	0.0045

Transcriptional Response to Hypoxia in A Priori Hypoxia-Related Genes

Results of a priori hypoxia-related functions of interest show over-representation of several functionalities in the gene lists that showed up-regulation in some or all treatments. These include antioxidants pathways, oxygen transporters (hemoglobins), HiF and HiF prolyl-hydrolases, MAPK-related genes, and heat shock proteins (Table 3). Lac/Pyr metabolism and NFkB-related genes proteins do show slight over-representation only within clones from intermittent hypoxia habitats. On the other hand, we observed no over-representation of AMPK-related genes among genes responding to hypoxia (Table 3).

Table 3: Representation of a priori hypoxia-related functions of interest in gene lists with uncorrected p-values <0.01 for CMIH, ASH, and their interactions in the whole data and estimated for clones originated separately from intermittent and permanent habitats. Values are FDR-adjusted Fisher exact test p-values of significant over-representation (bold: FDR<0.01).

Function of Interest	N in ref.	ASH	CMH	ASHxCMIH	ASH, habitat = intermittent	ASH, habitat = permanent	CMIH, habitat = intermittent	CMIH, habitat = permanent
Antioxidant pathways	33	**1.9E-08**	**7.0E-08**	**7.0E-08**	**6.8E-05**	**1.9E-06**	**0.0090**	**0.0006**
Hemoglobins and cytoglobins	9	**5.9E-08**	0.0756	0.0756	**1.2E-08**	**1.9E-06**	**2.0E-05**	0.0746
Respiration*	212	**8.9E-06**	n.s.	0.0617	**0.0050**	**0.0037**	n.s.	n.s.
HiF and HiF hydrolases	5	**0.0023**	0.0756	n.s.	0.0618	**0.0014**	n.s.	0.0672
MAPK-related	5	**0.0029**	n.s.	0.0567	n.s.	**0.0018**	n.s.	0.0896
Heat shock proteins	25	0.0260	n.s.	n.s.	n.s.	n.s.	**0.0072**	n.s.
Lac/Pyr metabolism	9	n.s.	n.s.	n.s.	0.0895	n.s.	n.s.	n.s.
NFkB-related	7	n.s.	n.s.	n.s.	0.0959	n.s.	n.s.	n.s.
TNF-related	5	n.s.	n.s.	0.0756	n.s.	n.s.	**0.0007**	n.s.
AMPK-related	2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

*Including membrane phosphorylation and ATP synthesis

Gluconeogenesis and Calcium Metabolism Genes

We tested the hypothesis that genes involved in GNG would respond to acute hypoxia stronger than expected by chance alone. The results for GNG are shown in (Table 4). Seven out of 27 transcripts with gluconeogenesis functionality based on b2g and/or PANTHER annotations show significant FDR, with three of these seven transcripts being specific to the GNG pathway (as opposed to glycolysis pathway; Figure 4). Among these three, at least one, the cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C) is known to be rate-limiting for the GNG pathway. All 27 GNG related transcripts are listed by FDR in Appendix Table 1).

For calcium metabolism, in contrast, the only protein that showed significant FDR was Aralar1, a calcium-dependent mitochondrial carrier also implicated in GNG metabolism (Fig. 3).

Table 4: Representation of Gluconeogenesis GO list by either blast2go (Götz et al. 2008) or PANTHER (Mi et al. 2013). Transcripts that show significant FDR in the log-ratio test are listed all gluconeogenesis related transcripts are listed in Appendix 1)

Blast2go or PANTHER description	FDR_LRT
Phosphoenolpyruvate carboxykinase, cytosolic	3.49E-11
Calcium-binding mitochondrial carrier protein Aralar1	1.85E-11
Sphingosine kinase 2-like	6.49E-08
Lactate dehydrogenase A	1.02E-05
Pyruvate kinase	1.64E-03
Fructose-1,6-bisphosphatase 1	2.93E-03
Pyruvate carboxylase	0.05
Pyruvate dehydrogenase (acetyl-transferring) kinase, mitochondrial	0.12

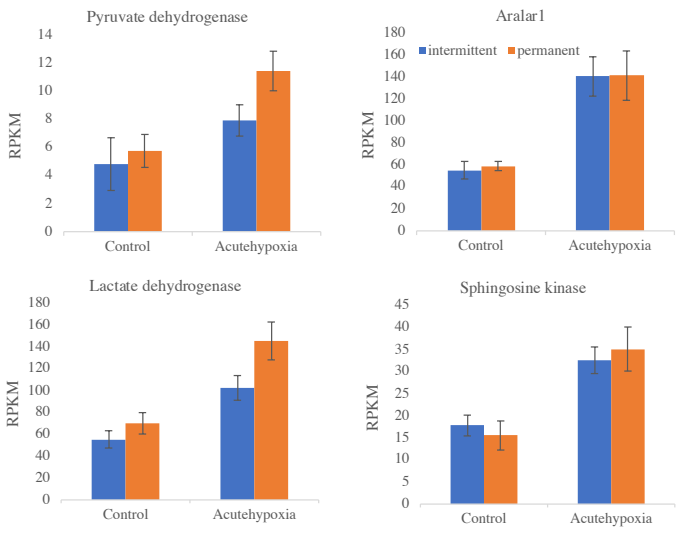
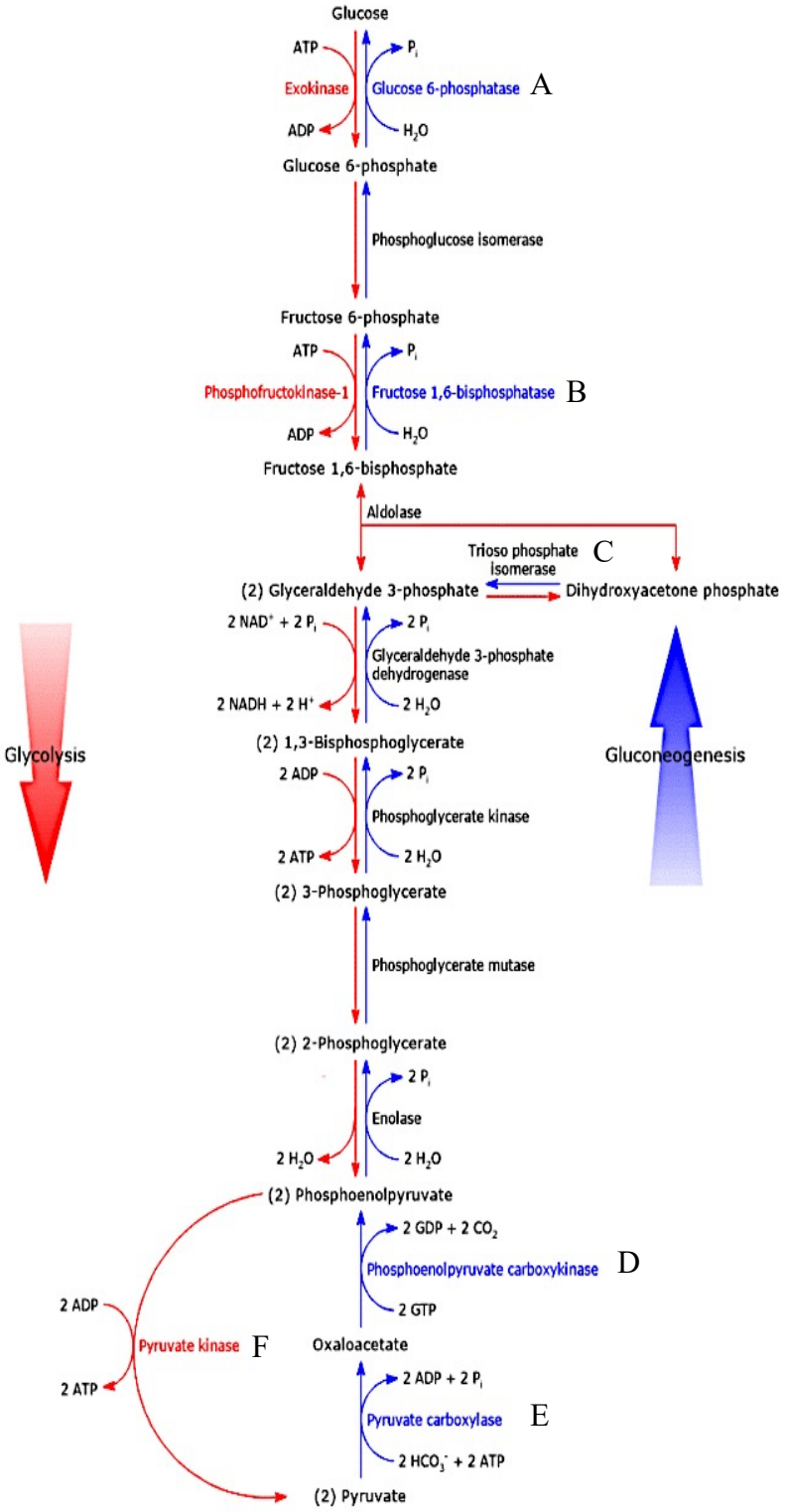
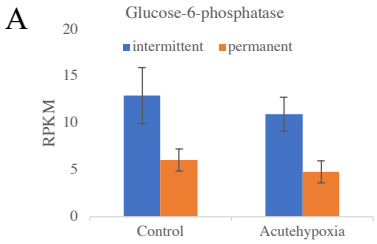
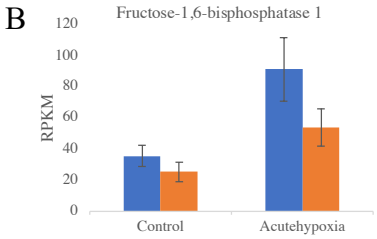


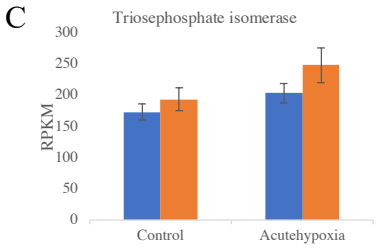
Figure 3: RPKMs of GNG-related proteins, which includes Aralar1, by control and acute hypoxia with intermittent (blue) and permanent (orange) clone types not shown on Fig x

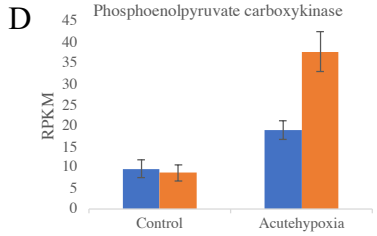
Figure 4: Gluconeogenesis/glycolysis pathways (GNG in blue letters), and RPKMs of enzymes identified in GNG GO, by control and acute hypoxia, with intermittent (blue) and permanent (orange) clone types. Pathway steps labeled A-F corresponding to RPKM bar graphs labeled A-F respectfully.

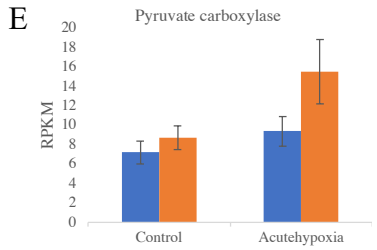


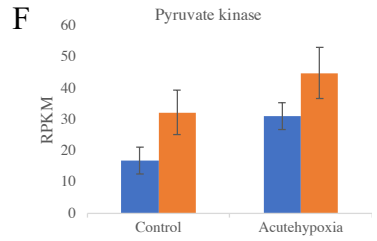












Response of Lactate and Pyruvate to Severe Hypoxia

Acute hypoxia increased the lac/pyr ratio across the four clones compared to control (Table 5 A, Fig. 5 A). This increase in lac/pry ratio was due to the increased response of lactate mM per mg of protein across all clones similarly (Table 5 B, Fig. 5 B). On the other hand, the response to pyruvate did not significantly change across treatments (Table 5 C, Fig. 5 C). There were clonal differences observed in the lac/pyr ratios and pyruvate measurements (Table 5 A and C). This can be explained by the IL clone having a lower pyruvate amount in both the control and acute hypoxia treatments (Fig. 5 C), thus resulting in a high lac/pyr ratio in response to acute hypoxia (Fig. 5 A).

Table 5: ANOVAs of the effects of clones, chronic hypoxia (cHyp), acute hypoxia (aHyp) and their interactions on lactate/pyruvate ratio (A) and protein content adjusted lactate (B) and pyruvate (C) concentrations.

A

Response Lac/Pyr				
Source	DF	Sum of Squares	F Ratio	Prob > F
Clone	3	13768.69	8.18	**0.0002**
cHYP	1	1320.84	2.35	0.13
Clone*cHYP	3	88.91	0.05	0.98
aHyp	1	16998.34	30.29	**<.0001**
Clone*aHyp	3	2018.44	1.2	0.32
cHYP*aHyp	1	0.02	0	0.996
Clone*cHYP*aHyp	3	1979.72	1.18	0.33
Error	48	26940.98		

B

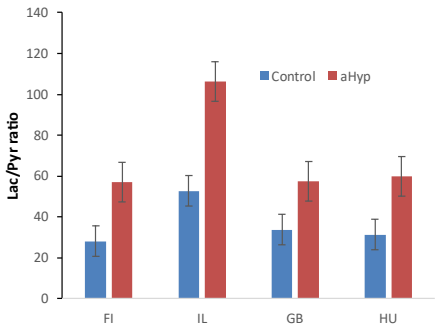
Response Lac, mM/mg Protein				
Source	DF	Sum of Squares	F Ratio	Prob > F
Clone	3	308.57	0.35	0.79
cHYP	1	721.85	2.42	0.13
Clone*cHYP	3	558.23	0.62	0.6
aHyp	1	11460.41	38.44	**<.0001**
Clone*aHyp	3	265.58	0.3	0.83
cHYP*aHyp	1	6.72	0.02	0.88
Clone*cHYP*aHyp	3	145.02	0.16	0.92
Error	48	14308.8		

C

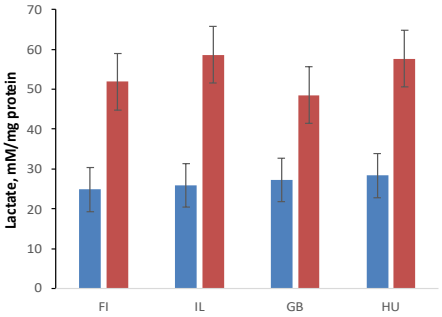
Response Pyr, mM/mg Protein				
Source	DF	Sum of Squares	F Ratio	Prob > F
Clone	3	1.5	5.01	**0.0042**
cHYP	1	0	0.01	0.91
Clone*cHYP	3	0.22	0.74	0.53
aHyp	1	0.06	0.56	0.46
Clone*aHyp	3	0.02	0.05	0.98
cHYP*aHyp	1	0.05	0.51	0.48
Clone*cHYP*aHyp	3	0.03	0.1	0.96
Error	48	4.78		

Figure 5: Lactate/pyruvate ratio (A) and protein content adjusted lactate (B) and pyruvate (C) concentrations in four clones in control (normoxia, blue) and acute hypoxia (red).

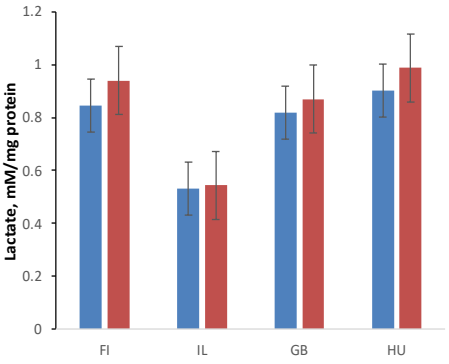
A



B



C



Discussion

We report the severe hypoxia survival of *Daphnia magna* originating from bodies of water identified as being either intermittent habitats, prone to experiencing low oxygenation (tolerant), or permanent habitats, not prone. Clones from intermittent habitats survived better in acute hypoxia than those from permanent ones. This is not surprising given that local adaptations to hypoxia-prone habitats are common and necessary for these clones' survival. What was surprising was the lack of a protective effect from the chronic mild intermittent hypoxia (CMIH) treatment, the hypothesis being that long-term adjustment of metabolic and oxygen provisioning could lead to better survival. In fact, we see the opposite: CMIH reduces *Daphnia* survival in both intermittent and permanent habitats (Fig.1). CMIH could thus be a constant stressful state for Daphnia, diminishing their ability to make the quick adjustments required to survive in severe hypoxia.

To elucidate the genetic basis of such differences in survival between clone types, we ran a transcriptomic analysis focusing on genes a-priori identified as being hypoxia/HIF related. None of the a-priori expected gene pathways showed any differences between clone types in the enrichment analysis. Indeed, antioxidant pathways, oxygen transport and storage, and respiratory and oxidative phosphorylation pathways responded just as strongly in clones from permanent habitats as in those from intermittent ones. However, HiF-and MAPK-related transcripts were up-regulated more in the less tolerant, permanent habitat clones, which could be indicative of greater damage and stress within such clones, concordant with their lower survival. Almost the only pathway that showed any stronger enrichment in the acute severe hypoxia (ASH)-tolerant clones was the Lac/Pyr metabolism (Table 3), consistent with the shifts in Lac/Pyr metabolism transcripts being adaptive. Therefore, additional analysis was performed to investigate Lac/Pyr metabolism by analyzing gene ontologies related to Lac/Pyr metabolism such as gluconeogenesis (GNG) and via Lac/Pyr analysis.

Severe hypoxia increased the Lac/Pyr ratios for each clone. Such increases in ratios are only due to significant increases in lactate amounts in response to ASH. While it is difficult to draw conclusions based on a small number of clones, we did observe the highest degree of changes in Lac and Pyr concentrations in the most severe hypoxia tolerant clone, namely the IL clone. IL showed the highest increase in Lac/Pyr ratio in severe hypoxia, as well as overall the lowest levels

of Pyr before and after ASH (Table 5, Fig. 5). Whether lower Pyr amounts overall and a higher Lac/Pyr ratio response led to better tolerance is an open question and should not be overlooked.

Individual transcripts involved in gluconeogenesis are significantly up-regulated in ASH, including the GNG-specific rate-limiting enzyme pyruvate carboxylase, PEPCK, fructose-1,6-biphosphotase (FBPase), and triosephosphate isomerase. In contrast, glucose-6-phospatase (G6Pase) shows some down-regulation in ASH. Of the up-regulated gluconeogenesis transcripts, one, namely FBPase, shows up-regulation consistent with the tolerant protective effect (stronger in clones from intermittent habitats, Fig. 4). Why is it that severe hypoxia reverses glycolysis by up-regulating GNG? Our experimental approach for determining tolerance was by observing daphnids' ability to remain swimming. At the cessation of swimming, daphnids will fall in the water column and may never recover in their natural habitats. Therefore, it is critical for *Daphnia* to keep moving, so GNG may serve to generate glucose, even if at an extra energic cost, to supply critical tissues, like the heart and muscles, with fuel for continued anaerobic respiration (glycolysis/Cori cycle). Additionally, considering the opposing directions of FBPase and G6Pase changes, perhaps the purpose is also to increase the concentration of either fructose-1,6-phosphate, or glucose-6-phosphate. One possible reason for this is the regeneration of NADPH in the oxidative stage of the pentose-phosphate pathway. NADPH is crucial for the hypoxia response as the activity of NADPH oxidases contributes to HiF-mediated responses (Nanduri et al., 2015).

FBPase and fructose-1,6-biphosphate are both regulated by hypoxia and may indicate protective effects and flexibility in some aquatic organisms (Cota-Ruiz et al., 2016; Crocker et al., 2013) or it may be reduced, indicating an inhibition of GNG, at least in animal livers (Gupta et al., 2021). Additionally, fructose phosphates control the transition from aerobiosis to hypoxia, in addition to the PH dependent activity of fructose 2,6-bisphosphate when in hypoxia (Ibarguren et al., 2000). FBPase also plays an important hypoxia-related role in cancer and cancer progression. While it is up-regulated in at least some cancers (Duda et al., 2020), it is down in many others (Fan et al., 2020; Li et al., 2014; Shi et al., 2017), indicating its function in hypoxia and its complex and often ambivalent role in health.

Calcium has significant effects on aquatic organisms, especially *Daphnia*. Here we examined two commonly used *Daphnia* media, ADaM and COMBO, the former having the least amount of Ca concentration. Severe hypoxia survival is dependent on Ca2+ availability, with longer survival dependent on Ca2+ being more than 0.1 uM of ions, as COMBO had the worst

survival (Appendix Table 3, Fig. 2). This was expected since Ca correlates positively with Daphnia abundance, with their survival threshold ranging between 0.1 and 0.5 mg Ca L^{-1} (Hessen et al., 2000; Tan & Wang, 2009). However, no transcripts annotated as being Ca2+-related show any significant up- or down-regulation in severe hypoxia. This is somewhat intriguing; another variable that effects survival positivity but does not translate to one specific gene or group being activated outright. However, it is important to note that the severe hypoxia survival and RNA-seq experiments were done only in COMBO, thus an additional RNA-seq experiment on ADaM should be explored to better understand the transcriptomic basis of Ca availability and severe hypoxia survival. Finally, Ca could simply assist in their general fitness and longevity. Fitter daphnia will fare better in hypoxia; thus, we propose the abandonment of COMBO as one of the de-facto *Daphnia* media in favor of ADaM.

Conclusion

Severe hypoxia survival within *Daphnia magna* shows genotype-by-environment interactions. Severe hypoxia tolerant clones from bodies of water prone to hypoxia have the longest survival than non-tolerant clones. Transcriptomic analysis reveals gluconeogenesis pathway enzymes are up-regulated in both tolerant and non-tolerant clones. Although fructose-1,6-biphosphotase does show stronger up-regulation in tolerant clones, suggesting its possible protective effects, it is the only one indicative of an adaptive hypoxia-mediated response. Additionally, the most tolerant clone, IL, shows the largest response in the lactate and pyruvate ratio, largely affected by the low pyruvate concentration in tissues. Contrary to predictions, prior exposure to mild intermittent hypoxia does not result in major transcriptome changes or increased survival in severe hypoxia. Finally, while calcium concentrations beyond the threshold of more than 0.1 uM of Ca2+ ions positively affect severe hypoxia survival, none of the transcripts annotated to calcium metabolism or signaling-related showed any significant differential expression in response to severe hypoxia. This indicates that while Ca2+ ions have protective effects in severe hypoxia, the lack of Ca2+ in COMBO does not equate to any clear transcriptional enrichment of a-priori Ca2+ related genes.

Appendix

Appendix 1. Blast2go or PTHR description of GNG or Ca2+ GO transcripts by FDR, with RPKM values of control and acute hypoxia treatments.

Blast2go or PTHR - description	Habitat	FDR_LRT	SE RPKM, Control	SE RPKM, AcuteExp1mg/L
Calcium-binding mitochondrial carrier protein Aralar1	intermittent	1.85E-11	8.183819022	17.86649958
Calcium-binding mitochondrial carrier protein Aralar1	permanent	1.85E-11	4.17437939	22.53892225
Phosphoenolpyruvate carboxykinase, cytosolic	intermittent	3.49E-11	2.095605735	2.211350056
Phosphoenolpyruvate carboxykinase, cytosolic	permanent	3.49E-11	1.9452858	4.872726522
Sphingosine kinase 2-like	intermittent	6.49E-08	2.37159194	3.062981631
Sphingosine kinase 2-like	permanent	6.49E-08	3.356395624	5.031741733
Lactate dehydrogenase A	intermittent	1.02151E-05	8.068148533	11.21139059
Lactate dehydrogenase A	permanent	1.02151E-05	9.991017137	17.27375608
Pyruvate kinase	intermittent	0.001643803	4.2527221	4.362166459
Pyruvate kinase	permanent	0.001643803	7.106293889	8.204172564
Fructose-1,6-bisphosphatase 1	intermittent	0.002932319	6.861865582	20.34442581
Fructose-1,6-bisphosphatase 1	permanent	0.002932319	6.257891737	12.05885644
Pyruvate carboxylase	intermittent	0.046273899	1.159095704	1.50919589
Pyruvate carboxylase	permanent	0.046273899	1.21253627	3.310771141
pyruvate dehydrogenase (acetyl-transferring) kinase, mitochondrial	intermittent	0.11581624	1.876238683	1.102043205
pyruvate dehydrogenase (acetyl-transferring) kinase, mitochondrial	permanent	0.11581624	1.180407925	1.418391455
Triosephosphate isomerase B	intermittent	0.172136537	12.93276645	15.90999682
Triosephosphate isomerase B	permanent	0.172136537	18.64066376	27.95482507

Appendix Table 2. Geographic origins *Daphnia* clones

Clone ID	Location	Habitat
IL-MI-8	Jerusalem	Intermittent Mediterranean pond
FI-FSPI-16-2	Suur-Pellinki, Finland	Intermittent summer rock pool
GB-EL75-69	London, UK	Permanent pond
HU-K-6	Hungary	Permanent lake

Appendix Table 3. Ca2+ and Mg++ concentrations of the four media types

Media types	uM of ions:	
	Ca2+	Mg++
ADaM	0.6	0.13
ADaM - Ca2+	0.1	0.13
COMBO	0.07	0.15
COMBO + Ca2+	0.5	0.15

Appendix Table 4. Likelihood Ratio Proportional hazards test of the effects of water medium with or without Ca2+ addition, clones, and ovaries status and body length as covariables on survival in acute hypoxia

Source	DF	L-R ChiSquare	Prob>ChiSq
Clone	3	2.874	0.4115
Treatment	3	15.996	0.0011
Clone*Treatment	9	17.948	0.0358
stage	4	13.729	0.0082
L, mm	1	2.059	0.1513

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