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Metabolic Phenotype of *Daphnia* Under Hypoxia: Macroevolution, Microevolution, and Phenotypic Plasticity

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Daphnia is a freshwater crustacean that is able to upregulate hemoglobin (Hb) in response to hypoxia, imparting a red color. We combine multiple field surveys across season with a lab experiment to evaluate changes in the metabolic phenotype of *Daphnia* in relation to environmental hypoxia. Looking at the zooplankton community, we found that *D. pulicaria* was restricted to lakes with a hypoxic hypolimnion. Comparing *D. pulicaria* with different amounts of Hb, red animals showed higher mRNA levels for several Hb genes, whereas most glycolytic genes showed red/pale differences of less than 50%. We also observed seasonal changes in the metabolic phenotype that differed between red and pale animals. Hb was upregulated early in the season in hypoxic lakes, and a relationship between Hb and lactate dehydrogenase only emerged later in the season in a temporal pattern that was lake specific. To evaluate whether these differences were due to specific lake environments or microevolutionary differences, we tested the induction of genes under controlled hypoxia in isofemale lines from each of four lakes. We found a strong response to 18 h hypoxia exposure in both Hb and lactate dehydrogenase mRNA, although the magnitude of the acute response was greater than the steady state differences in mRNA levels between pale and red *Daphnia*. The baseline expression of Hb and lactate dehydrogenase also varied between isofemale lines with different lake origins. These results, in combination with comparison of glycogen measurements, suggests that Hb functions primarily to facilitate oxygen delivery, mitigating systemic hypoxia, rather than an oxygen store. The combination of lab and field studies suggest that the metabolic phenotype of the animal is influenced by both microevolutionary differences (within and between lakes) as well as the spatial and temporal environmental heterogeneity of the lakes. The differences between *Daphnia* species, and the unexpected lack of hypoxia sensitivity of select glycolytic genes provide evidence of macroevolutionary differences in metabolic strategies to cope with hypoxia.

Keywords: anaerobic metabolism, transcriptome, gene-by-environment, microevolution, phenotypic plasticity, hemoglobin, lactate dehydogenase

INTRODUCTION

Clonal aquatic invertebrates, such as *Daphnia*, are helpful for disentangling genetic variation vs. phenotypic responses to complex natural environmental stress, which often include rapid evolution, phenotypic plasticity, and evolution of plasticity (Stoks et al., 2016). Since *Daphnia* has a well annotated genome (Colbourne et al., 2011), researchers have been able to identify the genetic basis for multiple phenotypic responses (e.g., Lyu et al., 2019; Frisch et al., 2020; Tams et al., 2020) making it a powerful model system to study the associations between genetics, molecular physiology, and the ecological phenotype (Miner et al., 2012).

Oxygen levels can vary tremendously over space and time in aquatic environments, and aquatic invertebrates often move across these gradients to utilize resources and avoid fish predation (Hanazato et al., 1989; Hembre and Megard, 2003). As such, aquatic invertebrates often experience periods of time under oxygen stress (Galic et al., 2019). To tolerate hypoxia, organisms remodel their physiology. The phenotypic remodeling to hypoxia often includes improved oxygen delivery (extraction, perfusion, and storage) and a greater capacity for anaerobic metabolism (substrate supply, glycolytic enzyme activities, and handling of end products). In vertebrates, oxygen delivery, including extraction, perfusion, and storage of oxygen, can be improved by increasing vascularization (via angiogenesis) and erythrocyte numbers (via erythropoiesis) (reviewed by Scott et al., 2015). Oxygen limitation can also lead to a metabolic shift from oxygen-dependent metabolism (oxidative phosphorylation) to anaerobic metabolism (glycolysis).

Central to all these responses is the ability of metazoan cells to sense hypoxia to remodel tissues. The transcriptional master regulator hypoxia-inducible factor (HIF) is central to coordination of the phenotypic plasticity that bolsters hypoxic tolerance (Semenza et al., 1994; Semenza, 2012). While HIF is ubiquitous amongst animals, the HIF-related network differs between taxa. Two of the most important targets of the HIF network in vertebrates are the capillaries and erythrocytes (Scott et al., 2015). However, these targets are absent in many invertebrates. Although *Daphnia* lack blood cells, HIF stimulates Hb genes directly (Gorr et al., 2004), leading to secretion of Hb into the circulation (Fox and Phear, 1953; Green, 1956; Kobayashi and Hoshi, 1982; Zeis et al., 2004). This extracellular hemoglobin confers greater hypoxia tolerance to hypoxia (Fox et al., 1951; Kring and O'Brien, 1976; Pirow et al., 2001). The other metabolic component of a typical vertebrate HIF response is coordinated induction of genes encoding proteins that support anaerobic glycolysis (Webster, 1987; Webster et al., 1990; Discher et al., 1998). Although acute hypoxic exposure of *Daphnia* causes lactate accumulation (Paul et al., 1997, 1998), it is not yet known if hypoxia also triggers coordinated induction of glycolytic genes.

Here we combine analysis of field collected animals with a controlled experiment in the lab to evaluate changes in the metabolic phenotype of *Daphnia* across a range of oxygen environments, and to disentangle the relative contribution of evolutionary differences from environmental drivers in phenotypic plasticity. The patterns we observe suggest important distinctions between *Daphnia* and better studied models. From species- and lake-specific metabolic phenotype, to seasonal shifts in enzyme relationships, and incoordinate regulation of anerobic pathways, our results demonstrate that *Daphnia* is a dynamic metabolic system that has been shaped by the complexity of its oxygen environment in both space and time. Hypoxic episodes will be more frequent in lakes as climate change and temperature increases. Our results are important to help understand how species and populations may adapt to these future environmental changes.

MATERIALS AND METHODS

Field Sampling

Our study utilizes data from two observational surveys. The first was done in June and July 2018 and collected zooplankton from twenty lakes in the Frontenac Arch Biosphere, Ontario, Canada (**Supplementary Table 1**). These lakes were selected because they have a number of *Daphnia* species and a depth of at least 9 m, which permits thermal stratification. Ten of the lakes develop a hypoxic hypolimnion ($<$1 mg/mL O_2) and ten have a normoxic hypolimnion ($\geq$ 1 mg/mL O_2) (**Figure 1**). The second survey was focused on five lakes with a hypoxic hypolimnion where *D. pulicaria* was the dominant zooplankton species.

Zooplankton were collected during the day from the deep point of each lake using a large macrozooplankton net (50 μm mesh size, 35 cm opening diameter). *Daphnia* were collected using vertical hauls that sampled the entire water column. Quantification of proteins vs. RNA requires different approaches to sampling and preservation. *Daphnia* collected for protein analyses were brought back alive to Queen's University for sorting and identification (Haney et al., 2013), then frozen in liquid nitrogen before storage at −80°C until further analysis. *Daphnia* collected for RNA analyses were sorted in the field and either flash-frozen or immersed in RNAlater. Sample sizes are given in **Supplementary Table 2**.

Lake-Dependent Microevolution in Hypoxic Gene Induction

Animals were collected from the field in June 2019 to establish replicate isofemale lines from four focal lakes: Doe, Round, Long, and Black. Twenty individuals were placed in approximately 100 mL of water from the lake from which it was sampled. The animals were fed *ad libitum* by adding *Chlamydomonas reinhardtii* (CPCC 243; Canadian Phycological Culture Centre University of Waterloo) every second day. These *Daphnia* were reared to a third generation by isolating and transferring batches of no less than 5 offspring to 7 vials of approximately 50 mL of filtered (0.22 μm) Lake Ontario water each generation. After 3 generations, individuals were prepared for oxygen treatments.

The isofemale lines were used to explore the impact of lake origin of *Daphnia* on their hypoxic response. A total of 6 isofemale lines from each of the four lakes were subjected to a 18 h treatment of hypoxia or normoxia. We used 3% oxygen because this concentration was sufficient to induce Hb induction in a closely related species (*D. pulex*) in other studies

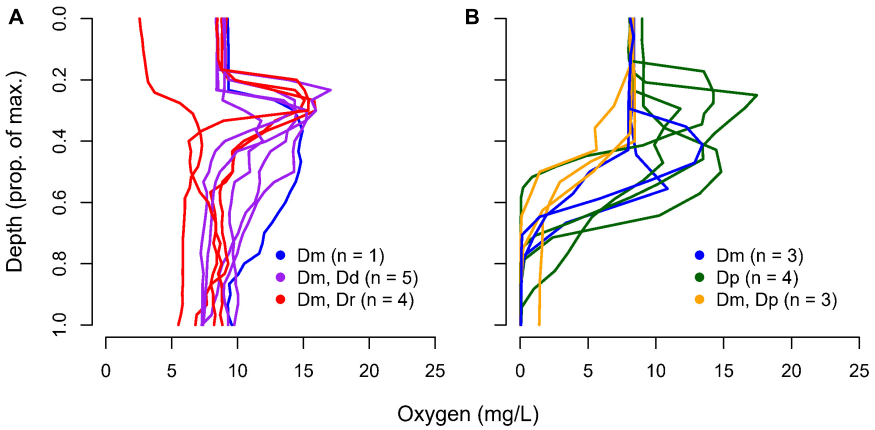

FIGURE 1 | Oxygen-depth profile and *Daphnia* species occurrence in 20 Frontenac Arch lakes. Lakes are divided into those with **(A)** normoxic or **(B)** hypoxic conditions at the bottom of the water column. Species found were *D. pulicaria* (Dp), *D. mendotae* (Dm), *D. dubia* (Dd), and *D. retrocurva* (Dr). The identity of the specific lakes with different *Daphnia* compositions can be found in **Supplementary Materials**.

(Zeis et al., 2003, 2009; Gerke et al., 2011). Animals were placed in 100 mL beakers with filtered (0.22 μm) Lake Ontario water, which was equilibrated at the desired oxygen pressure (3 or 21% O_2, balance nitrogen) for 24 h. Oxygen levels were set using an MCO-5M controlled environment chamber, thermostatted to 18°C (Sanyo Electric Biomedical Co., Japan). Individuals were preserved immediately after 18 h of treatment. For each replicate, 15 whole animals were stored in 1 mL of TRIzol (Thermo Fisher Scientific) and frozen at −80°C until assayed for the mRNA levels of lactate dehydrogenase (LDH), hemoglobin (Hb), pyruvate kinase (PK), and enolase (ENOL), as described in detail below.

Enzymes Analyses

Assays for enzymes and Hb were conducted on a Spectramax Plus plate spectrophotometer (Molecular Devices, United States) at 25°C. Animals used for enzyme analyses were individually placed in microcentrifuge tubes and frozen (−80°C) for storage. For enzyme analyses, animals were homogenized using a polypropylene disposable pestle in a 1.5 mL microcentrifuge tube with buffer composed of 20 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 1 mM EDTA (ethylene diamine tetraacetic acid), and 0.1% Triton X-100 (pH 7.4). Unless stated otherwise, the homogenate was centrifuged (10,000 × *g* for 5 min) then used for Hb, enzyme, and protein analyses. Centrifugation did not result in a measurable loss in activity of any enzyme, but removing particulates reduced light scatter. Enzyme assays were based on Davies et al. (2011).

For Hb quantification, extracts were transferred to a 96-well plate. Hb concentration was estimated using measurements at 560, 576, 600 nm (Yampolsky et al., 2014). The difference between A_{576} and the average of A_{560} and A_{600} was the Hb-specific absorbance. Without purified *Daphnia* Hb to use as a standard curve, we relied on bovine hemoglobin (64,500 g/mol) to approximate hemoglobin concentrations and expressed the values per nmol heme (4 mol heme per mol Hb). A 1 mg/mL solution of bovine crystalline hemoglobin (largely met-hemoglobin) was reduced by a 10-molar excess of ascorbic acid (27 μg per mL) for 6 h. Using 96-well plates, 0.2 mg Hb (= 12.4 nmol heme) resulted in an absorbance difference [A_{576} − (0.5 × (A_{560} + A_{600})] of 0.11 absorbance units. Hb levels were corrected for the fraction of sample analyzed and standardized per ng protein in the extract.

LDH catalyzes a reversible reaction of pyruvate + NADH + H^+ to lactate + NAD^+. Its activity was measured in 50 mM Hepes (pH 7.0) assay buffer with 0.2 mM NADH and 2.0 mM sodium pyruvate. NADH and pyruvate were prepared fresh every 4 h in assay buffer. The reaction rate was determined by the loss of NADH, which was measured at 340 nm over the linear phase, typically for 3 min. Early studies showed no indication of a decline in NADH absorbance in the absence of pyruvate, so this control was omitted.

PK catalyzes the irreversible transphosphorylation reaction of phospho-*enol*-pyruvate (PEP) + MgADP to MgATP + pyruvate. Its activity was assayed by coupling pyruvate production to LDH activity. PK was measured in assay buffer (50 mM Hepes (pH 7.4), 150 mM KCl and 5 mM $MgCl_2$) with 0.2 mM NADH, 0.5 mM PEP and 1 mM ADP. NADH and PEP were prepared fresh every 4 h in assay buffer. The reaction rate was determined by the loss of NADH, which was measured at 340 nm over the linear phase, typically 3 min. Early studies showed no indication of a decline in NADH in the absence of PEP, so this control was omitted.

ENOL catalyzes the conversion of 2-phosphoglycerate to PEP. The reaction (PEP production) was coupled to LDH and PK. The assay buffer contained 50 mM Hepes (pH 7.4), 50 mM KCl and 3 mM $MgCl_2$, with 0.2 mM NADH, 5 mM 2-phosphoglycerate, 5 mM ADP, 1 U/mL LDH, and 1 U/mL PK. As with the other enzymes, no activity was seen in the absence of the substrate, 2-phosphoglycerate. The reaction rate was determined by the loss of NADH, which was measured at 340 nm over the linear phase, typically 3 min.

Citrate synthase (CS) converts acetyl CoA + oxaloacetate to citrate + coenzyme A (CoASH). It was assayed in 50 mM Tris (pH 8.0), 0.12 mM acetyl CoA, and 0.5 mM oxaloacetate, with 0.1 mM DTNB (dithio-nitrobenzoic acid) included to detect

the production of CoASH, Preliminary studies showed that the rate in the absence of oxaloacetate (thiolase) was below detection limits, and thus this control was omitted in subsequent experiments. CS was detected by the increase in absorbance at 412 nm, and remained linear for at least 5 min.

Soluble protein in the homogenate was detected using Bio-Rad Protein Assay Dye Reagent (Bio-Rad), using bovine serum albumin as a standard, assayed 595 nm.

Glycogen Analysis

Animals were collected from the field and returned to Queen's University live. Since the delays between capture and sample preservation could impact glycogen levels, we returned animals to the lab and held them with abundant food and oxygen to permit them to replenish glycogen stores. Animals were separated into bins of red and pale and cultured for 3 days. Each vial contained 20 mL filtered Lake Ontario water and 6 individuals, supplemented daily with an *ad libitum* supply of *Chlamydamonas reinhardtii* (CPCC 243; Canadian Phycological Culture Centre for Algae, Cyanobacteria, and Lemna at the University of Waterloo). Individuals were harvested after 3 days of feeding and frozen at −80°C for glycogen measurements.

To measure glycogen, individuals were transferred to 200 μL of 3% perchloric acid (PCA) and homogenized using polypropylene pestle. Without centrifugation, 100 μL of the suspension were transferred to a new tube for glycogen determination and the remainder reserved for glucose measurements. To each tube, 100 μL of 2M sodium acetate (pH 4.8) and 50 μL 1M $KHCO_3$ were added. The glycogen tubes were also given 10 U amyloglucosidase, then incubated 2 h at 40°C. Both tubes were frozen at −80°C for subsequent glucose determinations. Samples were thawed, neutralized with 20 μL of 1 M KOH, then centrifuged 5 min at 10,000 × *g*. The supernatants were used for the glucose assay.

The glucose assay consisted of sample (unknown or standard) added to a solution of 1 mM ATP, 2 mM NAD^+, 1 U/mL hexokinase (HK), and 1 U/mL G6PDH (*Leuconostoc* glucose 6-phosphate dehydrogenase). The plate was incubated at room temperature for 60 min, at which point the reaction had reached its endpoint. Values of glycogen were translated back to levels per individual *Daphnia*. We could not measure protein directly in the acid-extracted *Daphnia*. Instead, 6 individual animals from each group (4 lakes, 2 color phenotypes) were used to determine protein per individual *Daphnia*, with the mean used to estimate glycogen per mg protein, which would correct for *Daphnia* of different sizes in the treatment groups.

Transcriptome Analyses

Thirty animals were collected from each of the four lakes and added directly to 8 mL of RNALater solution. Total RNA was extracted with TRIzol Reagent (Thermo Fisher) following the instruction. The RNA samples were then purified with NEBNext rRNA Depletion kit (NEB E6310). The libraries were constructed as per the manufacturer using the NEBNext Ultra II Directional RNA Library Prep kit for Illumina (NEB E7760) and the NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 1) (NEB E7335).

Sequencing was performed by The Centre for Applied Genomics (The Hospital for Sick Children, Toronto, Canada). The libraries were sequenced on a HighSeq 2500 using the single-end 150-bp mode. Following sequencing, data processing was done using the standard Illumina pipeline and Fastq files were generated for data processing and assembly.

Quantitative PCR

For RNA extraction, 500 μL of the TRIzol was discarded and the animals were homogenized in the remaining solution. RNA was then extracted following standard TRIzol protocol and checked and quantified based on 260/280 nm absorbance. The integrity of total RNA was assessed using gel electrophoresis. Total RNA was DNase-treated (DNA-free DNA Removal Kit, Thermo Fisher Scientific) and reverse transcription of 2 μg of total RNA was performed using OneScript cDNA Synthesis Kit (ABM). The cDNA was diluted to 20 ng/μL for qPCR and stored at −20°C.

Primers for qPCR (**Supplementary Table 3**) were based on sequences derived from the RNA-seq data and verified for use in qPCR. We identified three genes of interest based on the results of RNA-seq: *LDHA* and two Hb subunits selected based on high levels of mRNA and responsiveness to oxygen. We also measured mRNA levels for PK and ENOL to test for their responsiveness to hypoxia. PCR reactions were optimized for temperature and a dilution series was performed to verify amplification efficiencies. For qPCR reactions, 5 μl of diluted cDNA was mixed with 0.5 μl each of forward and reverse primers (10 μM), 4 μl of water, and 10 μl of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad). qPCR reactions were performed using a CFX96 (Bio-Rad) under the following conditions: 30 s at 95°C; 40 cycles of 95°C for 10 s, 55°C for 45 s, with a melt curve from 65°C to 95°C at 0.5°C increments for 5 s each. Each biological replicate was assayed in duplicate. No signal was detected in no template controls samples. The expression levels of each gene of interest were normalized by the expression of a reference gene (α -tubulin).

Data Analysis

Sequence Alignment and Annotation

FastQC (Andrews, 2010) was used to evaluate the RNA sequencing quality and Trimmomatic (Bolger et al., 2014) was used to trim low quality reads with a phred score < 20, within a window of 4 base pairs. The reads after trimming with length < 100 were removed from downstream analysis. The remaining high-quality reads were aligned to a *Daphnia pulex* reference genome (Gilbert, 2009)[1] using Bowtie (Langmead, 2010) with default parameters. Transcript expression data were obtained at both the gene and isoform level using RSEM (Li and Dewey, 2011). Expression level estimation was generated as fragments per kilobase of transcript sequence per million mapped fragments (FPKM) value. A small value (0.001) was added to the gene expression for the genes with expression value of 0 to statistically evaluate the data. PCA and cluster analysis were performed to identify outlier samples and no outliers were detected.

[1]http://arthropods.eugenes.org/EvidentialGene/daphnia/daphnia_pulex/daphnia_pulex_genes2017/genes/

Statistical Analyses

Normality of the data was tested by Shapiro-Wilk test. For each analysis, we compared AICc of models containing independent variables and that of a null model containing no independent variable (Burnham et al., 2011). If the model with the lowest AICc contains any independent variable, we followed with *post hoc* Tukey's all-pair comparisons test using the *glht* function (Hothorn et al., 2008) with Benjamini-Hochberg adjustments for multiple tests.

First, linear mixed models (*lme4*; Bates et al., 2015) were used to test for the main effect of species on LDH and PK activity, while accounting for the random effects of lake origins of the *Daphnia*. *D. mentodae* were separated into two groups based on whether the animals were collected from normoxic or hypoxic lakes. Second, linear models were used to test for the main effects of lake and phenotype on enzymatic activity (ENOL, PK, and LDH), glycogen, and Hb levels. Third, an analysis of covariance test (ANCOVA) was used to see if Hb influenced LDH activity with a lake or seasonal effect. The response variable was LDH activity. The categorical variables were lake and season, with Hb levels as a covariate.

Finally, we considered two response variables: baseline and response, when evaluating gene expression data in the lab experiment. The gene expression under normoxic treatments is considered "baseline," and the ratio of hypoxic/normoxic expression is considered "response," We used linear models to test for the effects of lake and line on the two response variables for each gene of interest.

All statistical analyses and graphs were done using R version 3.4.2.

RESULTS

Metabolic Phenotypes of *Daphnia* Amongst 20 Lakes

We collected *Daphnia* from 20 lakes in the region in June and July 2018, with the goal of surveying enzyme activities across lakes and *Daphnia* species (**Figure 1**). The early sampling was intended to target animals before seasonal accumulation of Hb occurred, although there were a few animals that appeared to be pink, suggesting low levels of Hb even early in the season. *D. dubia* and *D. retrocurva* were found only in lakes that possessed a normoxic hypolimnion. *D. mendotae* populations were found across both lake categories, though not all lakes. *D. pulicaria* were not found in lakes that possessed a normoxic hypolimnion and appeared in all but 2 lakes with a hypoxic hypolimnion.

To understand the metabolic phenotype of these animals, we measured enzyme activities in pools of individuals separated by species. The data were expressed relative to CS activity, which was found to not differ among lakes or species (**Supplementary Figure 1**). The enzyme data were separated by lake type (normoxic vs. hypoxic hypolimnion) to evaluate whether the LDH and PK activities differed between lake types across the four species (**Figure 2**). The main effect of species was not significant on LDH activity (**Figures 2A,B** and **Supplementary Table 4**). However, *D. mendotae*, which was found in both categories of lake, had a trend of showing higher LDH activities in lakes with a hypoxic hypolimnion (t = 1.91, df = 56.02, p = 0.06; **Figures 2A,B**). In contrast, the main effect of species was significant on PK activity (**Figures 2C,D** and **Supplementary**

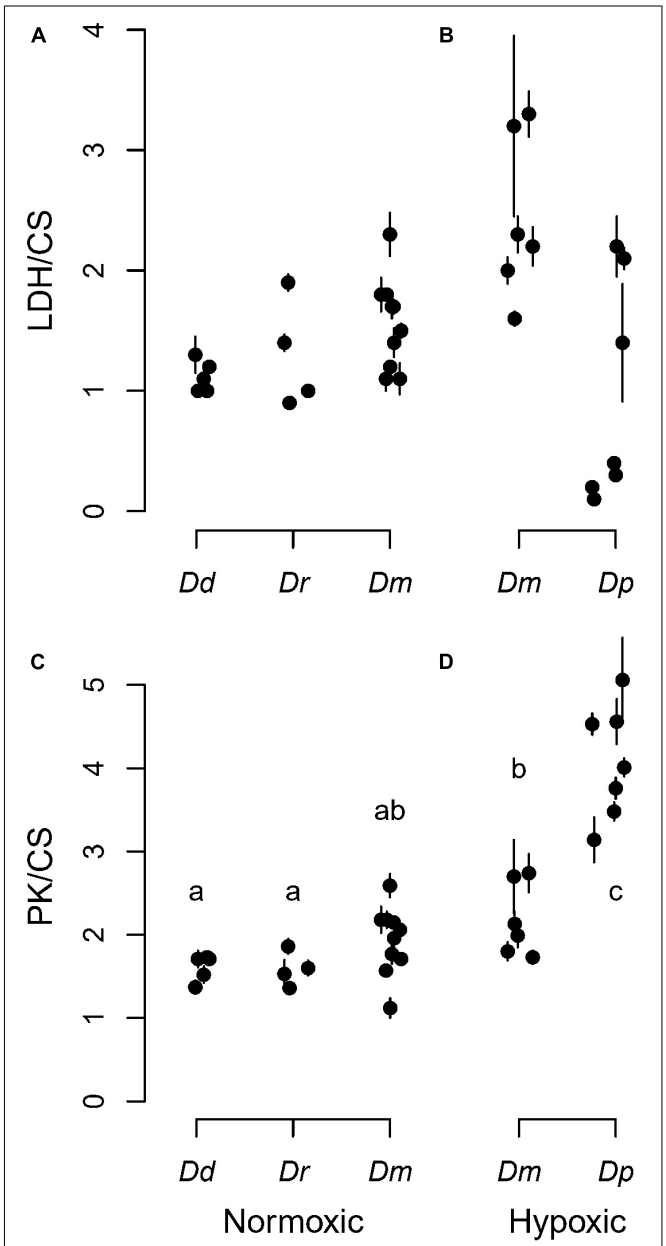

FIGURE 2 | Glycolytic enzyme activities across species and lake type. The top row shows lactate dehydrogenase (LDH) relative to the housekeeping gene citrate synthase (CS) for each **(A)** normoxic or **(B)** hypoxic lake. The bottom row shows pyruvate kinase (PK) relative to the housekeeping gene citrate synthase (CS) for each **(C)** normoxic or **(D)** hypoxic lake. Solid circles are individual lakes, and vertical bars are ± SD from six replicates. *Daphnia* species are identified in **Figure 1**. Data points are jittered horizontally to avoid visual overlap within a lake category. Different letters indicate significant differences ($p < 0.05$).

Table 4). *D. mendotae* that were found in hypoxic lakes had greater PK activity than *Daphnia* found in normoxic lakes (**Figures 2C,D** and **Supplementary Table 5**). *D. pulicaria*, only found in lakes with a hypoxic hypolimnion, showed PK activities that were higher than other species (**Figure 2D** and **Supplementary Table 5**). They also demonstrated higher among-lake variation in LDH levels compared to the other species (**Figure 2B**). Although no red animals were found during this sampling period, there were some animals that were pink, suggesting some history of hypoxic exposure that may have influenced glycolytic enzyme levels.

Exploring Glycolytic Enzyme Stoichiometries

To better understand the relationship between glycolytic enzymes and Hb expression, we conducted a separate study that focused on *D. pulicaria* in five hypoxic lakes (Black, Doe, Long, Round, South Otter). We collected samples of *D. pulicaria* with the goal of measuring the activities of select glycolytic enzymes to assess if they differed between red and pale, and whether the differences were stoichiometric amongst glycolytic enzymes.

Daphnia were collected in Fall 2017, at which point the differences between pale and red are typically greatest. Of the glycolytic enzymes we measured, ENOL was the least variable in *D. pulicaria* within and between lakes (**Figure 3A**). The greatest difference in ENOL activity between pale and red animals was in Round and South Otter Lakes, where reds had about 50% higher activity than pales. Although both LDH (**Figure 3B**) and PK (**Figure 3C**) showed a significant interaction between phenotype and lake, the interactive effect was stronger for LDH than PK (**Supplementary Tables 6**, **7**). Reds had 2–2.5 times greater LDH activity than pales in Black and Round Lakes, but activities were similar between red and pale animals in Doe and Long Lakes (**Figure 3C** and **Supplementary Table 9**). Thus, red *D. pulicaria* in Black and Round Lakes showed the clearest evidence of metabolic remodeling in these enzymes, whereas little metabolic differentiation was seen in red animals from Doe and Long Lakes.

To evaluate whether glycogen storage is used as a strategy to tolerate periods of hypoxia, we measured glycogen levels in animals with different color phenotype across the lakes. Animals might accumulate glycogen to high levels when food was available to prepare for hypoxia. However, we saw no significant difference between reds and pales in 3 of 4 lakes, while red animals from Black Lake had significantly lower glycogen levels (t = 4.34, df = 5.94, p = 0.005) than pale animals from the same lake (**Figure 3D**). Thus, animals with an established red phenotype were not predisposed to storing more glycogen than pale animals, when resources were plentiful.

Transcript Analysis

In Fall 2017, we collected animals to investigate differences in transcriptomic profiles using RNA-seq (**Supplementary Table 11**). These samples were collected in parallel with the animals used for **Figures 3A–C**. Among the 33,069 transcripts in the assembly (Gilbert, 2009), 14,355 transcripts were detected in all samples. Global transcript patterns (**Supplementary Figure 2**) clustered by lake rather than by color morphs, although Long Lake red animals showed a more unique transcriptional profile (i.e., did not cluster with any other group) compared to those from other lakes. Thus, the overall transcriptional profiles were more dependent on lake-by-lake differences rather than the effects of oxygen exposure history, inferred from color.

Focusing on transcripts of the enzymes associated with carbohydrate utilization, we found that pale animals generally had similar mRNA levels for each gene (**Figure 4**). The differences between colors were negligible for most of the glycolytic genes. Only phosphoglucomutase, glycogen phosphorylase and LDH showed elevations in reds more than 50% the values seen in pales, driven largely by elevations in Black and Round Lakes. Comparing LDH mRNA (**Figure 4**) and enzyme activities (**Figure 3C**), red animals in Black Lake had 2.9-fold more mRNA and twofold higher enzyme activity than pales from the same lake, while more modest 1.3-fold elevation in mRNA seen in Doe Lake was consistent with the 1.3-fold difference in enzyme activities. Interestingly, red animals in Long Lake had a relatively modest 1.6-fold increase in enzyme activity from pale animals but the second greatest difference in LDH mRNA (1.9-fold), whereas reds in Round Lake had the greatest increase in LDH activity from pales by 2.6-fold but only 1.5-fold more LDH mRNA.

Hb paralogs were annotated based on a previously published database (Gilbert, 2009). We focused on 10 paralogs among 23 that were expressed at the highest levels (**Figure 5A**). There were few patterns that were uniform across lakes, with a fair bit of heterogeneity in the nature of the patterns within color morphs. Some paralogs showed consistent patterns between lakes. For example, the mRNA for Daplx7pEVm013242 was present at similar levels in all lakes and both color morphs. However, Daplx7pEVm058987, Daplx7pEVm013392, Daplx7pEVm011494, Daplx7pEVm013338, and Daplx7pEV m012731 were all highly variable between lakes.

The patterns also differed lake by lake. When the mRNA levels were depicted as the ratio of red/pale for each paralog in all lakes, clearer patterns emerged (**Figure 5B**). Hb paralogs were differentially expressed the least between pales and reds of Doe Lake (∼2-fold), followed by Black Lake (∼4-fold). However, in Round and Long Lakes, the mRNA for Daplx7pEVm011494, Daplx7pEVm013338, Daplx7 pEVm012731, and Daplx7pEVm013713 were up to 40–100-fold higher in red *Daphnia*. Paradoxically, the mRNA levels for the collection of Hb paralogs did not reflect the differences in relative Hb protein levels seen in animals captured at the same time (**Figure 5C** and **Supplementary Tables 12, 13**). Reds in all lakes had a similar magnitude of difference in Hb levels than pales, yet the red/pale difference in Hb mRNA vary from up to 100-fold greater in red animals of Round Lake to two fold in Doe Lake (**Figure 5B**).

Effects of Season on Enzyme Patterns

The lack of stoichiometry between Hb and glycolytic enzymes prompted us to explore the impact of the history of hypoxic exposure. We returned to these same lakes the following year to characterize the patterns in Hb and LDH that emerge as the season progresses. To capture individual variation to a greater

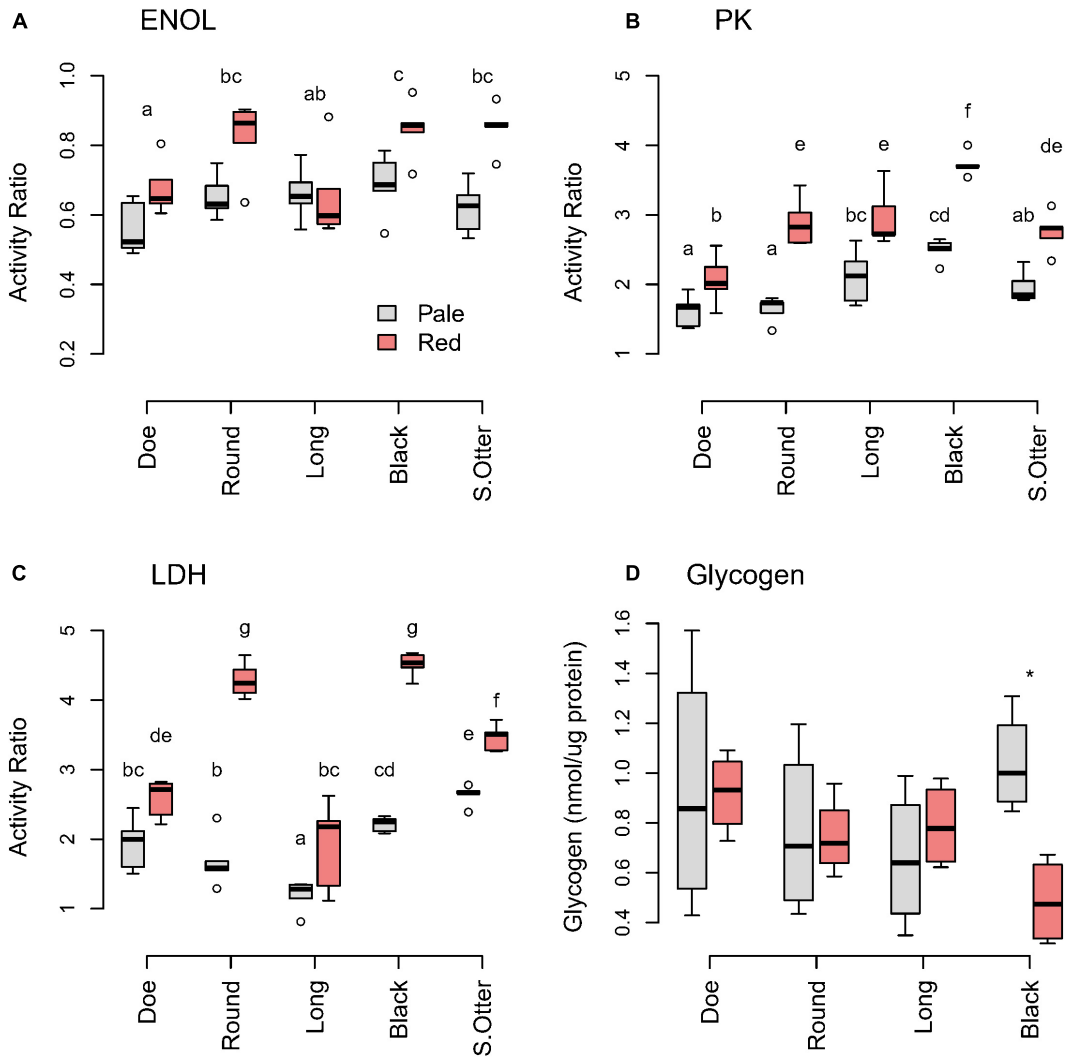

FIGURE 3 | Glycolytic enzyme (**A:** ENOL; **B:** PK; **C:** LDH) ratios and **(D)** glycogen of *D. pulicaria* by color morph across hypoxic lakes. Activities of glycolytic enzymes are expressed relative to the housekeeping gene citrate synthase (CS). Different letters indicate significant differences ($p < 0.05$) (**Supplementary Tables 6–10**). The asterisk (*) indicates significant difference between color morphs in Black Lake.

extent, our subsequent studies pooled 3 animals that were color-matched visually to obtain sufficient extract to measure both enzymes and Hb levels. In May 2019, we were unable to find *D. pulicaria* in Doe Lake, however in the other three lakes we found sufficient animals that ranged in color from pale to red (**Figures 6A–D**).

There was a lake and season interaction for changes in LDH activity with Hb (**Supplementary Tables 14, 15**). In Spring, there was no relationship between Hb levels and LDH activities in any lake. Although Hb varied from undetectable to 20x the detection limit, LDH variation did not correlate with Hb levels. In June, significant correlations between LDH and Hb emerged in Doe and Round Lakes (**Figures 6E,F**). By September a relationship was seen in these lakes as well as Black Lake (**Figures 6I,J,L**). By October, all 4 lakes showed a significant correlation between LDH and Hb (**Figures 6M–P**). Thus, it appears that early in the season, animals increase Hb but not LDH. As the season progresses, LDH activities increase, presumably as part of a metabolic response to hypoxic exposure in combination with warmer temperatures reducing oxygen solubility and increasing metabolic demands.

Gene Induction Under Controlled Hypoxia Exposure

To determine whether the differences in glycolytic enzymes and Hb among lakes was due to evolutionary variation in responsiveness to a similar environment, or exposure to a different environmental history, we exposed isofemale lines from each of the 4 lakes to hypoxia under the same conditions in the lab. Replicate isofemale lines from each experiment were cultured and exposed to normoxia or hypoxia and evaluated for changes in mRNA transcripts of Hb and LDH. Under normoxia, gene expression of one Hb

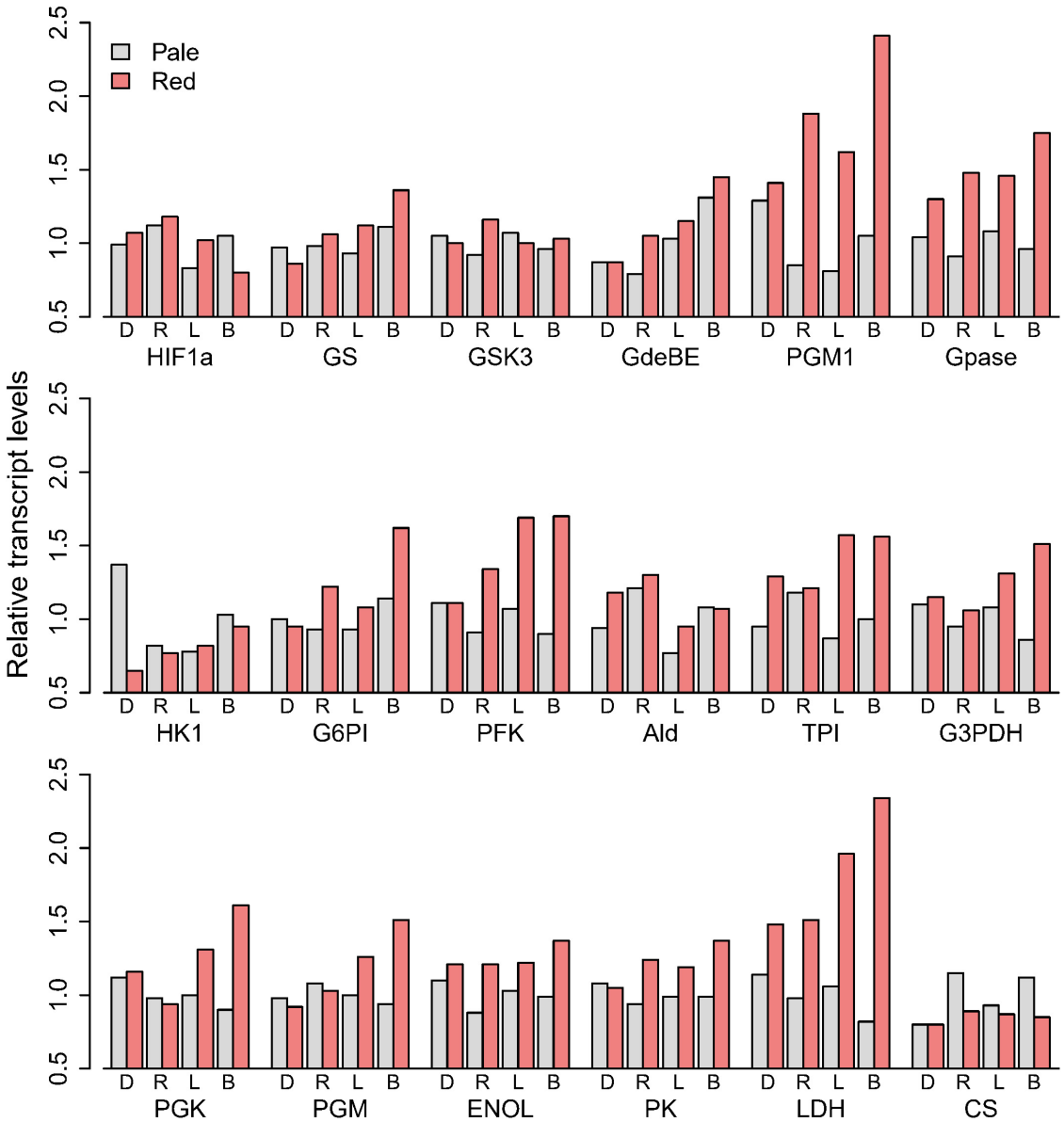

FIGURE 4 | Transcript levels of glycolytic enzymes of pale and red *D. pulicaria*, relative to the mean of the pale animals from the four hypoxic lakes: Doe Lake (D) Round Lake (R), Long Lake (L), and Black Lake (B).

subunit (Daplx7pEVm058987; **Figure 7A**, **Supplementary Table 17**) and LDHA (**Figure 7C**, **Supplementary Table 18**) varied significantly by up to 10- and 2-fold, respectively, between lakes, whereas expression of another Hb subunit (Daplx7pEVm011494; **Figure 7B**) did not vary significantly between lakes (**Supplementary Table 16**). Interestingly, isofemale lines may also exhibit microevolutionary variation within lakes, which is especially pronounced in the baseline expression of Daplx7pEVm058987 in Round Lake (**Figure 7A**) as well as the baseline expression of LDHA in Doe and Black Lakes (**Figure 7C**).

When exposed to hypoxia, isofemale lines from different lakes upregulated genes by a similar magnitude over normoxic expression; however, the genes differed in sensitivity (**Supplementary Table 19**). Expression of one Hb subunit Daplx7pEVm058987 increased by about 20-fold under hypoxia (**Figure 7A**), while another subunit Daplx7pEVm011494 increased by more than 200-fold (**Figure 7B**), although the sensitivity of these two genes did not differ by lakes. The expression of LDHA increased by about two-fold under hypoxia in most lakes (**Figure 7C**), with animals from Long Lake showing a significantly weaker response than Doe and Black Lakes (**Supplementary Table 20**). In comparing the mRNA changes in response to acute hypoxia with the differences seen in transcriptome analyses, it is worth noting that in the transcriptomes, these two Hb genes showed a

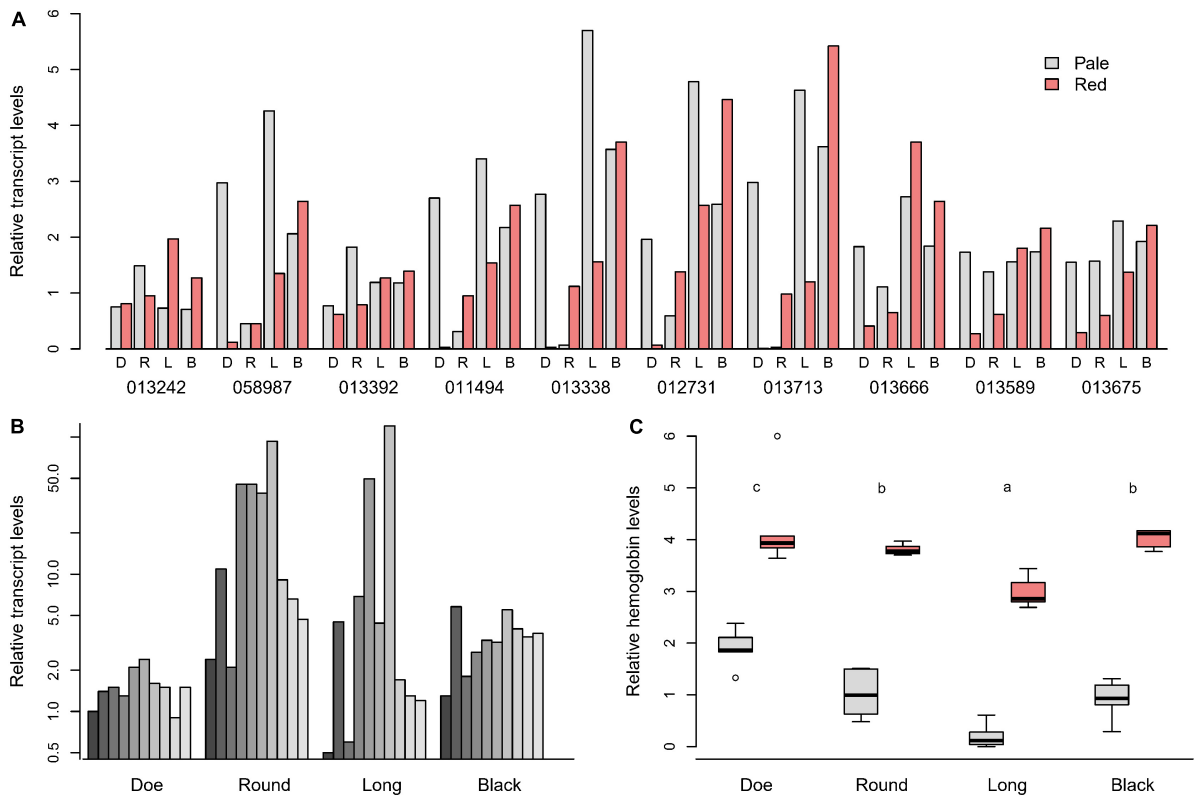

FIGURE 5 | (A) Transcript levels in *D. pulicaria* for the 10 most abundant hemoglobin transcripts (reported as Daplx7pEVm0#####), expressed relative to the mean of the pale animals. **(B)** Induction of Hb mRNA seen in red animals relative to the pale from the same lake for each gene in **(A)**. **(C)** Relative Hb protein levels, scaled to the mean of the pales. Different letters in **(C)** indicate significant differences ($p < 0.05$).

muted difference in pale vs. red: 2–10-fold higher in red *D. pulicaria* (**Figure 5B**).

We also analyzed gene expression of ENOL and PK, focusing on the impact of oxygen. Preliminary studies showed no effect of hypoxia in animals from Doe Lake. When expressed as a fold change for hypoxic/normoxic treatments, there was no effect of hypoxia for either PK (0.99 ± 0.09) or ENOL (1.26 ± 0.13) (**Supplementary Figure 3**).

DISCUSSION

As first proposed by Verne (1923), *Daphnia* turn red due to an increase in Hb. Fox (1948) showed that this was due to exposure to low oxygen, conferring greater tolerance to low oxygen environments (Fox et al., 1951; Kring and O'Brien, 1976; Pirow et al., 2001). A great deal has been learned about the subunit composition of Hb (Kobayashi et al., 1988, 1994; Kimura et al., 1999; Zeis et al., 2003), localization of Hb synthesis (Goldmann et al., 1999), and HIF dependency of the Hb response (Gorr et al., 2004). At the organismal level, ecological studies (Landon and Stasiak, 1983; Salonen and Lehtovaara, 1992) used the Hb response to explore differences between pale and red phenotype ecology (Prepas and Rigler, 1978; Duffy, 2010; Meyer and Nelson, 2019; Meyer et al., 2021). These studies collectively support the idea that Hb is essential to the ability of *Daphnia* to tolerate low environmental oxygen. While the trigger for Hb is undoubtedly hypoxia in the tissues that make Hb, it is unclear whether the Hb response is sufficient to preclude physiological compensation through metabolic remodeling. What was unappreciated at the outset of this study was the complex interplay between oxygen levels and exposure history, the confounding influence of Hb levels, all intertwined with the potential of macroevolutionary and microevolutionary variation in all aspects of the metabolic phenotype.

Connecting Hb Differences to Remodeling of the Metabolic Phenotype

We used *D. puIicaria* as the focal species to examine the connections between changes in Hb and glycolytic enzymes. Based on vertebrate models, we hypothesized that conditions that were sufficiently hypoxic to trigger Hb gene induction would also induce expression of other HIF-responsive genes, which in mammals include most glycolytic genes (Robin et al., 1984; Webster, 2003). Thus, we were surprised to see little evidence of coordination of genes that are expected to be HIF-responsive. In comparing red and pales from the four lakes, only 2 genes for enzymes associated with glycolysis (LDHA and PGM) showed elevated mRNA (**Figure 4**). ENOL and PK are HIF-responsive

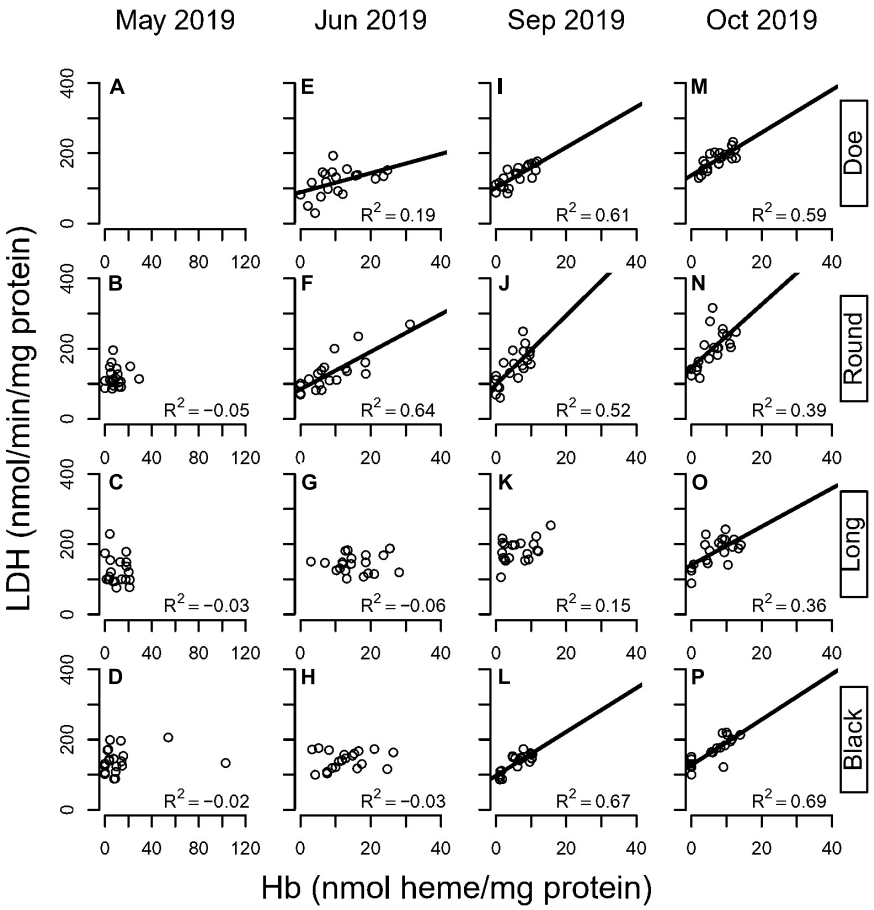

FIGURE 6 | Correlation between LDH and hemoglobin in four hypoxic lakes across months (**A–D**: May; **E–H**: June; **I–L**: September; **M–P**: October) in *D. pulicaria*. Each circle is a sample of three animals. Lines denote statistically significant relationships ($p < 0.05$).

in mammals (Webster et al., 1990; Semenza et al., 1994), but red and pale animals differed by less than 50% for in both enzyme activities (**Figure 3**) and mRNA levels (**Figure 4**). Zeis et al. (2009) acclimated *Daphnia pulex* (the pond relative to *D. pulicaria*) to 3kPa PO_2 and found that they only upregulated ENOL by 1.4-fold under hypoxia, consistent with our results. When exposed to acute hypoxia in the lab, animals from all lakes increased mRNA levels of two Hb genes we studied (Daplx7pEVm058987 and Daplx7pEVm011494) by 20- and 200-fold, respectively, within 18 h (**Figure 7**). However, the steady state mRNA levels in our transcriptome analysis of field-collected animals showed that the increase in Hb expression in reds varied from 1.4- to 10.9-fold, and 1.3- to 45.2-fold, respectively, for the same two genes between lakes.

There would seem to be a paradox between the major Hb differences, implying a hypoxic response, and the minor differences in the metabolic phenotype. The somewhat muted hypoxia response in field-collected animals that possess Hb compared to lab animals exposed to acute hypoxia suggests that red *Daphnia* experiences a lesser degree of hypoxia. Nevertheless, there are many confounding factors and alternative explanations to consider in explaining these observations.

Hb is synthesized in the epithelial cells of epipodites, and to a lesser extent the fat cells lining the vessels that carry hemolymph (Goldmann et al., 1999). The location on an external appendage means that the regulation of Hb genes may be more acutely sensitive to environmental hypoxia than other glycolytic genes. In both the transcriptome comparisons of field (**Figure 5**) and lab animals (**Figure 7**), it is clear that Hb gene expression is elevated in red animals, suggesting a recent exposure to hypoxia in that tissue. The presence of Hb in the circulation could then mitigate the systemic effects of low ambient oxygen. However, our seasonal sampling shows this to be a fluid relationship. Early in the season (May) when *Daphnia* undergoes strong population growth, they had little Hb. This could be attributed to Hb's dual function in reproduction as protein stores in eggs (Fox and Phear, 1953; Kobayashi et al., 1990), with animals transferring Hb to their eggs. As the season progressed, with warmer temperatures and the onset of stratification reducing oxygen solubility, first Hb levels then LDH activity increased, resulting in significant correlations between Hb and LDH levels (**Figure 6**). Furthermore, each lake reached that point at different timepoints.

The short life span and migratory behavior of *Daphnia* further complicates the kinetics of Hb. In mammals, the half-life of

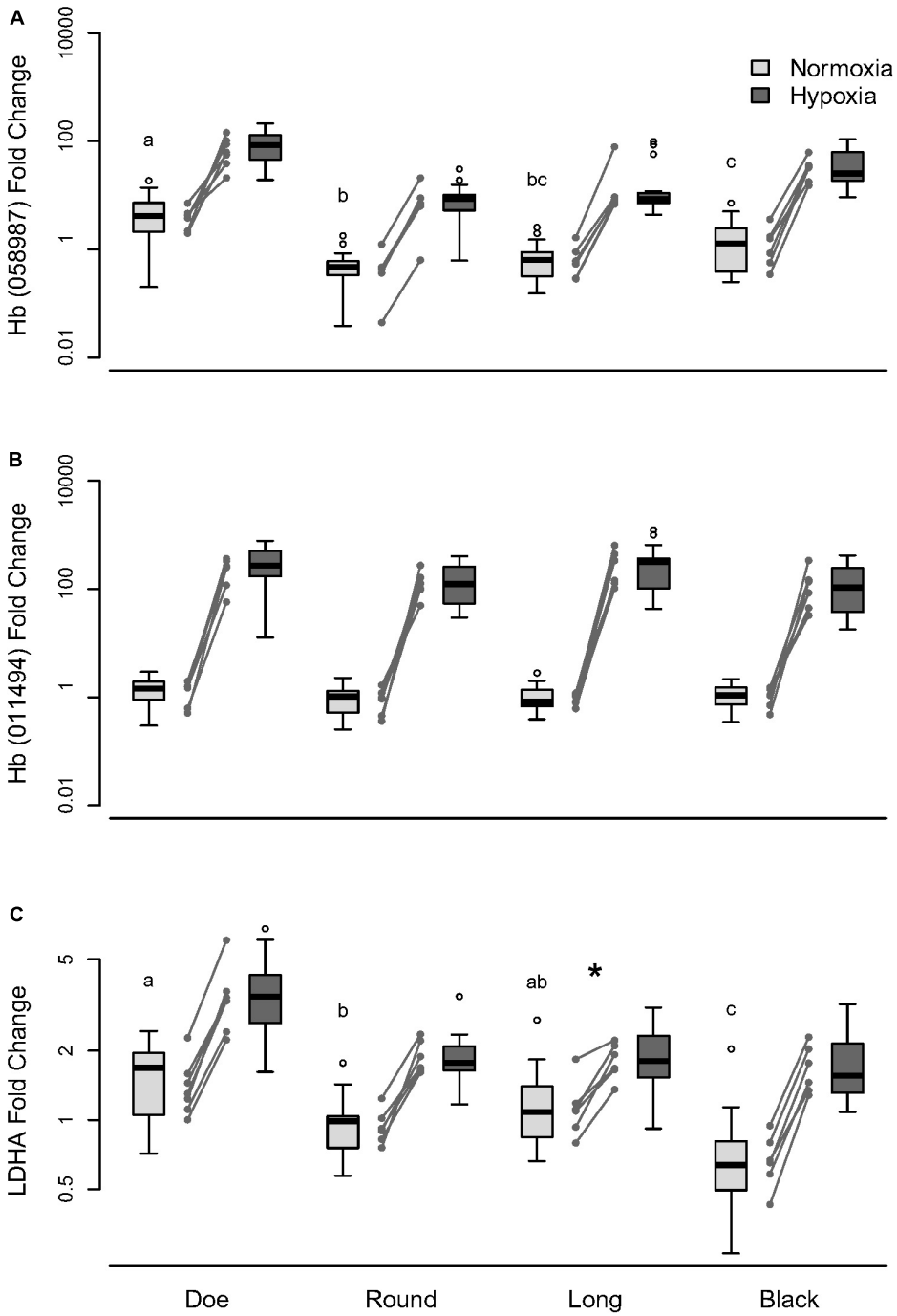

FIGURE 7 | Change in **(A)** Hb Daplx7pEVm058987, **(B)** Hb Daplx7pEVm011494, and **(C)** LDHA mRNA transcripts under hypoxic stress manipulation in *D. pulicaria* from four hypoxic lakes. *D. pulicaria* were isolated from each lake and reared in the lab under standard conditions for at least two generations prior to treatment. The treatment oxygen environment was either normoxia (ambient; 21% O2) or hypoxia (3% O2). Baseline expression labeled with different letters indicate significant differences ($p < 0.05$). The asterisk (*) indicates significantly lower LDHA sensitivity to hypoxia in Long lake compared to Doe and Black lakes.

Hb is on the order of weeks or months, corresponding to the lifespan of an erythrocyte. Hb protein in red *Daphnia* can decay within days when the animals are placed in normoxic waters (Fox and Phear, 1953). However, *Daphnia* undergoes diel vertical migrations in and out of hypoxic waters (Lampert, 1993; Meyer and Nelson, 2019; Meyer et al., 2021), which would certainly influence the behavior of Hb genes. Not only do pale and red *Daphnia* stay at and migrate through different depths that vary in oxygen levels, but red animals also exhibit more intraspecific variation in their diel vertical migration

pattern than pale animals (Meyer and Nelson, 2019). Such behavior can introduce extensive variability to the oxygen-deficiency signals that individuals experience, both spatially and temporally. The kinetics of Hb synthesis and degradation in animals that periodically experience hypoxia of different degrees and magnitudes makes it difficult to predict how Hb protein may change once Hb genes are induced. If Hb is maintained in red animals in hypoxic waters, the previously produced Hb may be able to outlive the animal. The benefits of the initial hypoxia-induced expression of Hb may then persist long after the hypoxic signal is removed, reducing cellular exposure to hypoxia. For example, it is possible that the animals in Black and Doe Lakes may have previously produced enough Hb (in response to hypoxia) to avoid hypoxia in the tissues (**Figure 5C**), resulting in a lack of induction of hypoxia responsive Hb genes (**Figure 5B**). Conversely, the modest increases in Hb protein in Long Lake may be insufficient to preclude hypoxia, triggering a greater response in hypoxia responsive Hb genes.

However, it is important to keep in mind that changes in relative quantities of individual Hb paralogs may not translate to changes in Hb protein levels, which represent all isoforms. The contribution of each paralog to the overall transcript and eventually protein pool, as well as the effect on Hb function under hypoxia, are unknown. Therefore, the connection between changes in individual Hb transcripts and effects at the protein level remains to be investigated. Nevertheless, Hb protein levels may not reflect the recent history of oxygen exposure in the environment or the degree to which the internal tissues experience hypoxia. In contrast, enzyme protein and mRNA are generally thought to have greater turnover rates than Hb proteins, and thus may be better indices of recent hypoxic history.

Interestingly, our lab experiment enabled us to show that, in contrast to better studied models, *Daphnia* PK and ENOL orthologs (**Supplementary Figure 3**) lack oxygen sensitivity, which may explain the smaller and variable difference in PK in the field. Although PK mRNA was not affected by acute hypoxia in the lab, red animals in several lakes showed elevated PK to varying degrees, and the responses were not as strong as LDH within the same lakes (**Figure 3**). Thus, it seems likely that elevated PK activities were induced by oxygen-independent mechanisms. We do note that PK activities were uniformly higher in *D. pulicaria* than other species (**Figure 2**), suggesting a constitutive difference in gene expression.

Are *Daphnia* Experiencing Systemic Hypoxia?

The seasonal changes in the Hb and LDH relationship suggests that early in the season Hb reduces systemic hypoxia, obviating any metabolic adjustments. This is consistent with the role of Hb in either facilitating diffusion from the periphery in hypoxia or acting as an oxygen store when the animals are anoxic. Kobayashi and Hoshi (1982) found that when animals were faced with a decline in oxygen, pales could sustain their oxygen consumption rate until oxygen levels fell by 80%. Pirow et al. (2001) examined the performance and redox state ([NADH]) of limb muscles in red and pale *Daphnia magna* that were exposed to a transition from normoxia to anoxia (< 1% atmospheric oxygen) over 51 min. Though both phenotypes were able to maintain limb beating frequency, the pale *Daphnia* showed a decline in NADH in hypoxia, suggesting that Hb permitted animals to avoid anaerobic metabolism (Pirow et al., 2001). Usuki and Yamaguchi (1979) measured survival time and lactic acid in red and pale *Daphnia magna* exposed to a gradient of oxygen concentrations. Red and pale animals showed a similar pattern in lactate production where both phenotypes had high production at low oxygen concentration and low lactate production at high oxygen concentration. However, the two phenotypes differed in survival time. Red *Daphnia* could survive up to 24 h when oxygen levels were approximately 5% air saturation. Pale *Daphnia* died after 1-h of oxygen levels less than 7% air saturation. Collectively, these studies suggest that Hb acts primarily to facilitate oxygen delivery.

Our analysis of metabolic parameters argues against a role for Hb to act purely as an oxygen store. In brief, there is not enough Hb to store enough oxygen to permit the animals to sustain their normoxic metabolic rate in the absence of oxygen for a realistic period. Based on previous studies (Ullrich and Millemann, 1983; Baillieul et al., 2005; Jensen and Hessen, 2007; Villarroel et al., 2013; Lari et al., 2017), we estimate *Daphnia* metabolic rate to be in the range of 0.7–1.7 nmol O_2 per mg per min, or 3.5–8.5 nmol ATP per min per mg (assuming ATP:O_2 = 5). Each of these studies reports units in a different manner, so our assumptions and calculations are provided in **Supplementary Table 21**. Our Hb measurements provide an index of Hb levels and oxygen carrying capacity. Using bovine Hb as a standard, we estimate that Hb levels in *Daphnia* are less than 40 nmol heme per animal, which would carry 40 nmol O_2. Thus, at a metabolic rate of 1.7 nmol O_2 per min, a fully loaded Hb could sustain aerobic metabolism for approximately 24 min at 20°C. Our glycogen measurements (**Figure 3D**) show these animals to have about 1,000 nmol glucosyl/mg protein, so a 1 mg animal (0.1 mg protein/mg animal) would have approximately 100 nmol glucosyl, which under anaerobic conditions would contribute 300 nmol ATP. Thus, at a metabolic rate of 8.5 nmol ATP per min, a 1 mg animal could survive on glycogen for 24 min without oxygen. These capacities for oxygen-dependent metabolism (using O_2 on Hb) and glycogen-based anaerobic metabolism would be extended at lower temperatures. With a Q_{10} of 2–3, and animal living at 4°C could extend the limits to approximately 1 h.

Putting these values together, a *Daphnia* with fully loaded Hb and storing a typical amount of glycogen could survive at its normal metabolic rate in the absence of external oxygen for approximately 48 min. However, the exact limits are difficult to predict for a free-living animal. On one hand, the limits could be extended with metabolic suppression (reducing demand) and feeding (increasing glucose), in combination with the benefits of lower temperature. Conversely, animals that needed to actively swim upwards in the water column to return to normoxic surface water would incur greater energetic costs associated with antennal movement. Since these animals live in and migrate through deep hypoxic zones, the implication is that Hb is present not as an oxygen store but rather as a means of extracting

oxygen from hypoxic waters. Furthermore, the ability of Hb to facilitate oxygen extraction may be sufficient for an animal to ensure its tissues remain normoxic, obviating a hypoxic response in terms of the metabolic phenotype. Increases in LDH mRNA and protein would only be necessary when oxygen demands, enhanced by warm temperatures, exceed oxygen supply. In our seasonal sampling, *Daphnia* in each lake seemed to reach this point of an induced metabolic response along a different time course, and those dynamics likely change from year to year.

Macroevolutionary and Microevolutionary Variation in the Hypoxic Response

Enzyme measurements can be used to characterize the metabolic poise of a tissue and animal (Newsholme and Crabtree, 1986). Previous studies have explored the differences in *Daphnia* species that may explain their distribution within and between lakes (Hanazato et al., 1989; Leibold, 1991; Leibold and Tessier, 1991; Lampert, 1993; Tessier and Leibold, 1997; Duffy, 2010; Meyer et al., 2021). The ability to produce Hb alters where the animals can move within the water column, but it also alters appearance, which can influence predation (Green, 1956; Leibold, 1991; Larsson and Lampert, 2011). High activities of glycolytic enzymes, particularly LDH, reflect a reliance on anaerobic glycolysis to produce ATP. Changes/differences in glycolytic enzymes between individuals/species usually implies some degree of specialization for either burst activity (where ATP demand outstrips aerobic ATP supply) or oxygen limitations (where aerobic ATP production is compromised). We explored the metabolic properties of *Daphnia* species within and between lakes of different physical and chemical properties. Although we discriminate based on oxygen levels arising from thermal stratification, there are also associated differences in salinity, conductivity, pH and nutrients, all of which may affect *Daphnia* populations (Aladin and Potts, 1995; Vijverberg et al., 1996; Elser et al., 2001; Dodson et al., 2009).

Given the lack of simple relationship between Hb and LDH levels within *D. pulicaria*, it is perhaps not surprising that there is no simple pattern in this relationship between species. However, a few comparisons stand out. First, when comparing species, we were surprised to find two species (*D. dubia* and *D. retrocurva*) excluded from the 10 lakes that possessed a hypoxic hypolimnion, each of which have a normoxic epilimnion. If this was a metabolic limitation, then it could be hypothesized that these species might be specialized for normoxic environments, and perhaps possess relatively low glycolytic enzyme activities. However, LDH and PK for these species were indistinguishable from *D. mendotae* in the same lakes (**Figure 2**), providing no evidence for metabolic limitation. Second, *D. mendotae* was found in both normoxic and hypoxic lakes, and they showed a trend toward having higher LDH activities in lakes with a hypoxic hypolimnion even before stratification arose within the lakes (**Figures 2A,B**), suggesting some degree of compensation in this species. Third, *D. pulicaria*, only found in hypoxic lakes, showed higher PK activity than other species (**Figures 2C,D**), suggesting some degree of metabolic specialization, yet LDH was not higher, and in some cases markedly lower, with considerable lake-to-lake variation (**Figures 2A,B**). Since our controlled experiment showed that the PK gene was not hypoxia sensitive, it is likely that a higher constitutive expression of PK benefits anaerobic glycolytic metabolism. Thus, the differences in PK activities between red and pale animals seen in select lakes is likely due to factors other than oxygen-sensitive PK expression. It also opens the possibility of lineage-specific losses in hypoxia responsiveness of glycolytic genes that, in other species, respond to HIF as part of phenotypic remodeling as part of hypoxic responses. Although HIF and the HIF network are thought to have evolved in early metazoans (Loenarz et al., 2011; Mills et al., 2018), there are many examples of evolutionary changes in the pathway. It is noteworthy that other crustaceans appear to have evolved differences in their HIF network. For example, the copepod *Tigriopus californicus* has lost multiple components of the HIF network (Graham and Barreto, 2019).

Our initial impressions were that the changes seen in *D. pulicaria* between lakes at a sampling point might be driven in part by microevolutionary variation. With *Daphnia* there is a great potential for population-level variation because of their reproductive cycle, where only a few genotypes survive the overwintering period then proliferate by parthenogenesis. Although much of the variation we see in metabolic traits can be attributed to the dynamics of changes in oxygen, we did see evidence of population level variation. Under controlled lab conditions, we saw clear differences in baseline expression of LDH and one inducible Hb gene between lakes (**Figure 7**). The basal transcriptional regulation of these genes is governed by many factors that act in tissue-specific ways, so it is not a contradiction that each gene seemed to be induced to a similar extent by hypoxia. The variation in LDH basal expression seen between lakes is consistent with the highly variable LDH activities seen amongst lakes in pale animals (**Figure 2**).

Isofemale line within lakes also appear to show variation in expression of Hb and LDH (**Figure 7**), which suggests the possibility for microevolution due to the availability of distinct ecological niches within lakes. However, the extent of within-lake variation in this study may be limited by the use of only six isofemale lines, propagated from one sampling attempt at the deep point of each lake. Nevertheless, our results provide preliminary evidence for microevolution within lakes, prompting further deliberate sampling from multiple parts of each lake, as well as incubation experiments testing for the ecological forces that may drive such microevolution.

CONCLUSION

Collectively, these studies provide evidence for a combination of macroevolutionary and microevolutionary variation in the relationship between ecological niche and the hypoxic metabolic phenotype. Hb accumulation in response to hypoxia appears to obviate the hypoxia-dependent expression of LDH early in the seasons, but inadequate to preclude a hypoxic response later in

the season. The metabolic phenotype also supports the idea that Hb is present primarily to extract oxygen from hypoxic waters, permitting the animal to be functionally normoxic.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, GSE159485. Further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

KW, WN, and CDM contributed to conception and design of the study. TL and KW conducted the experiments. TL, KW, CJM, and WN performed the statistical analyses. TL, KW, WN, and CDM wrote sections of the final manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.822935/full#supplementary-material

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