01 Sep 2005

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Recommended Citation

J. A. Guadagnoli et al., "Environmental Hypoxia Influences Hemoglobin Subunit Composition in the Branchiopod Crustacean Triops Longicaudatus," *Journal of Experimental Biology*, vol. 208, no. 18, pp. 3543 - 3551, The Company of Biologists, Sep 2005.
The definitive version is available at https://doi.org/10.1242/jeb.01794

The Journal of Experimental Biology 208, 3543-3551
Published by The Company of Biologists 2005
doi:10.1242/jeb.01794

Environmental hypoxia influences hemoglobin subunit composition in the branchiopod crustacean *Triops longicaudatus*

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Accepted 13 July 2005

Summary

Hemoglobin (Hb) is a highly conserved protein that provides a vital link between environmental oxygen and its use and/or storage within an organism. While ubiquitous among vertebrates, Hb occurs frequently in invertebrate phyla as well. Many arthropod species use the copper-binding pigment hemocyanin, but unique in this phylum are the branchiopod crustaceans, which express Hb. Branchiopod Hb concentration and structure are exquisitely sensitive to environmental oxygen availability. Hemoglobin concentration and oxygen-binding affinity increase with decreasing oxygen tension in *Daphnia*, *Artemia* and *Triops*. The change in binding affinity is attributed to differential Hb subunit expression in *Daphnia* and *Artemia* but remains unclear for *Triops*. This is the first study to demonstrate developmental plasticity of Hb subunit expression in a notostracan, *Triops longicaudatus*, reared under conditions of varying oxygen availability. In response to variable oxygen environments, *T. longicaudatus* differentially express four primary Hb subunits ranging between 30 and 34 kDa, with normoxic-reared animals expressing primarily the heavier subunits, and hypoxic-reared animals expressing increased proportions of the lower molecular mass subunits. Moreover, differential Hb subunit expression is induced upon transfer of normoxic-reared adults to a hypoxic environment, such that the distribution of Hb subunits in the transferred adults becomes similar to that of hypoxic-reared animals. Two-dimensional gel electrophoresis and follow-up analyses revealed several isoforms of Hb subunits that may represent differential gene expression and/or post-translational modification. Unlike *Daphnia* and *Artemia*, the Hb hypoxic response in *Triops* is not reversible in that there was no significant decrease in Hb concentration or change in Hb subunit expression pattern when hypoxic-reared adults were transferred to a normoxic environment.

Key words: hypoxia, hemoglobin, invertebrate, *Triops longicaudatus*.

Introduction

Hemoglobin (Hb) is a highly conserved protein that provides a vital link between environmental oxygen and its use and/or storage within an organism (Weber and Vinogradov, 2001). A ubiquitous oxygen transport molecule among vertebrates, Hb occurs frequently in invertebrate phyla as well. Among the arthropods, many animals use the copper-binding pigment hemocyanin, but unique in this phylum are the branchiopod crustaceans, which express Hb. Within this group, the concentration and structure of Hb is exquisitely sensitive to environmental oxygen availability, especially in the Anastraca (*Artemia*) and Cladocera (Daphnids) (Weber, 1980; Peeters et al., 1990; Terwilliger, 1998). However, little is known about the developmental and hypoxia-dependent kinetics of Hb expression in Notostraca, the subject of this study.

Hemoglobin structure varies widely among branchiopods. It is a large extracellular molecule ranging from 220 kDa in *Artemia salina* (Moens and Kondo, 1978) to nearly 800 kDa in *Lepidurus apus lubbocki* (Ilan and Daniel, 1979) and is composed of various sized subunits. Among the Cladocera and Notostraca, Hb subunit chains range from 30 to 37 kDa (Peeters et al., 1990), with two heme groups per chain (Ilan and Daniel, 1979). In the notostracans *Lepidurus apus lubbocki* and *Lepidurus bilobatus*, the native Hb molecules have molecular masses of approximately 798 kDa and 680 kDa, respectively, with subunits in the 33–34 kDa range (Dangott and Terwilliger, 1979; Ilan and Daniel, 1979). *Triops longicaudatus* Hb has a molecular mass of approximately 600 kDa, and Horne and Beyenbach (1974) estimated the molecular mass of the subunits at approximately 20.5 kDa.

The natural history of many crustaceans includes regular bouts of hypoxia, the response to which includes, but is not limited to, increased ventilation and perfusion over oxygen exchange tissues, increased cardiac output and/or reduction in metabolism and the demand for oxygen (Wheatly and Taylor, 1981; Hochachka and Lutz, 2001). During periods of chronic hypoxia, certain branchiopods such as *Daphnia magna* (Fox,

1955; Zeis, 2003), *A. salina* (Gilchrist, 1954; Heip et al., 1978) and *T. longicaudatus* (Scholnick and Snyder, 1996; Harper, 2003) increase Hb content; this response has been observed in both experimental and natural populations (Kobayashi and Hoshi, 1982; DeWachter et al., 1992). Moreover, branchiopods modify Hb structure and functional properties in response to hypoxia (Wolf et al., 1983; Zeis et al., 2003). In *D. magna* and *T. longicaudatus*, hypoxia induces an increased Hb oxygen-binding affinity (Wolf et al., 1983; Kobayashi et al., 1994; Harper, 2003; Zeis et al., 2003). The branchiopod hypoxic response may also include differential Hb subunit assembly (Kimura et al., 1999) and differential subunit expression, as demonstrated in *D. magna* (Zeis et al., 2003).

In this study, we assess the hypoxic response and plasticity of Hb expression in a notostracan by examining differences in Hb concentration and subunit expression in *T. longicaudatus* reared under hypoxic or normoxic conditions. We show that Hb subunit expression changes significantly during developmental hypoxia and when normoxic-reared adults are transferred to a hypoxic environment. Interestingly, the adult response is apparently fixed, as a reversal to normoxic Hb expression patterns does not occur when hypoxic-reared adults are returned to a normoxic environment.

Materials and methods

Animal rearing

Tadpole shrimp (*Triops longicaudatus* LeConte) cysts were collected from an ephemeral pool in Brownstone Canyon, Clark County, NV, USA. Cysts were dried for at least 30 days at 40°C and re-hydrated in 10-liter aquaria filled with either normoxic or hypoxic water. Normoxic conditions were maintained by bubbling room air into the aquaria (20 kPa O_2). A gas-mixing flow meter (GF-3; Cameron Instruments, Port Aransas, TX, USA), which provided a mixture of 90% nitrogen and 10% air, was used to maintain hypoxic conditions at 2–5 kPa O_2. Water temperature was maintained at 25±1°C using submersible aquarium heaters. Newly hatched larvae were fed ground spirulina flakes (Tetra) for the first 2 days and were then switched to finely ground goldfish pellets. All animals were exposed to a 14 h:10 h light:dark cycle.

Hemolymph collection, purification and protein content

Hemolymph samples were collected from sexually mature females (12–13 days after re-hydration). To induce a hypoxic response in adults, females were reared to sexual maturity under normoxic conditions and on day 12 were transferred to hypoxic conditions (5 kPa O_2). A normoxic hemolymph sample was collected on day 12 (prior to transfer), and hypoxic hemolymph samples were collected once every 24 h post-transfer for 7 days. To assess Hb changes in hypoxic-reared adults subsequently exposed to normoxia, the reverse experiments were conducted in which female *Triops* reared to sexually maturity under hypoxic conditions were switched to normoxic conditions.

Hemolymph samples were collected from adult animals (12–20 days old) *via* dorsal puncture of the heart. Specifically, animals were netted, blotted dry and the carapace folded back to reveal the dorsal location of the heart. The heart was punctured with a 28-gauge needle, and hemolymph samples were collected into capillary tubes, transferred into ice-cold micro-centrifuge tubes and frozen at –20°C until use. As previously demonstrated by Horne and Beyenbach (1971), the major detectable protein in *Triops* hemolymph is Hb. To confirm this, samples were partially purified on a 10 cm gel exclusion column packed with Sephacryl S-300 (Amersham Biosciences, Piscataway, NJ, USA) in buffer containing 0.05 mol l^{-1} Tris, 0.1 mol l^{-1} NaCl, 0.01 mol l^{-1} $MgCl_2$ and 1 mmol l^{-1} PMSF and collected in 0.5 ml fractions. Absorbance peaks of each fraction were measured at 280 nm for the determination of protein and 415 nm for determination of Hb. Hb samples from both normoxic- and hypoxic-reared animals were run through the column, and these partially purified samples were run on SDS-PAGE gels and compared with non-purified samples. After confirming that the primary protein in *Triops* hemolymph is Hb, protein concentrations of whole hemolymph samples were used to estimate changes in hemolymph Hb content. Protein content was determined colormetrically, based on the method of Bradford with a kit from BioRad (500-0001; Hercules, CA, USA).

One-dimensional gel electrophoresis

SDS-PAGE was performed using the Protean II xi Gel Electrophoresis system (BioRad) with a 10% separating and 4% stacking gel as described by Laemmli (1970). After determination of protein content, Hb was diluted in sample buffer containing 62.5 mmol l^{-1} Tris HCl (pH 6.8), 25% glycerol, 2% SDS, 0.01% bromophenol blue and 5% β-mercaptoethanol. Samples were heated at 95°C for 5 min and then loaded onto gels. One lane of each gel was loaded with Precision Plus Dual Color Protein Standards (Bio-Rad #161-0374) with a molecular mass range from 250 kDa to 10 kDa. Specifically, molecular mass markers at 25 kDa and 37 kDa were used for determination of the molecular mass of the Hb bands. Following electrophoresis, proteins were visualized using Coomassie Brilliant Blue and scanned on a Typhoon 9410 Phosphorimager (Amersham Biosciences).

Two-dimensional gel electrophoresis

Hemolymph samples containing 200 μg protein were added to 300 μl of rehydration media consisting of 7.9 mol l^{-1} urea, 4.0% CHAPS (w/v), 84.4 mmol DTT, 35 mmol Tris base, 0.0025% bromophenol blue, 2 mol l^{-1} thiourea and 0.8% 3.5–10 ampholytes and shaken for 2 h at 30°C. Isoelectric focusing was performed using pre-cast gel strips with a 3–10 immobilized pH gradient (Amersham Biosciences). The samples were then applied to the gel strips and the strips were rehydrated overnight. Proteins were focused (Multiphor IEF; Amersham Biosciences) at 500 V for 30 min, 1500 V for 1 h, 2500 V for 1 h and 3500 V for 48 h. Gel strips were removed and laid perpendicularly over a 10% SDS-PAGE to separate the proteins by molecular mass. Two-dimensional gels were

stained with Coomassie Brilliant Blue and scanned on a Typhoon 9410 Phosphorimager (Amersham Biosciences) for later analysis of the spots using ImageQuant software (Amersham Biosciences).

Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE)

The experimental design and protocol was followed as per manufacturer's instructions (Amersham Biosciences). Briefly, CyDyes were reconstituted in 1.5 volumes of high-grade *N*,*N*-dimethylformamide-d_7 (DMF) to a concentration of 400 pmol ml^{-1} CyDye. One hemolymph sample each from a normoxic-reared and a hypoxic-reared animal was labeled with a fluorescence dye at a ratio of 50 μg protein labeled with 400 pmol fluor. Samples were labeled as follows: Cy2, a mixture hemolymph from a normoxic-reared and a hypoxic-reared adult; Cy3, the normoxic-reared hemolymph sample; Cy5, the hypoxic-reared hemolymph sample. The Cy2-labeled pooled sample served as an internal control on the gel for the DeCyder analysis.

After labeling, 50 μg of each of the three labeled samples was added to sample buffer (as described above in 2D gel methodology) and loaded onto pre-cast gel strips with a 3–10 immobilized pH gradient. The samples were then subject to 2D gel electrophoresis as described previously and scanned with the phosphorimager in the 2D-DIGE mode at wavelengths appropriate for each of the CyDyes. All spot picking and image analysis of the gel was performed using DeCyder software (Amersham Biosciences) developed specifically for 2D-DIGE gel analysis using an internal standard experimental design. DeCyder software scans the entire gel and outlines all detectable areas. Areas that are upregulated greater than 2.5 times are outlined in blue and those downregulated greater than 2.5 times are outlined in red.

NH_2-terminal sequencing

Hemolymph proteins were isolated using 2D gel electrophoresis under denaturing conditions. Gels were electro-transferred to polyvinylidine fluoride (PVDF) membranes (TransBlot electrophoretic transfer cell; BioRad). Transferred spots were visualized with Coomassie Brilliant Blue, cut and sent to the Nevada Proteomics Facility (Reno, NV, USA). Sequence analysis was performed using automated Edman degradation on an Applied Biosystems (Foster City, CA) Precise 492 sequencer.

Statistical analysis

A Student's *t*-test was used to determine the differences in Hb content between rearing groups, and one-way analysis of variance (ANOVA; Sigma Stat 3.2) was used to determine differences in Hb content in the transfer experiments. ANOVA was used to determine significant differences in subunit expression between normoxic- and hypoxic-reared animals and the difference in the time course of subunit induction for the transfer experiments. All data passed the normality test and were equally distributed. *Post hoc* pairwise comparisons were made using Tukey's test. The level of significance was set at $P<0.05$ for all statistical analysis. Values are reported as means ± S.E.M., with *N* indicating the number of samples.

Results

Hemolymph purification and protein content

Absorbance of hemolymph fractions at 280 nm (absorbance value for protein) coincided with the absorbance peaks at 415 nm (absorbance value for Hb), suggesting that Hb is the predominant protein present in hemolymph samples (Fig. 1). We further confirmed this result by comparing one-dimensional SDS-PAGE gels of partially purified and non-purified hemolymph (data not shown). Since the primary bands on both gels were those of Hb, samples were not purified prior to 1D SDS-PAGE, 2D SDS-PAGE or 2D-DIGE.

Hypoxic-reared animals had a significantly greater [Hb protein] (37.7±1.3 mg ml^{-1}; $N=23$) than normoxic-reared animals (29.3±1.6 mg ml^{-1}; $N=20$; $P<0.001$) (Fig. 2). In the transfer experiments, normoxic-reared adults showed no change in [Hb protein] during the first 3 days after transfer to hypoxia but significantly increased [Hb protein] 4–7 days after transfer ($F_{2,25}=5.14$, $P=0.01$; Fig. 3A). In the reverse

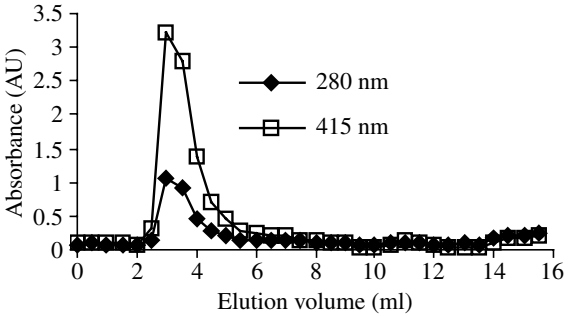

Fig. 1. Spectral analysis of *Triops* hemolymph fractions. The fraction with the greatest protein absorbance peak (280 nm) coincides with the fraction having the greatest Hb absorbance peak (415 nm). AU, arbitrary units.

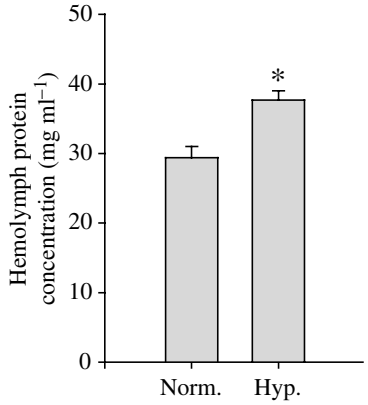

Fig. 2. Differences in hemolymph protein concentration between normoxic- and hypoxic-reared adult females. Hypoxic-reared females have significantly more Hb than normoxic-reared individuals (*$P<0.001$).

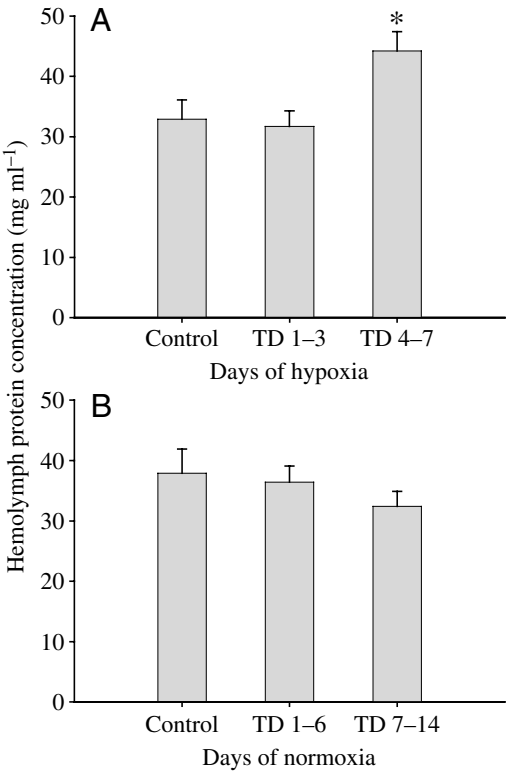

Fig. 3. Changes in hemolymph protein concentration when *Triops* were transferred to different environmental partial oxygen pressures. (A) Normoxic-reared females transferred to hypoxia. Pooled samples from 1–3 and 4–7 days after hypoxic transfer. (B) Hypoxic-reared females transferred to normoxia. Pooled samples from 1–6 and 7–14 days after normoxic transfer (*P<0.05).

experiments, there were no significant differences in [Hb protein] when hypoxic-reared animals were transferred to normoxic conditions even after 14 days (Fig. 3B; $F_{2,23}$=0.99; P=0.4).

One-dimensional gel electrophoresis

One-dimensional gel electrophoresis revealed that Hb from normoxic-reared *Triops* consisted of three primary subunits: two dark staining bands at 34 kDa (Hbα) and 33 kDa (Hbβ) and a lighter staining band at 32 kDa (Hbδ) (Fig. 4A, lanes 3 and 6). From ImageQuant analysis, the relative proportions of Hbα, Hbβ and Hbδ in normoxic animals were 45.5±1.3%, 37.3±2.1% and 14.5±1.1% of total Hb, respectively (N=7). A faint band at 30 kDa (Hbγ), which accounts for less than 3% of total Hb subunit composition, was present in some normoxic-reared animals. The same four bands are present in hypoxic-reared animals, but the relative contribution from each band changes dramatically (Fig. 4A, lane 12). The higher molecular mass subunits Hbα and Hbβ drop to 30.9±2.1% and 12.6±1.6% of total Hb, respectively, whereas lower-molecular-mass subunits Hbδ and Hbγ increase their contributions to 33.4±3.0% and 23.1±1.9%, respectively (N=8).

When sexually mature, normoxic-reared females were transferred to hypoxic water, induction of Hbγ was detected as early as 2 days post-transfer (Fig. 4A, lanes 4 and 5). With continued hypoxic exposure, the Hb composition of normoxic-reared adult females changed to match that of hypoxic-reared females, with a decrease in Hbα and Hbβ and an increase in Hbδ and Hbγ (Fig. 4A, lanes 7, 8, 10 and 11).

ImageQuant analysis of separated Hb subunits from 4–6 animals per treatment per day was used to determine the time course of Hb subunit induction in normoxic-reared animals that were transferred to hypoxia upon sexual maturity. The intensity of each band (Hbα, Hbβ, Hbδ and Hbγ) is expressed as a percentage of the total intensity of all the bands in each lane. The relative contribution of Hbα decreases significantly from normoxic controls by 2 days post-transfer, from 45.5±1.3% to 34.8±1.3% ($F_{8,36}$=13.17, P<0.001), and thereafter is not different from hypoxic control values (Fig. 5A). After 3 days of hypoxic exposure, the contribution of Hbβ decreases significantly from the normoxic value of 37.3±2.1% to 14.1±1.9% ($F_{8,36}$=37.59, P<0.001) and thereafter is not different from hypoxic-reared animals (Fig. 5B). Hbδ increases significantly by 2 days post-transfer, from 14.5±1.1% to 25.3±2.1% ($F_{8,36}$=12.27, P<0.001) (Fig. 5C). The mean value of Hbδ from 3 days post-transfer to 7 days post-transfer is 30.8±1.4%, a value not significantly different from hypoxic controls. The induction of Hbγ occurs by 3 days post-transfer, when it increases significantly from normoxic values of 2.78% to 25.1±1.3% ($F_{8,36}$=23.01, P<0.001), which is not significantly different from hypoxic-reared animals (Fig. 5D). These data collectively show that the Hb subunit composition of normoxic-reared, sexually mature animals becomes indistinguishable from hypoxic-reared animals by 3 days after transfer to chronic hypoxia (Fig. 4A).

Similar plasticity in Hb subunit expression was not observed in hypoxic-reared *Triops*. Hypoxic-reared animals transferred to normoxia did not alter Hb subunit composition after 6 days; therefore the experiment was repeated and extended to 14 days. Even after 14 days of normoxic exposure, hypoxic-reared animals retained Hbγ and did not exhibit the Hb subunit pattern of normoxic reared animals (Fig. 4B).

Two-dimensional gel electrophoresis

Hemolymph samples collected from normoxic-reared, hypoxic-reared and hypoxic-transferred *Triops* were run on 2D gels to further elucidate changes in Hb subunit expression. Representative gels of individual animals are shown in Fig. 6. Based on an average of four 2D gels from each group, Hbδ and Hbγ increase from 12% and 0% in normoxic-reared animals to 28% and 22% in hypoxic-reared animals, respectively. Each 2D gel revealed several isoelectric forms of Hb subunits, suggesting a typical phosphorylation train (Halligan et al., 2004).

2D-DIGE and image analysis

The use of 2D-DIGE allows samples from different treatments to be run on the same gel, eliminating the problem of gel-to-gel variation, and allows comparisons to be made using DeCyder

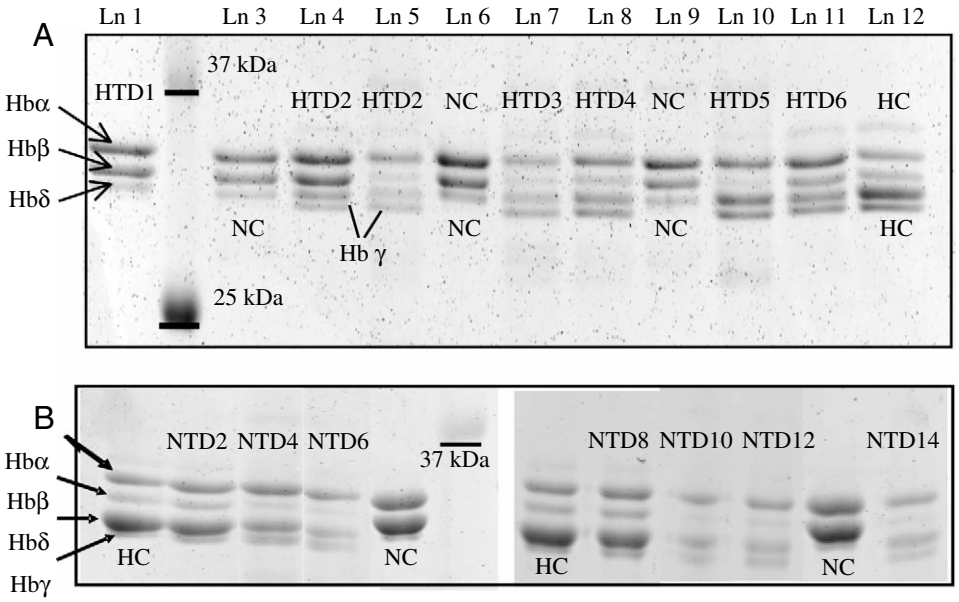

Fig. 4. Representative one-dimensional gels of changes in Hb subunit expression with time spent in either a normoxic or hypoxic environment. (A) Variation in Hb isoforms in normoxic-reared control (NC), hypoxic-reared control (HC) and hypoxic-transferred animals [HTD = hypoxic transfer day (*n*)]. (B) Variation in Hb isoforms in NC, HC and normoxic-transferred animals [NTD = normoxic transfer day (*n*)].

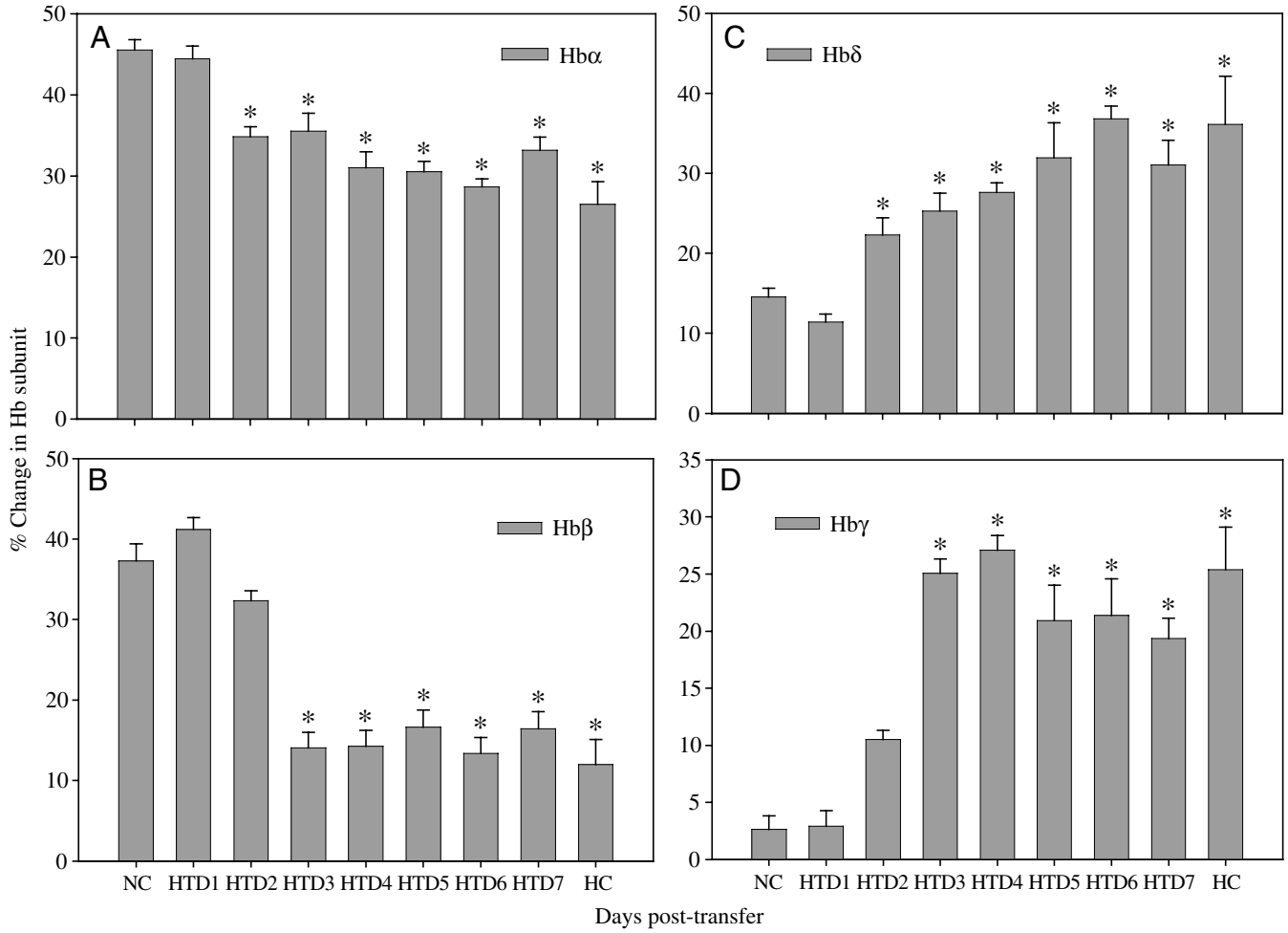

Fig. 5. ImageQuant Analysis of changes in Hb subunit isoforms in animals reared in normoxia and transferred to hypoxia: (A) Hbα; (B) Hbβ; (C) Hbδ and (D) Hbγ. The intensity of each band (Hbα, Hbβ, Hbδ and Hbγ) is expressed as a percentage of the total intensity of all the bands in each lane. Asterisks indicate significantly different from normoxic-reared control (NC) and hypoxic transfer day 1 (HTD1) (P<0.001).

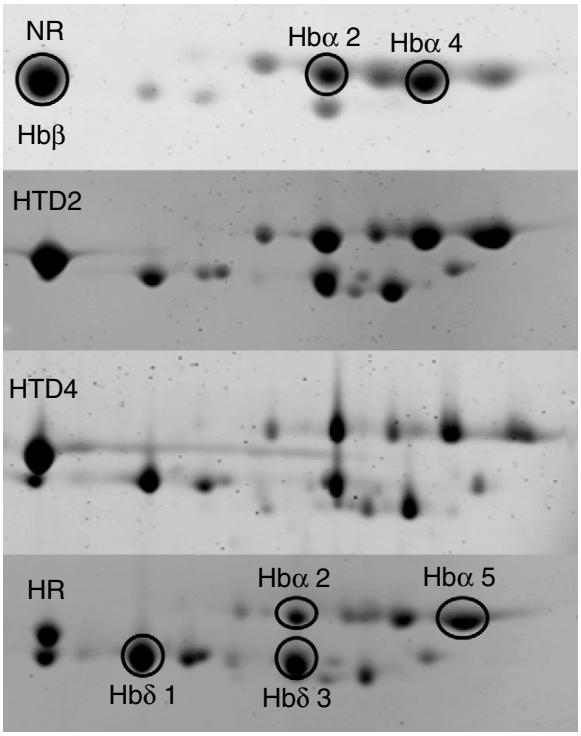

Fig. 6. Two-dimensional gel electrophoresis of the hypoxic induction of *Triops* Hb. Normoxic-reared (NR), hypoxic transfer day 2 and 4 (HTD2, HTD4) and hypoxic-reared (HR). A train of spots corresponded with the molecular masses of Hbα and Hbδ; therefore, more than one of the spots was removed and sequenced for comparison. The lettered circled spots were selected for NH_2-terminal amino acid sequencing.

software (Amersham Biosciences) designed specifically for the analysis of 2D-DIGE. An individual gel scan is shown in Fig. 7A. When the normoxic and hypoxic wavelength scans are combined, hypoxic animals clearly show differential expression of lower molecular mass subunits. The yellow spots (Fig. 7A, top panel) are expressed in both normoxic- and hypoxic-reared animals, while the green spots (those induced by hypoxia) are of lower molecular mass. These are the same lower molecular mass subunits that are significantly increased in both the 1D and 2D gels shown in Figs 4 and 6. DeCyder analysis quantitatively confirms the upregulation of lower molecular mass subunits. The spots outlined in blue (Fig. 7B, top panel) are upregulated 2.5 times or greater. The lower panel of Fig. 7B shows an example of DeCyder analysis of the spots. The spot outlined in pink shows a dramatic increase in volume in the hypoxic gel *versus* the normoxic gel.

NH_2-terminal sequencing

Spots were removed from 2D gels of normoxic- and hypoxic-reared animals for NH_2-terminal sequencing, as shown in Fig. 6. Examination of NH_2-terminal amino acid sequences (Table 1) revealed differences in amino acid sequences between Hb subunits, with the sequences for Hbα, Hbβ and Hbδ being distinctly different. The 'trains' of spots that are of similar molecular mass have similar NH_2-terminal sequences, supporting the hypothesis that these trains of spots are derived from post-translational events, perhaps phosphorylation. Both spots of Hbδ have identical sequences, while there is minor Hbα spot sequence variation.

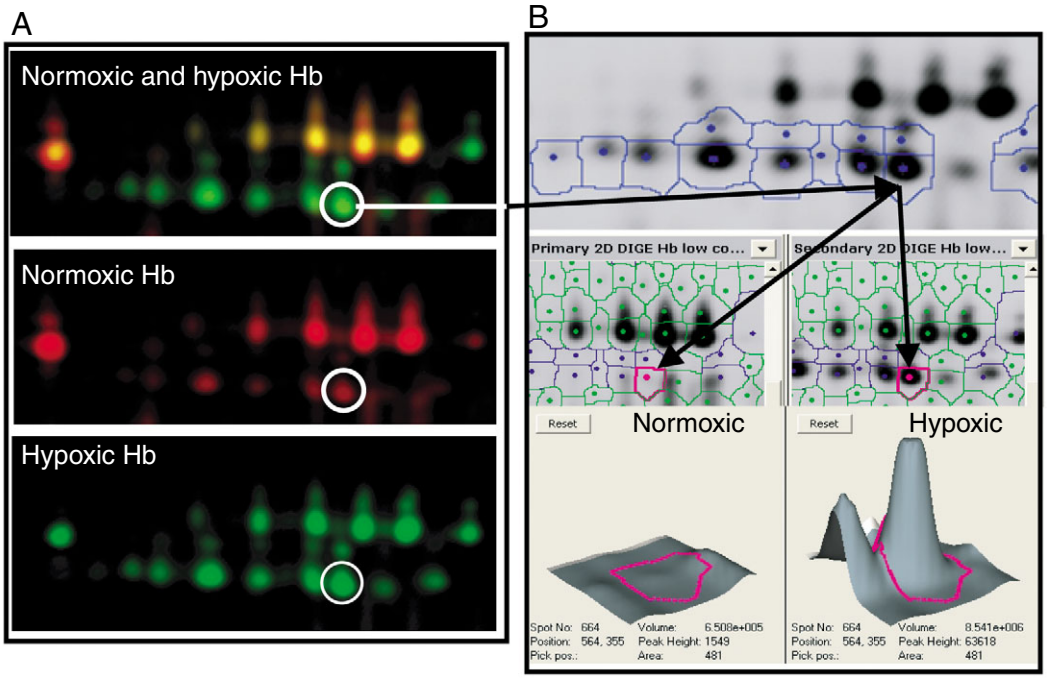

Fig. 7. (A) An example of a 2D-DIGE gel obtained by combining samples of normoxic and hypoxic Hb on the same gel. The gel was scanned at the appropriate wavelength for each fluor. Top panel, combined scan; lower two panels are the separated scans. (B) An example of DeCyder analysis of spots. Areas outlined in blue represent an increase of at least 2.5 times normoxic values. The spot enclosed in pink is the specific area being analyzed by DeCyder software. The lower panels demonstrate the difference in volume between the spot on the normoxic *vs* the hypoxic gel.

Table 1. *NH_2-terminal amino acid sequences of Hb chains isolated from two-dimensional gels*

	Amino acid(s) at position:															
Spot	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Norm Hbα 2	H	P	E	C	A	G	D	S	V	V	V	T	E	T	R/M/T	
Norm Hbα 4	H	R/P	E	C	A/T	G	D	S/V	V	V	V	T	E	T	R	T
Norm Hbβ			G	D	C	G	D	S	V	V	V	T	E	T	R	T
Hyp Hbα 2	H	P	E	C	A	G	D	S	V	V	V	T	ND	T	R	ND
Hyp Hbα 5	H	R	E	C	T	G	D	S	V	V	V	T	E	ND	R	ND
Hyp Hbδ 1				G	R	L/G	D	S/Q	V	V/L	V	T	E	ND	ND	ND
Hyp Hbδ 3				G	R	L/G	D	S	V	V	V	T/I	E	ND	ND	ND

Spots were sequenced from both normoxic and hypoxic-reared animals. Spots are labeled in Fig. 6. If more than one amino acid was detected at the same position, they are shown with a solidus in between. ND, not determined.

Discussion

This study clearly demonstrates that both developing and adult *T. longicaudatus* respond to hypoxia by altering Hb concentration and subunit composition. The correlation between lower oxygen tension and increased Hb concentration has been documented in many animal phyla including the branchiopods *Daphnia* (Green, 1956; Kobayashi and Hoshi, 1982; Kobayashi and Tanaka, 1991; Zeis et al., 2003) and *Artemia* (Gilchrist, 1955; DeWachter et al., 1992) and thus is not surprising in *Triops*. Our values for total hemolymph protein are greater than the [Hb] values previously reported in the literature for field-captured *Triops*: 16.2 mg ml^{-1} by Horne and Beyenbach (1971) and 7 mg ml^{-1} by Scholnick and Snyder (1996). Interestingly, when *Triops* were reared in laboratory conditions, Hb values were elevated compared with field values to 14 mg ml^{-1} in normoxic-reared animals and 20–24 mg ml^{-1} in hypoxic-reared animals (Scholnick and Snyder, 1996). Since the primary hemolymph protein is Hb (Horne and Beyenbach, 1971), total hemolymph protein concentration was used to estimate relative changes in Hb content. This would necessarily elevate the values reported in this study since previous reports determine hemolymph Hb levels based on the spectral properties of heme in hemolymph samples (Horne and Beyenbach, 1971; Scholnick and Snyder, 1996).

Increasing Hb concentration affords the animal an increase in oxygen-carrying capacity (Gilchrist, 1955; Pirow et al., 2001) and, in both *A. salina* and *D. magna*, the increase in Hb concentration during hypoxic stress is accompanied by an increased oxygen affinity (Heip, 1978; Kobayashi et al., 1988). Similarly, using whole hemolymph samples, under physiological conditions, *T. longicaudatus* Hb demonstrates an increase in O_2 binding affinity from P_{50}=1.14 kPa O_2 in normoxic-reared animals to P_{50}=0.5 kPa O_2 in hypoxic-reared animals (1–3 kPa; Harper, 2003). Horne and Beyenbach (1971) reported a P_{50} value of ~0.91 kPa O_2 for hemolymph samples collected from field populations of *T. longicaudatus*.

The differential Hb subunit expression between normoxic- and hypoxic-reared individuals may account for the differences in Hb oxygen-binding affinity in hypoxic-reared individuals. When exposed to chronic hypoxia, normoxic-reared adult *Triops* alter their Hb subunit structure to match that of hypoxic-reared animals. During this transition, hypoxia induces a nearly 10-fold increase in Hbγ by 3 days post-transfer and a mean 3-fold increase in Hbδ. Variation in the concentration of these two subunits largely explains the increases in Hb concentration during hypoxia (Figs 5C,D, 7). DeCyder analysis indicates no downregulation of the heavier subunits, Hbα and Hbβ, and supports the findings of the ImageQuant analysis that the relative drop in Hbα and Hbβ is due to the upregulation of the lower molecular mass subunits, Hbδ and Hbγ.

The relative contribution of Hbα drops 2 days post-transfer but then stabilizes and continues to be an important component of Hb, contributing over 30% to total Hb. Alternatively, the contribution of Hbβ drops sharply from 35% of total Hb to a mean of only 15%. These changes, combined with those of Hbδ and Hbγ, result in Hb profiles that do not differ between normoxic animals 3 days post-transfer to a hypoxic environment and hypoxic-reared animals. Hypoxic induction of differential Hb subunit expression has been demonstrated in the cladoceran *D. magna*, with both up- and downregulation of different Hb subunits (Kobayashi et al., 1988; Zeis et al., 2003). In *A. salina*, all three Hb subunits are upregulated when adult animals are exposed to hypoxia, with the greatest increase in the subunit with the greatest O_2 affinity, Hb III (van den Branden et al., 1978), which is not normally present in adult animals (Heip et al., 1978; Vandenberg et al., 2002).

Separation of Hb on 2D gels revealed a number of isoelectric forms for three of the four different molecular mass subunits. The observed pattern was characteristic of post-translational phosphorylation (Halligan et al., 2004) and was similar to a train of spots that has been reported for *Moina macrocopa* and *D. magna* (Kimura et al., 1999; Kato et al., 2001; Zeis et al., 2003). While post-translational modification may play a role in regulating Hb oxygen affinity, these spots may just as likely be due to differential gene expression, as already demonstrated in *D. magna* exposed to hypoxia (Kimura et al., 1999).

Sequencing of the NH_2-terminal was performed in an effort to assess whether the isoelectric forms were due to post-translational modification or differential gene expression. The differences in NH_2-terminal sequences between Hbα, Hbβ and HBδ suggest that these Hb subunits are produced from

different genes that may be differentially expressed upon hypoxic exposure. The similarity in sequence between Hbδ spots 1 and 3 suggests that these two spots have different isoelectric points due to post-translational modifications. The slight amino acid differences between Hbα 2, 4 and 5 are inconclusive and may be due to either post-translational modification and/or additional gene regulation. NH_2-terminal sequences of Hb in the daphnid species *M. macrocopa* indicate at least three different subunits represented by three genes (Kato et al., 2001). This was confirmed by comparing the amino acid sequences with the amino acid sequences derived from the translation of nucleotide sequences of *M. macrocopa* Hb-encoding genes (Kato et al., 2001). There are currently no genetic data available for such a comparison in *Triops*.

While differential Hb subunit expression is plastic during development and inducible in adulthood, the response was not reversed upon transfer of hypoxic-reared *Triops* to normoxia. Hypoxic-reared *Triops*, when returned as adults to a normoxic environment, showed no changes in the pattern of Hb subunit expression up to 14 days after return to normoxia, nor did Hb concentration decrease significantly (Figs 3B, 4B). In *D. magna* and *A. salina*, differences in Hb concentration are often associated with changes in coloration (Gilchrist, 1954; Kobayashi and Gonoi, 1985). Hypoxic-reared adult *Triops* have a visibly deeper red coloration of their ventral appendages compared with their normoxic-reared counterparts. When normoxic-reared animals are transferred to hypoxia, there is a visible increase in the redness of their ventral appendages; however, there is no obvious decrease in redness when hypoxic-reared animals are transferred to normoxia. In our experiments, coincident with the hatching of *Triops* we observed hatching of the anastrocan *Thamnocephalus platyurus*, and we therefore transferred hypoxic-reared *T. platyurus* along with *Triops*. The *T. platyurus* response is bidirectional in that hypoxic-reared individuals show a decrease in color and reduction in hemolymph protein concentration from 28.3±3.6% to 8.4±1.1% after 10 days in normoxia; hypoxic-reared *T. platyurus* not transferred to normoxic conditions remain dark red (J.A.G., unpublished). Similarly, *A. salina* and *D. magna* lower Hb concentration after a return to high oxygen concentration (Kobayashi and Hoshi, 1982). The mechanism of Hb turnover and/or degradation in branchiopods is not well understood, although hypoxic-reared *Triops* are clearly less responsive to a return to normoxia than *T. platyurus*, *A. salina* or *D. magna*.

Even so, *Triops longicaudatus* demonstrates remarkable developmental plasticity when reared in different oxygen environments, and a hallmark of this response is differential Hb subunit expression. Adults transferred to hypoxia are sensitive to changes in oxygen tension, which induce a change in Hb subunit composition that is similar to the Hb subunits expressed by hypoxic-reared animals. These subunits may be crucial to increasing Hb oxygen-binding affinity of hypoxic animals. The recent discovery that hypoxia-induced Hb synthesis in *D. magna* is HIF (hypoxia inducible factor) dependent (Gorr et al., 2004) could explain the mechanism by which hypoxia induces differential subunit expression in *Triops* as well. The finding that Hb concentration and subunit expression are not reversed upon a return to normoxia in *Triops* merits further investigation into the possible mechanisms and regulation of Hb turnover in branchiopods.

The authors would like to thank Dr Frank van Breukelen for his knowledge and assistance in 2D gel electrophoresis and protein transfer, Ms Kate Shen for her technical assistance in the laboratory techniques used in these experiments and Mr Dan Harkness at Amersham Biosciences for his assistance in the DyCyder analysis. Edman sequencing was performed by the Nevada Proteomics Facility, part of the Nevada Biomedical Research Infra-structure Network (BRIN), which is supported by an award from the National Center for Research Resources (NCRR) (grant # 5P20RR016464-03). This research was also supported by NSF grant EPS-0132556 to S.P.R. and C.L.R. and NSF EPS Graduate Research Grant to J.A.G.

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