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RESEARCH ARTICLE

Hypoxia delays hematopoiesis: retention of embryonic hemoglobin and erythrocytes in larval rainbow trout, *Oncorhynchus mykiss*, during chronic hypoxia exposure

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SUMMARY

In rainbow trout development, a switch occurs from high-affinity embryonic hemoglobin (Hb) and round, embryonic erythrocytes to lower-affinity adult Hb and oval, adult erythrocytes. Our study investigated the early ontogeny of rainbow trout blood properties and the hypoxia response. We hypothesized that hypoxia exposure would delay the ontogenetic turnover of Hb and erythrocytes because retention of high-affinity embryonic Hb would facilitate oxygen loading. To test this hypothesis we developed a method of efficiently extracting blood from individual embryos and larvae and optimized several techniques for measuring hematological parameters on microliter (0.5–2.0 µl) blood samples. In chronic hypoxia (30% of oxygen saturation), stage-matched embryos and larvae possessed half the Hb concentration, erythrocyte counts and hematocrit observed in normoxia. Hypoxia-reared larvae also had threefold to sixfold higher mRNA expression of the embryonic Hb α-1, β-1 and β-2 subunits relative to stage-matched normoxia-reared larvae. Furthermore, in hypoxia, the round embryonic erythrocytic shape persisted into later developmental stages. Despite these differences, Hb–oxygen affinity (P_{50}), cooperativity and the Root effect were unaltered in hypoxia-reared *O. mykiss*. The data support our hypothesis that chronic hypoxia delays the ontogenetic turnover of Hb and erythrocytes, but without the predicted functional consequences (i.e. higher than expected P_{50}). These results also suggest that the Hb–oxygen affinity is protected during development in chronic hypoxia to favor oxygen unloading at the tissues. We conclude that in early trout development, the blood–oxygen transport system responds very differently to chronic hypoxia relative to adults, possibly because respiration depends relatively more on oxygen diffusion than convection.

Key words: embryo, erythrocytes, hemoglobin, hypoxia, larva, rainbow trout.

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INTRODUCTION

All vertebrate species undergo ontogenetic changes in Hb isoform expression (Wood, 1988). In the rainbow trout [*Oncorhynchus mykiss* (Walbaum 1792)], two distinct Hb polymorphs are present during ontogeny: embryonic Hb (Hb_E) and adult Hb (Hb_A) (Iuchi and Yamagami, 1969). Hb_E is only found inside round, embryonic erythrocytes, which are produced in the intermediate cell mass and blood islands on the yolk sac (Iuchi and Yamamoto, 1983). Embryonic erythrocytes are the only erythrocyte in the circulation until 1 day before hatching, when a turnover of embryonic for oval, adult erythrocytes begins. By 1 day post-hatch (15°C), erythropoiesis is initiated in the kidney and spleen, which produce immature adult erythrocytes that ultimately grow into mature adult erythrocytes (Iuchi and Yamamoto, 1983). Post-hatching alevins thus possess a mixture of both Hb polymorphs and as development proceeds, the proportion of Hb_E decreases and that of Hb_A increases (Iuchi, 1973a; Iuchi and Yamamoto, 1983).

Previous research has shown that the blood–O_2 binding properties in early *O. mykiss* development differ from those in adults. At 25–28°C, Hb_E exhibits a higher O_2 affinity (lower P_{50}) and cooperativity (Hill coefficient, n_H), a lower Bohr effect and no Root effect compared with Hb_A (Iuchi, 1973b). However, the O_2-binding properties of Hb_E at lower temperatures, more representative of *O. mykiss*'s typical physiological temperatures, are unknown. Decreased temperature is known to lower the P_{50} and n_H and to raise the Bohr effect in Hb_A (Irving et al., 1941; Cameron, 1971; Eddy, 1971; Weber et al., 1976; Vorger, 1985; Willford and Hill, 1986). Whether a lower temperature would have a similar effect in Hb_E has yet to be investigated. It is important to note that Hb_E is an anodal Hb and is distinct from the cathodal Hb isoforms of adult trout, which also possess a high O_2 affinity and no Bohr effect (Iuchi and Yamagami, 1969; Iuchi, 1973a; Maruyama et al., 1999; Fago et al., 2001). Therefore, like the anodal Hbs in adult rainbow trout (Jensen et al., 1998), it is possible that Hb_E is allosterically modified by organic phosphates.

Traditionally, it has been assumed that the initial ontogenetic appearance of convective Hb–O_2 transport is temporally synchronized with the onset of the heart beat and blood circulation. However, a growing body of work questions the role of Hb in O_2 uptake in early development. In larval rainbow trout and zebrafish (*Danio rerio*), functional ablation of Hb with carbon monoxide had little effect on heart rate, ventilation frequency or O_2 consumption (Holeton, 1971; Pelster and Burggren, 1996). In addition, *O. mykiss* larvae survive until the fry stage following phenylhydrazine-induced hemolysis (Iuchi, 1985). This raises the question: what is the physiological role of Hb prior to the need for convective O_2 transport?

Alternate roles for Hb_E during embryonic and larval development have been proposed. Pelster and Burggren (Pelster and Burggren, 1996) suggested that Hb is required for the initial inflation of the swim bladder *via* the Root effect in fishes that do not gulp air; however, the absence of a Root effect in Hb_E (Iuchi, 1973b; present

study) points to an alternative function in *O. mykiss*. Rombough and Drader (Rombough and Drader, 2009) found that when zebrafish larvae [7–14 days post fertilization (dpf)] gradually depleted the dissolved O_2 in a closed respirometer, the residual O_2 level (the amount of O_2 remaining in the respirometer that the fish was unable to consume) was higher in larvae poisoned with carbon monoxide. They concluded that Hb_E assists with O_2 uptake in larval zebrafish during extreme hypoxia. It is possible that in less hypoxia-tolerant species, such as *O. mykiss*, Hb_E contributes to O_2 binding and transport during hypoxia, but this is unknown. Alternatively, Hb_E may also play a role in embryonic vasodilation *via* nitric oxide (NO). In adult vertebrates, NO is transported and may also be produced by Hb_A (Jensen, 2004). It is released from circulating erythrocytes in response to low tissue O_2 levels and triggers vasodilation (Allen and Piantadosi, 2006). Similar to adults, NO induces vasodilation in zebrafish larvae (Fritsche et al., 2000); it is thus possible that similar to Hb_A, Hb_E is also involved in NO transport and production (Pelster et al., 2010). Pelster et al. (Pelster et al., 2010) also suggest that because of its higher O_2 affinity, Hb_E may play a role in O_2 storage and buffering in early development. Hb_E and myoglobin have similar O_2 affinities (Wittenberg and Wittenberg, 2007) and therefore, like myoglobin, Hb_E may only unload O_2 during extreme hypoxia or help regulate intracellular O_2 levels in order to maintain P_{O_2} gradients between the sarcoplasma and mitochondrion (Ordway and Garry, 2004). The high-affinity Hb_E may also help to scavenge reactive oxygen species (Pelster et al., 2010). This would be of particular importance in early embryonic development, as embryos are more sensitive to reactive oxygen species than adults (Massabuau, 2001; Hassoun et al., 2005). Another suggestion by Iuchi (Iuchi, 1985) is that Hb_E is a byproduct of embryonic erythropoietic stem cell production, which is required for the subsequent development of adult erythropoietic stem cells, but no follow up studies have been performed.

The first objective of this study was to investigate the early ontogeny of rainbow trout blood properties and the hypoxia response. Hypoxia has the potential to elicit plasticity in the timing of the onset of ontogenetic events, a phenomenon termed heterokairy (Spicer and Burggren, 2003). This has been observed previously in rainbow trout, where chronic hypoxia (30% of O_2 saturation from the day of fertilization) delayed the onset of cardiac cholinergic control (Miller et al., 2011). We hypothesized that *O. mykiss* are also capable of plasticity in the ontogenetic onset of Hb turnover when O_2 is limiting in the environment. Presumably, the properties that distinguish Hb_E from Hb_A, such as the higher O_2 affinity relative to Hb_A (Iuchi, 1973b), could be exploited in hypoxia to help with O_2 loading. Rombough and Drader (Rombough and Drader, 2009) postulated that Hb assists with O_2 uptake in severe hypoxia in early development. Therefore, we predicted a delay in the ontogenetic turnover of Hb_E for Hb_A in hypoxia. We thus anticipated higher concentrations of Hb_E during hypoxia exposure and, consequentially, an elevated Hb–O_2 affinity and cooperativity and lower Bohr and Root effects in hypoxia-reared relative to normoxia-reared larvae. The second objective of this study was to determine the O_2-binding properties of Hb_E at a lower, more physiologically representative temperature.

MATERIALS AND METHODS

Experimental animals

Rainbow trout embryos were obtained on the day of fertilization from Rainbow Springs Trout Farm (Thamesford, ON, Canada) and were transferred to the Hagen Aqualab (University of Guelph, Guelph, ON, Canada). Embryos were held on a mesh-bottom insert within custom-built 4 l treatment tanks, which were shielded from light and supplied with a continuous flow (~20 ml min^{-1}) of local well water [10°C, 10 mg O_2 l^{-1}, pH 7.9, water hardness 411 mg l^{-1} as $CaCO_3$, ion concentrations (mmol l^{-1}): 2.6 Ca^{2+}, 1.5 Cl^-, 1.5 Mg^{2+}, 0.06 K^+ and 1.1 Na^+]. Over a 14 month period, eight different batches of embryos were used. Each batch was derived from a separate spawning event between three females and three males. Embryos were staged according to Vernier (Vernier, 1969).

Experimental protocol

Treatment conditions

From the day of fertilization, each batch of embryos was randomly divided into four tanks (4 l); half were supplied with normoxic water (100% of O_2 saturation) and half were subjected to chronic hypoxia (30% of O_2 saturation). This level of O_2 saturation has been observed previously in salmonid redds (Coble, 1961; Peterson and Quinn, 1996; Youngson et al., 2004) and was shown to significantly affect metabolic rate and cardiac development in embryonic rainbow trout (Miller et al., 2008; Miller et al., 2011). Hypoxia was generated by introducing N_2 gas into a header tank (~16 l). Dissolved O_2 was monitored daily in the normoxic (98.7±0.4%) and hypoxic (29.7±0.5%) treatment tanks throughout the experiment (Hach LDO101 electrode connected to Hach HQ30d meter, Hach Company, Mississauga, ON, Canada).

Because hypoxia exposure delays salmonid development (Shumway et al., 1964; Hamor and Garside, 1976; Ciuhandu et al., 2005; Miller et al., 2008), embryo sampling was stage-matched for the two treatment groups. Embryos were sampled at Vernier Stages 27 (prehatch; circulatory system is formed and functioning), 30 (hatch), 32 and 33 (in preliminary experiments a large increase in P_{50} was observed between these stages), and 35 (near-complete yolk absorption; Fig. 1). These sampling points allowed for an assessment

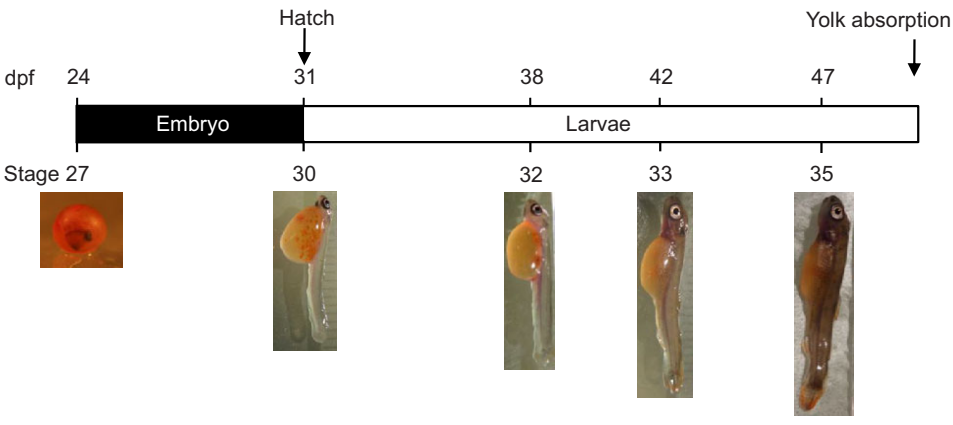

Fig. 1. Timeline in days post fertilization (dpf) of early rainbow trout (*Oncorhynchus mykiss*) developmental stages (Vernier, 1969) in normoxia (10°C).

of blood properties before and after the commencement of Hb turnover from embryonic to adult isoforms, which begins 1 day before hatch (Vernier stage 29) (Iuchi and Yamamoto, 1983). With yolk removed, embryo tissue mass and length were recorded at each stage.

Blood collection

Embryos were dechorionated and embryos and alevins were kept on ice during blood collection to prevent blood clotting. The tail was severed with a razor blade and whole blood was drawn by capillary action into either a 60 mm heparinized microhematocrit tube (VWR International LLC, Mississauga, ON, Canada) or into a 5 µl unheparinized glass micropipette (Drummond Scientific Company, Broomall, PA, USA). Ammonium heparin and EDTA were found to affect O_2 dissociation curves in microliter blood samples. Therefore, untreated tubes were used to collect blood for O_2 affinity measurements and heparinized tubes were used to collect blood for all other measurements; however, we found that cooling the blood was sufficient to prevent clotting and only unclotted samples were used.

Analyses

Blood–O_2 affinity

O_2 equilibrium curves (OEC) were determined using a custom-built spectrophotometer ($P_{wee}50$, La Trobe University, Melbourne, Australia), as described previously (Clark et al., 2008). The $P_{wee}50$ is composed of two parts: a gas mixer and an analyzer. The gas mixer mixes compressed O_2, CO_2 and N_2 according to user-defined parameters. Constant, low flows of gas mixtures from the gas mixer to the analyzer are controlled by a solenoid. Gas delivered to the analyzer is humidified before it enters a temperature-controlled, gas-tight sample chamber. The sample chamber contains light-emitting diodes (LEDs) at 435 and 390 nm, approximately the peak absorption for deoxygenated Hb (Iuchi, 1973b) and the isosbestic point [i.e. the wavelength at which absorption is independent of O_2 saturation (Hoxter, 1979)] between oxy- and deoxy-Hb, respectively. Whole-blood samples (~0.5 µl) were smeared between two 6 µm gas-permeable polyethylene membranes (Glad Go-Between Freezer Film, Hobart, Tasmania, Australia) held taut over a ring-shaped sample holder with a neoprene O-ring. The sample holder was placed into the sample chamber, with the blood sample positioned between the LEDs and a spectrophotometer, which records the sample absorbance at each wavelength. Within the sample chamber, OEC measurements were performed at 10°C. First, the P_{100} (absorbance at maximum percentage O_2 saturation, using P_{CO_2} 0.2 kPa, P_{N_2} 77.8 kPa and P_{O_2} 22 kPa) and P_0 (absorbance at P_{O_2} 0 kPa, using P_{CO_2} 0.2 kPa and P_{N_2} 99.8 kPa) were recorded. The sample was then flushed with stepwise changes in O_2 and N_2: O_2 saturation was increased by 0.5 kPa until 22 kPa was reached and total gas pressure was balanced with decreasing levels of N_2. At each O_2 saturation increment, the absorbance at 390 and 435 nm was recorded and used to construct an O_2 saturation curve. This was repeated at P_{CO_2} 0.4 and 1.2 kPa in order to determine the extent of the Bohr and Root shifts in Hb from each blood sample. The three CO_2 levels 0.2, 0.4 and 1.2 kPa represent the arterial, venous and post-exercise P_{CO_2}, respectively, in adult rainbow trout (Stevens and Randall, 1967; Nikinmaa and Soivio, 1979; Currie and Tufts, 1993). Using these O_2 saturation curves, the P_{50}, n_H and Root effect were automatically calculated using a manufacturer-provided Excel spreadsheet.

The extent of the Bohr effect was calculated from P_{CO_2} 0.2 to 0.4 kPa and from 0.2 to 1.2 kPa using the formula (Bohr et al., 1904):

$$\text{Bohr effect} = \Delta\log P_{50} / \Delta\text{pH} . \quad (1)$$

Because of the small volumes of each blood sample, measurements of P_{50} and pH were not possible in the same sample. Therefore, individual changes in P_{50} within the same treatment group and developmental stage were divided by an averaged ΔpH value from stage- and treatment-matched embryos.

Whole-blood pH was measured following methods similar to those of Patrick et al. (Patrick et al., 1997) and Wood et al. (Wood et al., 2010). Blood was extracted from six embryos (~6 µl total collected over ~2 min), pooled in a 0.5 ml microcentrifuge tube and covered with Parafilm (Fisher, Markham, ON, Canada). Blood samples were sequentially equilibrated to humidified P_{CO_2} 0.2, 0.4 and 1.2 kPa (10°C) using Wöstoff gas-mixing pumps (Bochum, Germany). In preliminary experiments, it was found that 5 min incubation at each P_{CO_2} was sufficient to allow for thorough gas mixing with the blood. Between incubation periods, a micro pH electrode (MI-710 combination pH electrode, Microelectrodes, Inc., Bedford, NH, USA) was used to measure pH and was calibrated using pH 4.0, 7.0 and 10.0 buffers (Fisher Scientific, Fair Lawn, NJ, USA).

Erythrocyte measurements

All microscopic measurements were made using a digital camera mounted on a light microscope (Leica DM1000, Leica Microsystems, Inc., Concord, ON, Canada) and OpenLab software (Improvision Incorporated, Lexington, KY, USA). To determine erythrocyte morphology, whole-blood samples (~1 µl) were diluted on a glass slide in Cortland's isotonic saline (1 µl) (Wolf, 1963). Slides were examined immediately (<5 min). The height and width of 20 erythrocytes per slide were measured, and erythrocyte shape was quantified by calculating erythrocyte height to width ratios (H:W). Preliminary results showed that the round embryonic erythrocytic shape persisted into larval stages in hypoxic *O. mykiss*. In adult *O. mykiss*, erythrocyte morphology is altered by elevated levels of catecholamines in hypoxia (Nikinmaa and Huestis, 1984). Therefore, we investigated whether catecholamines were responsible for the observed variations in erythrocytic morphology in early development by incubating blood samples in a 3× volume of adrenaline (10^{-4} mol l^{-1}; Sigma-Aldrich, St Louis, MO, USA) dissolved in Cortland's saline (Wolf, 1963) or in Cortland's saline without adrenaline for 30 min at room temperature. Preliminary experiments [as well as previous work (Tufts and Randall, 1989; Nikinmaa and Huestis, 1984)] revealed that this incubation time was sufficient to allow for significant erythrocytic swelling in adult *O. mykiss* blood. The projected erythrocyte surface area was then measured using OpenLab software. All measurements were calibrated using a stage micrometer (Nikon Stage Micrometer A MBM11100 1 mm, Nikon, Mississauga, ON, Canada).

The erythrocyte counts were determined by preparing a blood dilution (1:300) using Cortland's isotonic saline (Wolf, 1963). Erythrocytes were counted using a standard hemocytometer (Strober, 1997) where every red blood cell (RBC) in the counting area was tallied.

For hematocrit (Hct) measurements, blood was centrifuged at maximum speed for 2 min in an International Clinical Centrifuge (model CL, International Equipment Co., Needham, MA, USA). To accurately determine Hct values on microliter blood samples, pictures of the microhematocrit tubes were taken using a digital camera (Lumix DMC-ZS3, Panasonic Canada Inc., Mississauga, ON, Canada) and analyzed as the percentage of erythrocytes in the sampled blood volume using ImageJ imaging software (US National Institutes of Health, Bethesda, MD, USA).

Mean corpuscular volume (MCV), mean corpuscular Hb (MCH) and mean corpuscular Hb concentration (MCHC), corresponding

to the size of the erythrocytes, the amount of Hb per erythrocyte and the amount of Hb per unit volume, respectively, were calculated using the standard formulae (Sarma, 1990).

Hb isoform expression

To determine the expression of Hb_E mRNA, total RNA was extracted from whole embryos and larvae. RNA extraction and cDNA synthesis were performed following the methods of Essex-Fraser et al. (Essex-Fraser et al., 2005), where an extra TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA) extraction step was added to remove extra insoluble materials associated with yolk proteins. Real-time PCR was performed on cDNA products using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). Each PCR reaction contained 12.5 µl 2× QuantiTect SYBR Green PCR Master Mix (Qiagen, Toronto, ON, Canada), 5.5 µl RNase-free water (Sigma-Aldrich), 5 µl cDNA template or no-RT controls, and 1 µl each of forward and reverse primers. PCR was performed according to the manufacturer's protocol. A dissociation cycle was performed from 60 to 90°C to ensure that the amplification signal resulted from a single PCR product. To account for differences in amplification efficiency, standard curves were constructed for each primer using serial dilutions of pooled cDNA from randomly selected whole rainbow trout embryos. The relative dilution of each unknown sample was extrapolated by linear regression of the target-specific standard curve using the sample's threshold cycle. To correct for differences in RNA loading and reverse transcriptase efficiencies, each sample was normalized to the expression level of the housekeeping gene elongation factor 1 alpha (EF1-α), which did not significantly vary across developmental stages or with hypoxia treatment and has been used previously as a housekeeping gene in early rainbow trout development (Coulibaly et al., 2006). Samples were run in triplicate with only one target gene assayed per well. Non-reverse transcribed RNA and water-only controls were run to ensure that reagents were not contaminated and that no genomic DNA was being amplified.

Primer sets were designed based on the coding sequences for *O. mykiss* EF1-α (Aegerter et al., 2004) and rainbow trout Hb_E subunits α-1, α-2, β-1 and β-2 (Maruyama et al., 1999) using PrimerExpress 2.0 software (Applied Biosystems). Primer pairs and accession numbers are listed in Table 1. We were unable to design primers specific for Hb_A as there are discrepancies in the literature regarding the number of Hb_A isoforms that exist in adult rainbow trout, reporting anywhere from four to nine distinct isoforms (Binotti et al., 1971; Weber et al., 1976; Ronald and Tsuyuki, 1971; Fago et al., 2001). In addition, there is too much sequence similarity between the Hb_A isoforms and the Hb_E isoforms to specifically target Hb_A and not Hb_E (Bossa et al., 1976; Maruyama et al., 1999).

Whole-blood Hb concentration was quantified by the cyanmethemoglobin method modified for small volumes (0.5–2.0 µl). Blood samples were transferred to 1.5 ml centrifuge tubes containing 1 µl EDTA. For each set of samples, a standard curve was made by serially diluting Hb standard (Pointe Scientific, Inc., Canton, MI, USA) in Cortland's isotonic saline (Wolf, 1963); each standard dilution also contained 1 µl EDTA. Samples and standards were mixed with 0.5 ml modified Drabkin's reagent (Nestel and Taylor, 1997). Following incubation at room temperature for 5 min, absorbance was measured spectrophotometrically (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA) at 540 nmol l^{-1}. The concentration of each sample was extrapolated by linear regression using the Hb standard curve, while correcting for the initial blood volume.

Statistical analysis

Because only small blood volumes could be collected from rainbow trout embryos, it was not possible to perform repeated measurements on each individual. As a result, averaged Hb, Hct and erythrocyte counts were used to calculate the RBC indices MCV, MCH and MCHC. Consequently, raw data were not available for these variables and statistical comparisons were not possible.

Three-way ANOVAs were used to determine the overall effects of developmental stage, O_2 treatment and P_{CO_2} on pH, P_{50} and n_H. A three-way ANOVA was also used to determine the overall effects of developmental stage, O_2 treatment and adrenaline treatment on erythrocyte surface area. A one-way repeated-measures ANOVA was used to determine statistical differences in time to reach each developmental stage among O_2 treatments. Two-way ANOVAs were employed to assess the overall effects of developmental stage and O_2 treatment on the mean values of all remaining data. In the case of the Bohr and Root effects, this involved analyzing the data separately for each CO_2 level. Data for mass, Hb concentration, P_{50} and Hb_E mRNA expression were log transformed in order to meet the assumptions of normality and equal variance. Where significant effects were present, factors were compared using Tukey's *post hoc* analyses. Statistical analyses were performed using SigmaPlot version 11.0 (Systat Software, Inc., San Jose, CA, USA). All statistical tests were performed at the 0.05 level. Estimates are reported as means ± s.e.m.

RESULTS

Oncoryhynchus mykiss growth and metabolism

In normoxia-reared *O. mykiss*, tissue mass (without yolk) increased ninefold between Stages 27 (24 dpf) and 35 (47 dpf; Fig. 2). In all except Stage 33, tissue mass was significantly reduced (20–50%) in hypoxic embryos and larvae relative to stage-matched normoxic embryos and larvae ($P<0.01$); however, there was still an eightfold increase in tissue mass from Stage 27 (29 dpf) to Stage 35 (62 dpf) in hypoxia. Overall, development was delayed by ~15 days to reach Stage 35 in hypoxia-reared relative to normoxia-reared rainbow trout ($P<0.05$). There was no effect of hypoxia treatment on tissue lactate levels at any developmental stage ($P=0.2$, data not shown).

Hb–O_2 binding and carrying capacity

The shape of the OEC was altered by development and P_{CO_2}, but not by hypoxia (Fig. 3). The OEC shifted to the right as development progressed. In addition, maximum blood O_2 saturation was reduced

Table 1. Primer set sequences used for real-time PCR

Target	GenBank accession no.	Forward primer	Reverse primer	Size (bp)
EF1-α	AF498320	AGCGCAATCAGCCTGAGAGGTA	GCTGGACAAGCTGAAGGCTGAG	160
Hb_E α-1	AB015448	GCCCTGGCTCTGTCTGAGAA	ATTGGCACCCTACAAAGATTGTC	74
Hb_E α-2	AB015449	CTTCAAGATCATCAACCACAACATC	GGGTAAAGTCGTCAGGGAACAG	64
Hb_E β-1	AB015450	CACTAGAGCCCCTCCACTCAGA	AACATGTGCCTCTAAACAAATGATCT	146
Hb_E β-2	AB015451	CTTCAAGATCATCAACCACAACATC	GGGTAAAGTCGTCAGGGAACAG	97

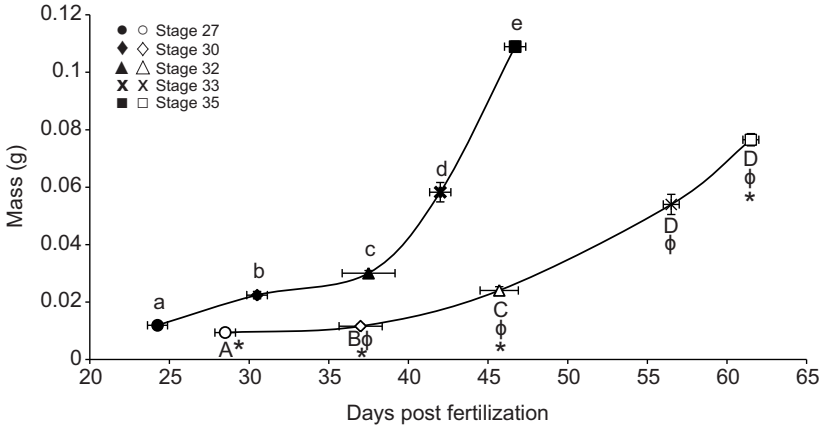

Fig. 2. Changes in tissue mass (yolk removed) and developmental rate in rainbow trout (*Oncorhynchus mykiss*) embryos and larvae reared in normoxia (100% of O_2 saturation; filled symbols) and hypoxia (30% of O_2 saturation; open symbols). Different letters (lowercase, normoxia; uppercase, hypoxia) indicate significant differences in mass among developmental stages, and asterisks indicate significant differences in mass from the normoxic group. ϕ indicates significant differences in time of development to a specific stage from the normoxic group. Data are presented as means ± s.e.m. (N=10 for mass and 3 for developmental rate).

with increasing P_{CO_2} and these reductions became more pronounced in later developmental stages.

Developmental stage and CO_2 level significantly affected P_{50} (three-way ANOVA, developmental stage $F_{4,33}$=142.01, P<0.001; CO_2 level $F_{2,22}$=45.89, P<0.001; Table 2). There was a twofold increase in P_{50} between Stages 27 and 35 (P<0.001). Changes in P_{CO_2} from 0.2 to 0.4 kPa significantly increased P_{50} at Stages 33 and 35 (P<0.001), while changes in P_{CO_2} from 0.2 to 1.2 kPa significantly increased P_{50} at all developmental stages (P<0.001). P_{50} was unaffected by hypoxia treatment (P=0.1; Table 2).

From Stage 27 to 35, there was a 30% (P_{CO_2} 0.2 to 0.4 kPa, P<0.03) to 40% (0.2 to 1.2 kPa, P<0.002) decrease in maximum O_2 saturation (Root effect; Table 2). There was a significant increase in the Bohr effect between Stages 27 and 35 at both P_{CO_2} levels (0.2 to 0.4 kPa P<0.02; 0.2 to 1.2 kPa P<0.001; Table 2). Hypoxia treatment did not alter the Root effect at either P_{CO_2} level (P=0.3) or the Bohr effect at higher P_{CO_2} (P=0.8). However, at 0.4 kPa, the Bohr effect was twofold to threefold higher in hypoxia-reared larvae at Stages 32 (P=0.012) and 33 (P<0.001) relative to normoxia-reared larvae.

Between Stages 27 and 35 in normoxic development, there was a decrease in n_H of 27% at P_{CO_2} 0.2 kPa (P<0.001), 37% at 0.4 kPa (P<0.001) and 34% at 1.2 kPa (P<0.001; Table 2). We also observed an 18% reduction in n_H at Stage 30 when P_{CO_2} was raised from 0.2 to 1.2 kPa (P=0.02). Hypoxia treatment had no effect on n_H at any P_{CO_2} level (P=0.28).

Blood parameters and RBC indices

In normoxia, whole-blood Hb concentration and Hct significantly decreased at Stages 30–32 relative to earlier stages (P=0.04), but recovered by Stages 33–35 (whole-blood Hb P<0.001, Fig. 4A; whole-blood Hct P<0.04, Fig. 4B). In contrast, erythrocyte counts significantly increased (+50%) between Stages 27 and 35 (P<0.001; Fig. 4C). In hypoxia-reared embryos and larvae, there was a profound reduction in whole-blood Hb concentration (38–53%), Hct (17–65%) and erythrocyte counts (33–65%) relative to normoxia (P<0.001; Fig. 4). Hb concentrations and erythrocyte counts followed a similar ontogenetic pattern in hypoxia relative to normoxia (Fig. 4A,C). We also observed a 1.7-fold increase in Hct between Stages 27 and 32 in hypoxia (P=0.02), and Hct remained stable from Stage 32 onward (P>0.96, Fig. 4B).

MCV and MCH decreased 2.5- and 1.7-fold, respectively, between Stages 27 and 35 during normoxic development (Table 3). However, MCHC showed no clear ontogenetic pattern, but increased by 45% between Stages 33 and 35. Hypoxia treatment did not appear to alter RBC indices relative to the normoxic group. From Stage 27 to 35 in hypoxia, we observed a decrease in MCV (–67%) and MCH (–75%). Additionally, MCHC decreased between Stages 27 and 30 in hypoxia-reared embryos (–28%) and there were no notable changes in later developmental stages.

Erythrocyte morphology

The H:W of erythrocytes from normoxia-reared embryos and larvae was low and stable between Stages 27 and 32, but then significantly increased (+12%, P<0.001; Fig. 5A). In hypoxia, the H:W of erythrocytes was significantly reduced at Stages 33 (–7%) and 35 (–9%, P<0.001) relative to erythrocytes from normoxia-reared individuals. Fig. 5B and 5C show oval erythrocytes from normoxia-

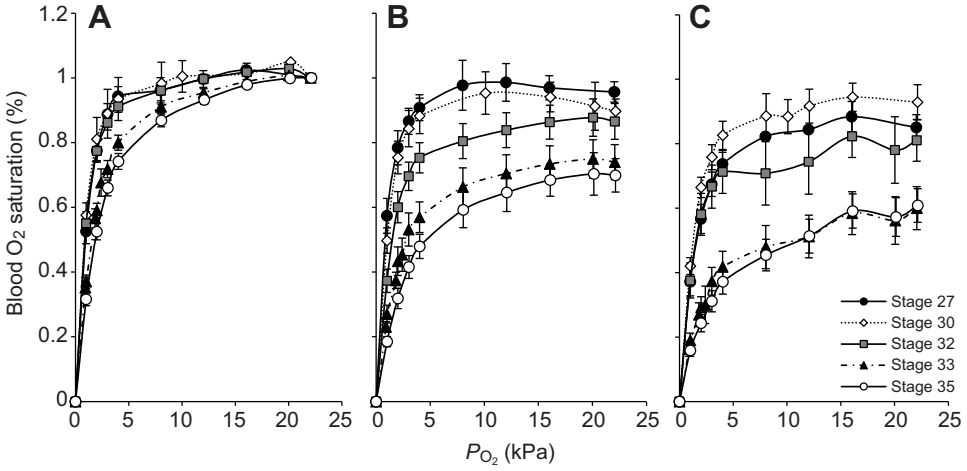

Fig. 3. Changes in Hb–O_2 dissociation curves in rainbow trout (*Oncorhynchus mykiss*) embryos and larvae with development and with P_{CO_2} increases from 0.2 kPa (A) to 0.4 (B) and 1.2 kPa (C). O_2 treatment had no effect on dissociation curves and therefore normoxia (100% of O_2 saturation) and hypoxia (30% of O_2 saturation) data were pooled. Data are presented as means ± s.e.m. (N=6–10).

Table 2. Oxygen transport variables (P_{50}, Hill coefficients, and Bohr and Root effects) at 0.2, 0.4 and 1.2 kPa CO_2 in rainbow trout (*Oncorhynchus mykiss*) reared in normoxia (100% of O_2 saturation) or hypoxia (30% of O_2 saturation) over a developmental series

		Normoxia			Hypoxia		
	Developmental stage	0.2 kPa CO_2	0.4 kPa CO_2	1.2 kPa CO_2	0.2 kPa CO_2	0.4 kPa CO_2	1.2 kPa CO_2
P_{50} (kPa)	27	1.13±0.13^{a}	0.90±0.08^{a,b}	1.37±0.16$^{a,+}$	0.93±0.07	0.87±0.08	1.41±0.16
	30	0.91±0.04^{a}	1.15±0.16^{a}	1.18±0.07$^{b,+}$	0.79±0.03	0.95±0.05	1.16±0.09
	32	0.98±0.07^{b}	1.27±0.12^{b}	1.33±0.11$^{a,b,c,+}$	1.00±0.08	1.31±0.07	1.29±0.13
	33	1.58±0.12^{c}	1.67±0.13$^{c,+}$	2.15±0.13$^{c,d,+}$	1.60±0.10	1.80±0.14	2.26±0.10
	35	2.10±0.13^{c}	2.67±0.19$^{c,+}$	3.17±0.14$^{d,+}$	1.89±0.11	2.19±0.15	2.84±0.14
Hill coefficient	27	1.88±0.10^{a}	2.02±0.13^{a}	1.87±0.14^{a}	1.88±0.15	1.86±0.29	1.65±0.17
	30	1.94±0.11^{a,b}	1.74±0.15^{a,b}	1.60±0.08$^{a,b,+}$	1.59±0.11	1.64±0.13	1.43±0.16
	32	1.44±0.07^{b,c}	1.39±0.07^{b,c}	1.37±0.05^{b}	1.69±0.07	1.48±0.07	1.46±0.06
	33	1.49±0.07^{c}	1.34±0.05^{c}	1.23±0.05^{b}	1.34±0.04	1.23±0.03	1.22±0.07
	35	1.38±0.06^{c}	1.27±0.07^{c}	1.23±0.07^{b}	1.49±0.04	1.35±0.04	1.26±0.04
Bohr effect	27		0.02±0.10^{a}	−0.47±0.13^{a,b}		−0.43±0.12^{A}	−0.41±0.14
	30		−0.51±0.10^{a,b}	−0.68±0.14^{a,b}		−0.63±0.18^{A,B}	−0.65±0.11
	32		−0.42±0.18^{a,b}	−0.50±0.10^{a}		−1.0±0.2^{A,B,*}	−0.38±0.07
	33		−0.43±0.04^{a,b}	−0.90±0.15^{b,c}		−1.4±0.3^{B,*}	−0.99±0.04
	35		−0.79±0.12^{b}	−0.99±0.14^{c}		−0.82±0.14^{A,B}	−1.1±0.1
Max. Hb saturation (%) (Root effect)	27		97.0±1.1^{a}	81.1±2.3^{a}		90.6±2.8	87.6±4.9
	30		87.7±3.3^{a}	89.4±3.4^{a}		87.0±4.2	94.3±3.8
	32		79.3±7.3^{a,b}	80.6±7.5^{a}		88.8±1.8	80.4±5.0
	33		71.4±5.9^{b,c}	58.4±6.7^{b}		71.0±4.2	61.5±6.6
	35		66.0±4.7^{c}	62.0±6.0^{b}		73.0±5.0	59.8±4.4

Data are presented as means ± s.e.m. (N=6–10).

Different superscripted lowercase and uppercase letters represent significant differences in the normoxic and hypoxic groups, respectively. + Indicates significant differences from 0.2 kPa CO_2. * Indicates significant differences from the normoxic group.

reared Stage 35 larvae and round erythrocytes from hypoxia-reared Stage 35 larvae, respectively.

In normoxic development, the projected surface area increased after Stage 27, was highest at Stage 30 and decreased thereafter (P<0.001; Fig. 6). Adrenaline caused erythrocytes to swell in normoxia-reared *O. mykiss*, which was significant at Stage 32 (P<0.001). In hypoxia, the projected surface area significantly increased after Stage 27, was highest at Stage 32 (P<0.001) and returned to initial values by Stage 35 (P=1.00). The projected surface area of erythrocytes from hypoxia-reared *O. mykiss* was greater at all developmental stages, from 11% at Stage 27 to 38% at Stage 33, relative to Cortland's-treated normoxic erythrocytes (P<0.001). No adrenaline-induced swelling was apparent at any developmental stage in hypoxia-reared rainbow trout.

Hb isoform expression

In normoxia-reared embryos and larvae, mRNA expression of embryonic Hb isoforms was significantly higher earlier in development relative to later in development (P<0.001; Fig. 7). In hypoxia-reared larvae, mRNA levels of Hb_E β-2 at Stage 33 (P=0.002; Fig. 7D) and of Hb_E α-1 and β-1 at Stages 33 and 35 were threefold to sixfold greater (P<0.01; Fig. 7A,B) than in the normoxic group. Hb_E α-2 mRNA levels were unaffected by hypoxia treatment (P>0.2; Fig. 7C).

DISCUSSION

The retention of rounder, embryonic erythrocytes and elevated Hb_E mRNA concentrations in hypoxia support the hypothesis that *O. mykiss* are capable of plasticity in the ontogenetic onset of Hb turnover. However, higher concentrations of the embryonic erythrocyte and Hb_E in hypoxia-reared relative to normoxia-reared larvae did not result in a higher Hb–O_2 affinity, as might be expected, suggesting that other factors may be involved. The hypoxia-induced delay in Hb and erythrocyte turnover may occur either as a result of a general reduction of protein synthesis in hypoxia – which is also suggested by the concomitant decrease in growth and developmental rate in hypoxia-reared embryos and larvae – or as a result of a more specific and regulated decrease in erythropoiesis (see below). Chronic hypoxia exposure dramatically reduced Hb concentration, Hct and erythrocyte counts in embryos and larvae relative to stage-matched normoxic controls. Thus, the enhancement of blood–O_2 transport previously observed in adult trout exposed to chronic hypoxia (Soivio et al., 1980; Nikinmaa, 1983; Lai et al., 2006) was absent or altered in early life stages prior to complete yolk absorption. However, the retention of Hb_E and of rounder erythrocytes, along with developmentally appropriate P_{50} values (i.e. similar to stage-matched normoxic group) in larvae exposed to chronic hypoxia suggests that other factors (e.g. intraerythrocytic adenylate levels) may be changed to achieve the best balance between O_2 loading and unloading of Hb at this stage of development.

The ontogeny of rainbow trout blood properties

Our study is the first to outline the ontogenetic sequence of blood–O_2 binding characteristics in early *O. mykiss* development at physiological temperatures using individual blood samples. Iuchi (Iuchi, 1973b) described the O_2 binding properties of *O. mykiss* Hb_E but the measurements were taken between 25 and 28°C, which is higher than the rainbow trout's normal physiological temperatures. At a lower temperature, Hb_E exhibited an increased O_2 affinity, reduced cooperativity, and greater Bohr effect relative to values reported previously by Iuchi (Iuchi, 1973b) at 25–28°C, which is also observed in Hb_A as temperature decreases (Weber et al., 1976; Vorger, 1985). Therefore, trout Hb_E and Hb_A responded in a similar manner to a reduction in temperature. In addition, Iuchi (Iuchi, 1973b) only investigated the Hb–O_2 binding properties in newly

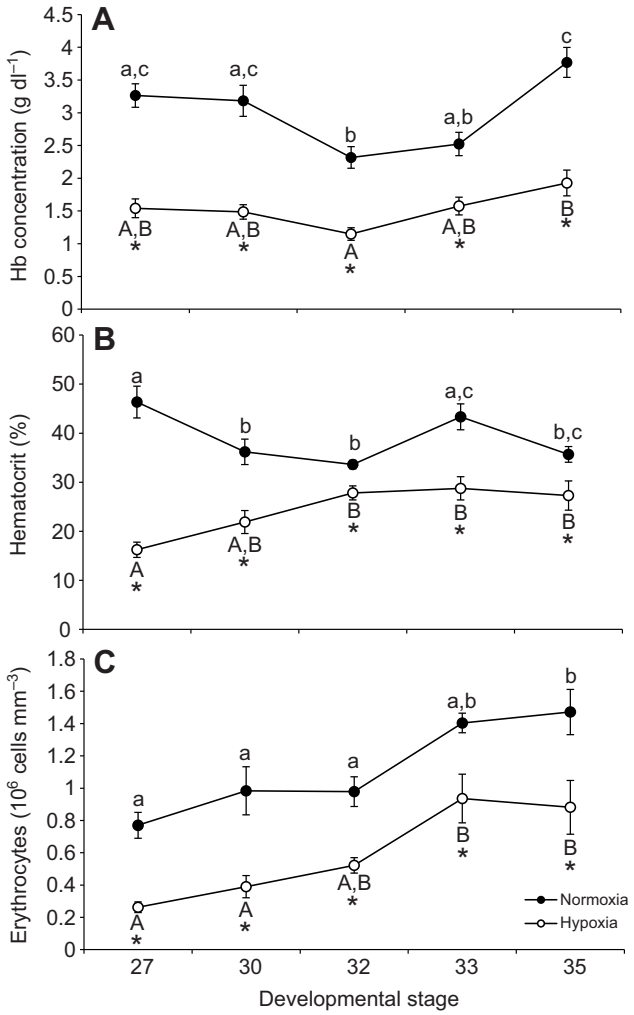

Fig. 4. Whole-blood Hb protein concentration (A), hematocrit (B) and erythrocyte counts (C) in various developmental stages of rainbow trout (*Oncorhynchus mykiss*) embryos and larvae reared in normoxia (100% of O_2 saturation; black circles) or hypoxia (30% of O_2 saturation; white circles). Different letters (lowercase, normoxia; uppercase, hypoxia) indicate significant differences among developmental stages and asterisks indicate significant differences from the normoxic group. Data are presented as means ± s.e.m. (*N*=6–10).

hatched larvae and compared this with the binding properties of 2-year-old trout. In contrast, we measured Hb–O_2 binding properties in prehatch embryos and in several larval stages during the transition from Hb_E to Hb_A. Furthermore, Iuchi (Iuchi, 1973b) euthanized 100–3000 individuals for each measurement. Presumably samples were pooled because of the difficulties in extracting sufficient blood volumes from small individuals. This approach would disguise the true biological variability among individuals. We developed a method of efficiently extracting blood from *O. mykiss* embryos and larvae and optimized several techniques for assaying numerous hematological parameters on microliter (0.5–2 µl) blood samples. These techniques can now be applied to more fully characterize blood homeostasis in response to a variety of environmental perturbations during early development and in species of small fishes.

Although Iuchi (Iuchi, 1973a) previously tracked the turnover of embryonic erythrocytes for adult erythrocytes, our study is the first to illustrate how Hb turnover affects blood–O_2 binding characteristics over an ontogenetic series. Our data indicate that the Hb_E to Hb_A turnover occurred between Stages 32 and 33 in normoxia: the OEC shifted to the right, P_{50} increased (lower affinity) and n_H decreased (lower cooperativity) after Stage 32. In addition, the erythrocyte H:W substantially increased between Stages 32 and 33, which signifies a predominance of the oval-shaped adult erythrocyte (Iuchi, 1973a) relative to earlier ontogenetic stages. Furthermore, Hb_E α-1, β-1 and β-2 mRNA expression was highest in Stage 27 embryos and significantly decreased between Stages 32 and 33. These changes were not associated with a decrease in overall Hb protein concentration, implying a replacement of Hb_E for Hb_A at the protein level. The Bohr and Root effects also became more pronounced with development. The P_{50} and Bohr and Root effect values in Stage 35 larvae were similar to those of adult rainbow trout (Eddy, 1971; Weber et al., 1976; Nikinmaa and Soivio, 1979). Taken together, these results indicate that Hb_A predominates at Stage 35.

We have characterized the detailed ontogenetic changes in Hb concentration, Hct and erythrocyte morphology in rainbow trout. In prehatch embryos, we observed a low Hb concentration and high Hct relative to *O. mykiss* adults. Hct was nearly 50%, which is 20% greater than the optimal Hct for O_2 delivery in adult rainbow trout (Wells and Weber, 1991), and may raise blood viscosity and impede circulation (Rand et al., 1964). The erythrocyte volume (MCV) data indicate that the elevated Hct is the consequence of a greater erythrocytic volume in prehatch embryos relative to rainbow trout adults (Johansson-Sjöbeck and Larsson, 1979; Řehulka and Adamec, 2004), probably reflecting the relatively larger, more spherical shape of the embryonic erythrocyte (Yamamoto and Iuchi, 1975). In addition, both MCH and MCHC were reduced at Stage 27 relative to values observed in adult rainbow trout (Johansson-Sjöbeck and Larsson, 1979; Boutilier et al., 1988; Řehulka and Adamec, 2004), suggesting a dilution of the embryonic erythrocytic contents. In adult fishes, such a dilution causes a dissociation of ATP–Hb complexes,

Table 3. Developmental changes in mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) in normoxia- (100% of O_2 saturation) and hypoxia-reared (30% of O_2 saturation) rainbow trout (*Oncorhynchus mykiss*) embryos and larvae

		Developmental stage				
	O_2 treatment	27	30	32	33	35
MCV (fl)	Normoxia	902.7±113.2	551.9±92.5	515.2±50.1	462.9±34.6	363.7±38.3
	Hypoxia	927.6±146.6	841.9±173.8	799.3±83.7	458.7±86.5	309.5±67.5
MCH (pg cell^{-1})	Normoxia	63.6±7.5	48.5±8.2	35.5±4.2	27.0±2.2	38.4±4.3
	Hypoxia	88.0±13.7	57.1±10.9	33.0±4.1	25.2±4.6	21.9±4.7
MCHC (g dl^{-1})	Normoxia	7.0±0.6	8.8±0.9	6.9±0.5	5.8±0.5	10.6±0.8
	Hypoxia	9.5±1.3	6.8±0.9	4.1±0.4	5.5±0.7	7.1±1.1

Data are presented as means ± s.e.m. (*N*=6–8).

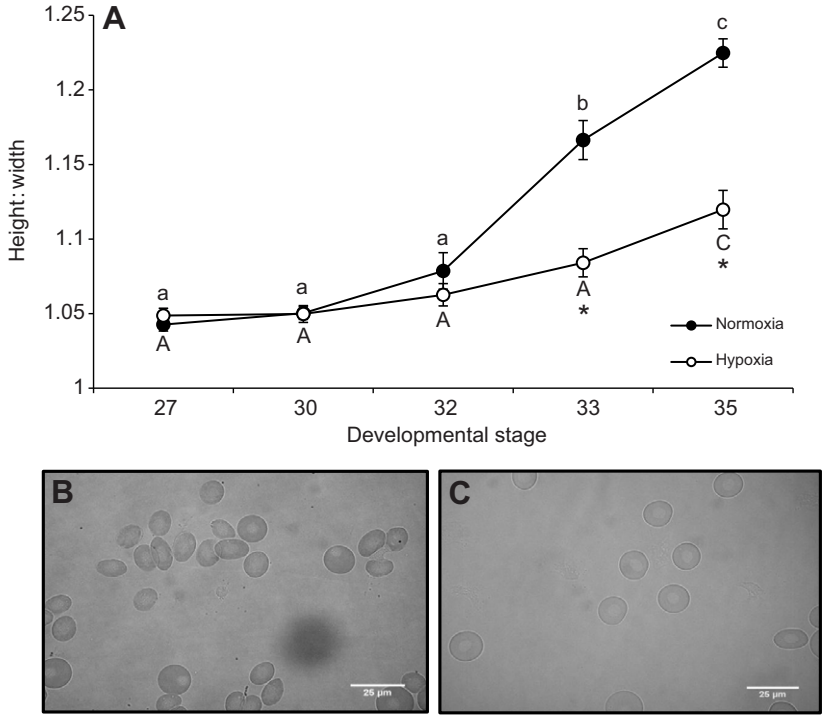

Fig. 5. Changes in erythrocyte shape (A) with development in normoxia (black circles) and hypoxia (white circles) and representative light micrographs of erythrocytes from Stage 35 *Oncorhynchus mykiss* larvae (B, normoxia, 100% of O_2 saturation; C, hypoxia, 30% of O_2 saturation). Different letters (lowercase, normoxia; uppercase, hypoxia) indicate significant differences among developmental stages and asterisks indicate significant differences from the normoxic group. Data are presented as means ± s.e.m. (*N*=9).

enhancing Hb–O_2 affinity (Nikinmaa, 1983; Nikinmaa and Huestis, 1984), consistent with our embryonic P_{50} values.

Although Stage 35 larvae possessed primarily Hb_A, their RBC indices differed from those of adult *O. mykiss*. At Stage 35, MCV resembled that of adult rainbow trout, while MCH and MCHC were ~50 and ~60% reduced relative to adult trout, respectively (Johansson-Sjöbeck and Larsson, 1979; Boutilier et al., 1988; Řehulka and Adamec, 2004). Therefore, how do *O. mykiss* larvae maintain adequate O_2 uptake with a low concentration of the lower affinity Hb_A? The direct diffusion of O_2 to the tissues *via* cutaneous respiration contributes up to ~40% of total O_2 uptake prior to exogenous feeding (Rombough and Ure, 1991; Wells and Pinder, 1996). These results suggest that the turnover of Hb_E for Hb_A precedes the complete dependence on Hb_A for gas exchange.

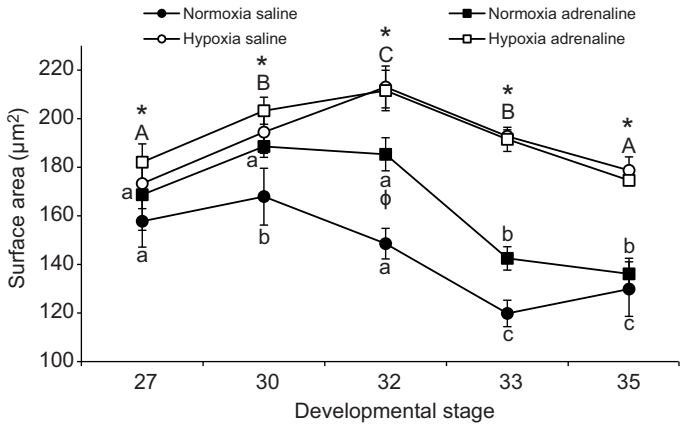

Fig. 6. The effects of adrenaline on erythrocyte swelling. Blood samples from normoxia (100% of O_2 saturation; black symbols) and hypoxia-reared (30% of O_2 saturation; white symbols) *Oncorhynchus mykiss* embryos and larvae at various developmental stages were incubated for 30 min in a 3× volume of 10^{-4} mol l^{-1} adrenaline (squares) or Cortland's saline (circles). Different letters (lowercase, normoxia; uppercase, hypoxia) indicate significant differences among developmental stages. Within the adrenaline-treated normoxic group, ϕ indicates a significant difference from the Cortland's-treated, normoxic group. Within the hypoxic group, asterisks indicate significant differences from the Cortland's-treated, normoxic group. Data are presented as mean ± s.e.m. (*N*=6).

Early ontogenetic strategies for coping with hypoxia

Moderate chronic hypoxia had profound negative impacts on developmental rates and growth, consistent with previous studies (Fig. 1) (Garside, 1966; Miller et al., 2008), as well as distinct alterations of the blood–O_2 transport system. The first possibility is that the decrease in overall Hb concentration and retention of embryonic erythrocytes in stage-matched hypoxia-reared larvae may be associated with a general metabolic depression. Previous work by Miller et al. (Miller et al., 2008) has confirmed that stage-matched trout embryos reduce their metabolic rate during chronic, moderate hypoxia (50% dissolved O_2). Protein production is typically one of the first metabolic functions to be limited by hypoxia (Buc-Calderon et al., 1993; Land et al., 1993; Land and Hochachka, 1994; Hochachka et al., 1996). In hypoxia it is also possible for the amount of Hb to exceed O_2 availability, and thus the benefit of increasing Hb concentration in hypoxia does not always outweigh the energetic cost of Hb production (Roesner et al., 2006). Moreover, a lower Hct would lower blood viscosity and in turn may lower cardiac energetic requirements (reviewed by Gallaugher and Farrell, 1998) at a time when O_2 is environmentally limited. It should be noted that the parsimonious explanation for the elevation of Hb_E mRNA levels in hypoxic larvae with lower Hb protein content is that this group has retained embryonic erythrocytes, which continue to express Hb_E mRNA.

The second possibility is that in hypoxia, embryos and larvae initiate a specific, coordinated response to low O_2 in order to conserve energy. In rainbow trout cells, hypoxia increases the concentration of HIF-1α protein (Soitamo et al., 2001), which is thought to mediate the increase in Hb and erythrocyte concentrations observed in adults during hypoxia exposure (Lai et al., 2006). However, in our study, chronic hypoxia resulted in a profound

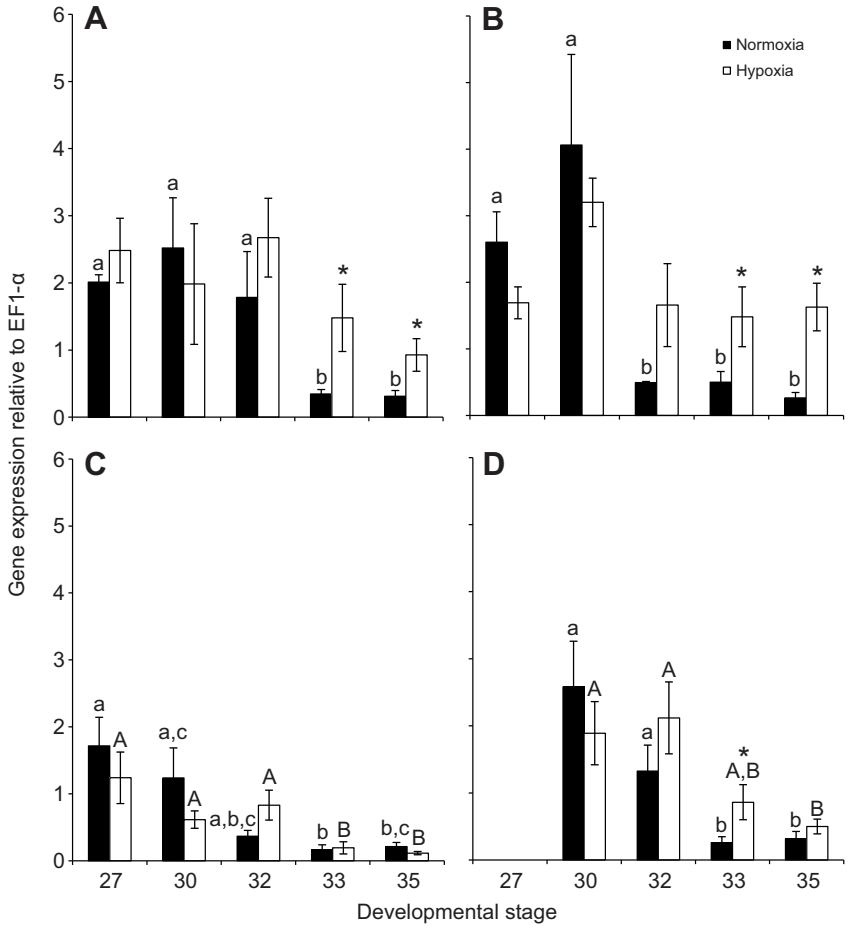

Fig. 7. Developmental pattern of Hb_E α-1 (A), Hb_E β-1 (B), Hb_E α-2 (C) and Hb_E β-2 (D) mRNA expression in whole rainbow trout (*Oncorhynchus mykiss*) embryos and larvae reared in normoxia (100% of O_2 saturation; black bars) or hypoxia (30% of O_2 saturation; white bars). Different letters (lowercase, normoxia; uppercase, hypoxia) indicate significant differences among developmental stages, and asterisks indicate significant differences from the normoxic group. Data are presented as means ± s.e.m. (*N*=4–9).

reduction in Hb and erythrocyte concentrations in embryos and larvae. Although HIF-1α is expressed in Baltic salmon (*Salmo salar*) and lake trout (*Salvelinus namaycush*) embryos (Vuori et al., 2004; Vuori et al., 2009), there is a lack of information on the HIF-1 mediated response during early salmonid development. It is possible that, similar to zebrafish (Ton et al., 2003), the hypoxia-induced HIF-1 pathway differs between embryonic and adult stages.

The reduced Hb protein and erythrocyte levels in hypoxia may also reflect the dependence on direct diffusion to the tissues for gas exchange, rather than blood convection, during early *O. mykiss* development. Holeton (Holeton, 1971) and Iuchi (Iuchi, 1985) suggested that Hb may not be necessary for O_2 consumption in early rainbow trout development (reviewed by Rombough, 1988). Therefore, reduced Hb production in early ontogenetic stages would conserve energy without seriously impeding O_2 uptake. Furthermore, because of their smaller size relative to the normoxic group, hypoxic embryos and larvae may be capable of prolonging the use of diffusion and cutaneous respiration for gas exchange. Indeed, chronic hypoxia exposure (25% of O_2 saturation, 38–47 days) slows gill development, in particular the proliferation of secondary lamellae (McDonald and McMahon, 1977). Thus a delay in the ontogenetic onset of branchial respiration during chronic hypoxia may offset low Hb concentrations.

It is surprising that there were no significant differences in Hb–O_2 affinity, cooperativity or the Root effect in hypoxia relative to the normoxic group. If Hb_E protein were retained, then we would expect a higher O_2 affinity and cooperativity and no Root effect, which are characteristic of Hb_E (Iuchi, 1973b). One explanation for this discrepancy is that a developmentally appropriate P_{50} value may be guarded in early life stages regardless of environmental O_2 levels to ensure adequate unloading at the tissues, as seen in some adult teleosts (e.g. Cook et al., 2013). Although hypoxic larvae are developmentally delayed and smaller than their normoxic counterparts, they still experience an eightfold increase in mass between Stages 27 and 35, and their mass is only 7 and 29% less than stage-matched normoxic Stage 33 and 35 larvae, respectively. However, this growth appears to be unaccompanied by a turnover to the lower-affinity Hb_A, and therefore O_2 unloading at developing tissues could potentially be compromised. Therefore, it is possible that allosteric modification of Hb_E occurs, which would be an energetically more favorable strategy compared with erythropoiesis in a low-O_2 environment. Furthermore, as others have argued (Brauner and Wang, 1997; Wang and Malte, 2011), a lower Hb–O_2 affinity may elevate venous O_2 content, supplying the heart with more O_2 during hypoxia. Unfortunately, the development of a method to measure RBC adenylates on <2 µl samples was beyond the scope of this study.

In rainbow trout adults, hypoxia initiates a stress response and stimulates the release of catecholamines that induce erythrocytic swelling and subsequently raise Hb–O_2 affinity (Nikinmaa, 1983; Nikinmaa and Huestis, 1984; Nickerson et al., 2003). Therefore, we investigated whether the dissimilarities in erythrocyte morphology (H:W) between O_2 treatments were due to endogenous catecholamines. In normoxia, the projected surface area increased upon adrenaline treatment, but this was not observed in hypoxia-reared embryos and larvae. The projected surface area of erythrocytes from hypoxia-reared *O. mykiss* was greater at all developmental stages, but the MCV data suggest that this was not

the result of erythrocytic swelling. It is also unlikely that prolonged hypoxia would chronically elevate catecholamines and maintain erythrocytic swelling, as catecholamines return to resting levels 1.5 h after hypoxia exposure (~25% of O_2 saturation for 90 min) in adult rainbow trout (Van Raaij et al., 1996). Therefore, the mechanism behind the higher projected surface area in hypoxia remains unknown.

We observed a greater Bohr effect in Stage 32 and 33 hypoxia-reared larvae relative to the normoxic group. This result was unexpected given that there was no significant change in whole-blood pH (data not shown) or in P_{50} in hypoxia and that Hb_E displays little Bohr effect (Iuchi, 1973b). Although the whole-blood pH values were not significantly different between treatments, when P_{CO_2} increased from 0.2 to 0.4 kPa, the change in pH was two to eight times smaller in hypoxia-reared compared with normoxia-reared larvae at Stages 33 and 32, respectively. These results imply greater blood buffering capacity in hypoxia-reared larvae, but this point will require further investigation.

Conclusions

Overall, rainbow trout exposed to chronic low O_2 showed a delay in Hb and erythrocyte turnover and thus demonstrated heterokairy (Spicer and Burggren, 2003). In addition, Hb concentrations and erythrocyte counts were not maintained at normoxic levels during low O_2 treatments, while Hb_E mRNA levels and round, immature erythrocytes were retained. Despite the presence of embryonic erythrocytes in larvae reared in hypoxia, Hb–O_2 affinity was similar to that of normoxic controls. We propose that Hb–O_2 affinity is set to optimize the balance between O_2 loading and unloading in fast-growing larval stages regardless of environmental O_2 levels, possibly through increased adenylates within the erythrocytes. We conclude that early hypoxia exposure has unique influences on the blood–O_2 transport system in developing trout in contrast to adults, which may be partly related to their heavier reliance on cutaneous respiration and direct O_2 diffusion.

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AUTHOR CONTRIBUTIONS

K.B. and P.A.W. conceived and designed the study. K.B. carried out the experimental work and analyzed the data. K.B. and P.A.W. interpreted the results and wrote the manuscript.

COMPETING INTERESTS

No competing interests declared.

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