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Effects of moderate and substantial hypoxia on erythropoietin levels in rainbow trout kidney and spleen

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Summary

Erythropoietin (EPO) is a glycoprotein hormone that regulates the proliferation and differentiation of erythroid progenitor cells in mammals. Although EPO has been identified in fish, the specific function and effects of hypoxia have not been investigated previously. In this study, we have demonstrated a relationship between increases in renal EPO levels and decreases in spleen EPO levels and spleen-somatic index (SSI), with increases in haemoglobin (Hb) concentration in the blood during hypoxia exposure in rainbow trout. Splenic contraction and the subsequent red blood cell release accounts for the initial increase in Hb concentration in the blood, whereas EPO action probably accounts for the later increases in hemoglobin concentration in the blood. Our data indicate that fish and mammalian erythropoietic systems are similar in response to hypoxia, in that erythropoiesis in fish is influenced by EPO.

Key words: erythropoietin, erythropoiesis, hypoxia, rainbow trout, *Oncorhynchus mykiss*, red blood cells, spleen somatic index.

Introduction

Hypoxia is a common event in aquatic environments. Fish respond to hypoxia by attempting to maintain oxygen delivery, downregulating aerobic processes, and upregulating anaerobic metabolism. Over the short term, fish try to maintain tissue oxygen levels by increasing ventilation, altering blood flow patterns, and increasing red blood cell (RBC) numbers and haemoglobin (Hb) concentration (Randall, 1982; Randall and Perry, 1992). Initial increases in RBC numbers are related to splenic contraction (Yamamoto, 1987; Randall and Perry, 1992), but over the long term, RBC increases are probably related to increases in the glycoprotein hormone, erythropoietin (EPO). Human erythropoiesis occurs in the bone marrow (Jelkmann, 1992) and is regulated by EPO levels, which increase slowly in response to increasing HIF-1 (Semenza, 2004) and remain elevated throughout the hypoxic period (Jelkmann, 1992).

In most fish, the kidney is the site for erythropoiesis (Fänge, 1986), which has been found to be enhanced by human urinary EPO (Pradhan et al., 1989). An *EPO* gene was recently identified in the blowfish *Takifugu rubripes* (Chou et al., 2004), encoding a protein probably similar in function to mammalian EPO. Furthermore, a protein that demonstrates competitive binding with antibodies raised against human EPO has been identified in kidney, spleen, plasma and serum from rainbow trout *Oncorhynchus mykiss* (Wickramasinghe, 1993), indicating that an EPO or EPO-like protein may be present. However, to date, no information exists regarding the effects of hypoxia on fish EPO levels. In this study, we use an ELISA kit against human EPO and western blot analysis targeting an EPO conserved region, to examine the effects of hypoxia on tissue EPO levels in rainbow trout.

Materials and methods

Rainbow trout *Oncorhynchus mykiss* Walbaum were reared at the University of British Columbia, Vancouver, Canada, at 12°C under natural light conditions for 2 months in an outdoor aquarium with running dechlorinated Vancouver tapwater and fed commercial trout pellets twice daily (2% body mass). Prior to hypoxia experiments, fish were transferred to indoor aquaria with running dechlorinated tapwater at densities no greater than one fish per 33 liters, and maintained for 2 weeks at 12°C under a 12 h:12 h L:D photoperiod and original feeding conditions. Experiments measuring Hb concentration, spleen somatic index, and erythropoietin ELISA assay were performed between November and December, 1999 using fish weighing 350–700 g. Western blot analyses for erythropoietin were done between November and December, 2004 using fish weighing 500–1000 g.

Hypoxia experimental protocol

At the start of the experimental period, the holding aquaria were covered and fish were starved for the entire exposure period. Oxygen levels in the inflowing water were reduced by passing water against a set flow of N_2 gas (Smale and Rabeni, 1995). Fish were exposed to two hypoxic treatments: 30% saturation (substantial hypoxia) and 55% saturation (moderate hypoxia). Fish were sampled after 24 h exposure or at 4, 8, 12, 24, 48, 72 and 144 h exposure to mild hypoxia and after 24, 72, 144 and 216 h exposure to substantial hypoxia. A blow to the head was used to stun fish, at which time blood was collected quickly from the heart using an EDTA-treated syringe. Spleen and kidney were removed and immediately frozen in liquid N_2 for future analysis. The formula (g spleen mass/g fish mass)$\times 10^4$ was used to calculate spleen somatic index (SSI). Hb concentrations were measured by the cyanmethaemoglobin method (Matsubara, 1972). Plasma cortisol was measured using a competitive binding ELISA assay (Neogen, Lexington, Kentucky, USA). To obtain plasma samples, blood was immediately centrifuged at 3000 ***g*** for 10 min at 4°C, and plasma was removed and stored at –80°C for later analysis.

EPO protein assay

Tissue samples were homogenized in extraction buffer (a cold phosphate-buffered saline, with proteinase inhibitor) and then centrifuged at 10 000 ***g*** for 20 min at 4°C. Total protein in the supernatant was measured with a protein assay kit (Bio-Rad, Hong Kong SAR), using bovine serum albumin (BSA) as a standard. An aliquot of each plasma sample was concentrated 5 times using a micropore filter to separate substances smaller than molecular mass of 5000, and then the plasma sample was assayed for EPO using an ELISA kit (Quantikine IVD; R&D systems, Minneapolis, MN, USA). To verify that the ELISA was specifically detecting EPO, protein sequences from various fish and mammalian species, including a partial sequence from trout (GenBank DQ288854) (J. C. C. Lai, unpublished), were aligned to find a conserved region. A synthetic peptide of the conserved region (KEAWDAEAAMR) was injected into rabbit to obtain a custom made polyclonal antibody against the EPO conserved region, and it was used as the primary antibody in western blot analysis.

Western blot analysis

Protein supernatant was combined with an equal volume of 2× sample buffer (62.5 mmol l^{-1} Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue, and 5% β-mercaptoethanol) and heated to 100°C for 5 min. Samples (30 μg total protein) were resolved by SDS-PAGE and transferred to Hybond-P PDVF membrane (Amersham Biosciences, Hong Kong SAR). The membrane was blocked with 1% BSA in 0.1% Tween-20 in phosphate buffered saline (TPBS) overnight at 4°C. The primary antibody was used in western blot analysis with 1:500 dilutions in TPBS and 1% BSA. After hybridizing the membrane with the primary antibody for 1 h at room temperature, membranes were washed with TPBS three times and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit *Ig*G (Zymed Laboratories, Hong Kong SAR) (1:10 000 dilution with 1% BSA in TPBS) for 1 h at room temperature. Following incubation, membranes were washed again three times before a chemiluminescent HRP substrate (ECL Plus Western Blotting Detection System, Amersham Biosciences) was added. Membranes were exposed to ECL film for various lengths of time to optimize signal intensities, and the film was ultimately scanned so band intensities could be quantified.

Statistical analyses

All data in this study are presented as mean ± s.d. Statistical differences between means of dependent variables were determined by Duncan's *post-hoc* analysis. Differences were accepted as significant at the 95% level of confidence ($P<0.05$).

Results

Effects of hypoxia on erythropoietin levels

EPO levels in rainbow trout kidney and spleen under normoxic conditions (control group) were 37.1±8.84 and 4.95±1.34 mIU 100 mg^{-1} protein, respectively. That is, kidney EPO concentrations were approximately sevenfold greater than levels detected in spleen. EPO was detected in all regions of the kidney, but the increase in EPO with hypoxia was greater in the head and middle regions. In rainbow trout exposed to substantial hypoxia (30% saturation), renal EPO levels increased significantly at 8, 12, 24 and 144 h exposure, compared to control levels (Fig. 1A). Conversely, spleen EPO levels showed significant decreases at 4, 8, 12, 24, 72 and 144 h exposure to substantial hypoxia (Fig. 1B). After exposure to moderate hypoxia (55% saturation), renal EPO increased at 24, 144 and 216 h (Fig. 2A), whereas spleen EPO levels decreased at 24 h but then increased at 144 and 216 h (Fig. 2B). There was an increase in kidney EPO within 8 h, and then a later secondary increase after several days. There was also an increase, following the initial drop in spleen EPO, after several days exposure to moderate hypoxia but not to substantial hypoxia.

We measured much lower levels of EPO in gills, blood and liver than in the kidney and spleen. EPO levels in blood and gills increased with moderate hypoxic exposure (data not shown). We were unable to detect EPO in plasma using ELISA, even after concentrating the plasma 5 times.

An EPO signal at ~25kDa was detected in rainbow trout kidney (Fig. 3) using western blot analysis. Kidney EPO levels increased significantly in rainbow trout after exposure to substantial hypoxia for 24 h (Fig. 3). We were unable to detect spleen EPO levels using this western blot protocol.

Effects of hypoxia on SSI and hemoglobin (Hb) concentration

Spleen somatic index (SSI) decreased in rainbow trout at 4, 8, 24 and 144 substantial hypoxia exposure, and at 24h after exposure to moderate hypoxia (Fig. 4).

In rainbow trout held at 30% oxygen saturation (substantial hypoxia), Hb concentrations increased at 4, 8, 12, 24, 48 and

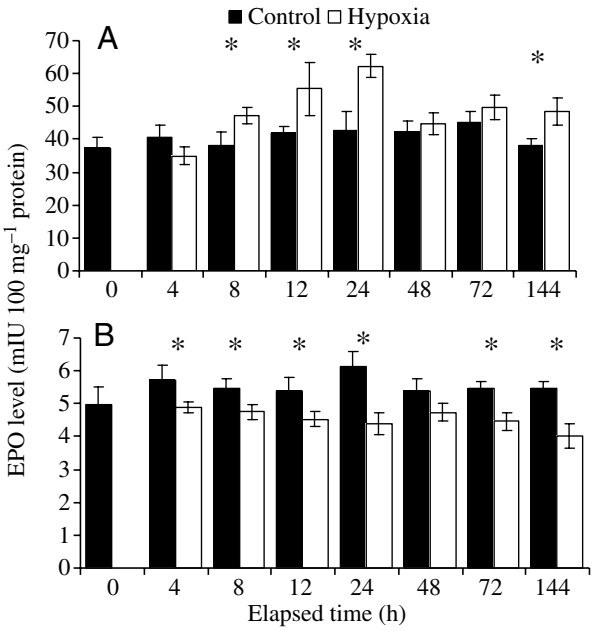

Fig. 1. Erythropoietin (EPO) levels measured (*via* ELISA) for (A) rainbow trout kidney and (B) spleen during exposure of the fish to substantial hypoxia. Values are mean ±s.d., *N*=6. *Significantly different from control (*P*<0.05).

144 h of exposure. Hemoglobin levels also increased significantly in fish exposed to 55% oxygen saturation (moderate hypoxia) at 24, 72 and 216 h exposure (Fig. 5). That is, there was an initial increase in hemoglobin levels followed by a later increase after several days.

Effects of hypoxia on plasma cortisol concentration

Plasma cortisol levels increased when fish were exposed to hypoxia. Measured cortisol levels doubled (from 20.05 ng ml^{-1} to 41.53 ng ml^{-1}) after 2 h exposure to moderate hypoxia but increased tenfold (from 14.73 ng ml^{-1} to 169.5 ng ml^{-1}) after 2 h exposure to substantial hypoxia. Plasma cortisol returned to control levels after a day in fish exposed to either moderate or substantial hypoxia (not shown).

Discussion

We used an ELISA kit based on (1) a monoclonal (murine) antibody against recombinant human EPO and (2) a rabbit polyclonal anti-recombinant human EPO to detect EPO in rainbow trout tissues. We found high levels in kidney and spleen homogenates and lower levels in the liver, gills and blood. We performed western blot analysis, using a polyclonal antibody against a vertebrate EPO conserved region, to ensure that we were, indeed, detecting trout EPO with the ELISA kit. Western blot analysis generated similar results for kidney tissue when compared to ELISA; it is likely, therefore, that we have been measuring EPO levels in trout. The western protocol was not sensitive enough to detect spleen EPO.

We were able to detect EPO in blood, but not in plasma, indicating that EPO may bind to red blood cells, preventing us from detecting it in plasma samples. Wickramasinghe was able to detect EPO in plasma (Wickramasinghe, 1993). Our inability to detect plasma EPO may be due to methodological limitations such as antibody specificity, the extent of non-specific reactions, and/or blood collection techniques, and particularly the use of heparin as an anti-coagulant by Wickramasinghe *versus* EDTA used by us.

Rainbow trout is generally considered to be a hypoxia-sensitive species that requires at least 60–70% O_2 saturation for normal growth and reproduction but can survive acute exposure to substantial hypoxia (30% saturation). Various physiological responses, such as enhancement of oxygen delivery, suppression of aerobic metabolism, and activation of anaerobic metabolism, are rapidly activated in trout exposed to substantial hypoxia (Boutilier et al., 1988). Hypoxia was observed to cause a rapid increase in HIF-1 in cultured hepatocytes (Soitamo et al., 2001). In our study of the whole-organism, we did not observe an increase in EPO after 4 h exposure but did record an increase after 8 h exposure to substantial hypoxia. Our first measurements at 24 h of exposure to moderate hypoxia also showed an increase in kidney EPO. The difference in time course between increases in HIF-1 and EPO is to be expected, since production of EPO requires first transcription and consequent translation (in the heart, see below) after HIF-1 activation, and then transport to the kidney. Studies of Fugu *Epo* promoter constructs, however, failed to find a hypoxia responsive region in the *Epo* promoter and the 3′ flanking region, but higher levels of spliced transcripts were found in cells subjected to hypoxic and anaerobic conditions (Chou et al., 2004). These observations suggest that, although hypoxia induces EPO production in both

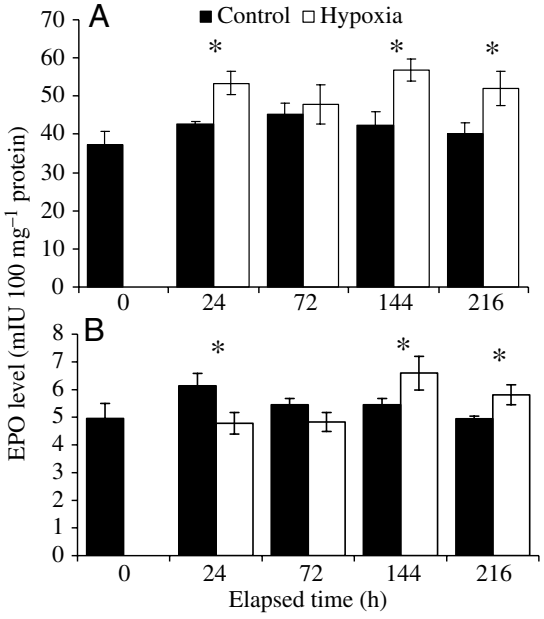

Fig. 2. EPO levels measured (using ELISA) for rainbow trout kidney (A) and spleen (B) upon exposure to moderate hypoxia. Values are mean ± s.d., *N*=6. *Significantly different from control (*P*<0.05).

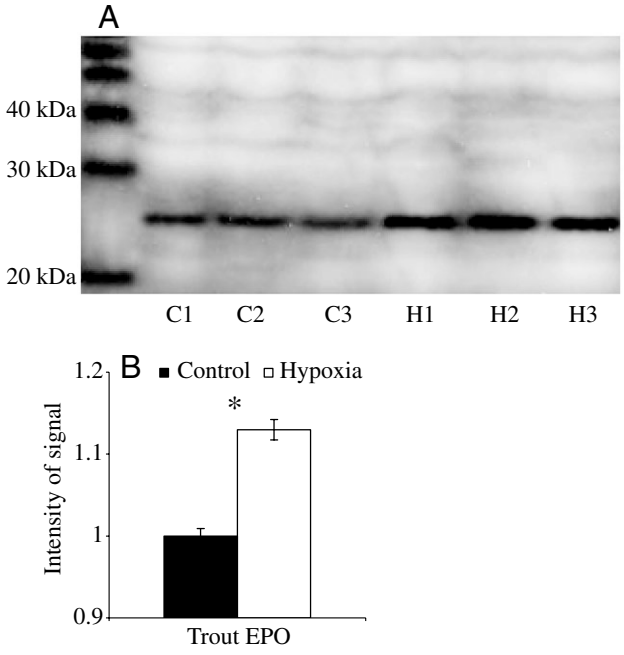

Fig. 3. (A) Control (C1–C3) and 24 h hypoxia-exposed (H1–H3) rainbow trout renal EPO levels (obtained *via* western blot analysis) and (B) the relative EPO levels in control and hypoxic rainbow trout kidney. Values are mean ± s.d., *N*=3. *Significantly different from control (*P*<0.05). The positions of molecular mass markers are shown in A.

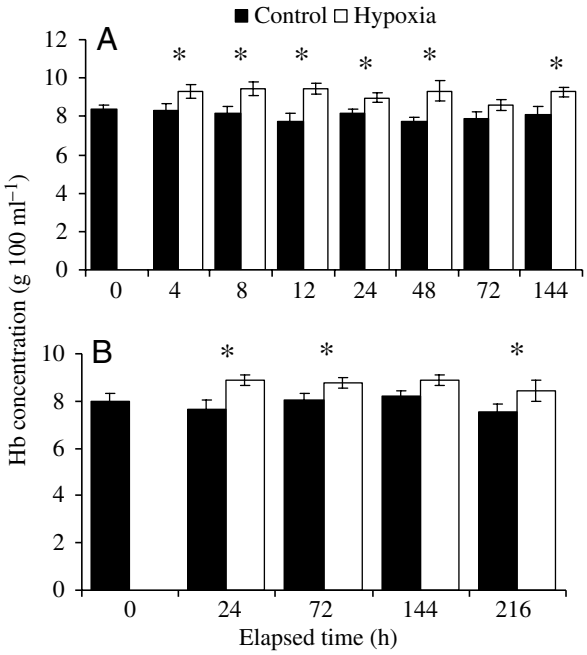

Fig. 5. Hb concentration for rainbow trout exposed to substantial (30% air saturation) hypoxia (A) and moderate (55% air saturation) hypoxia (B). Values are mean ± s.d., *N*=6. *Significantly different from control (*P*<0.05).

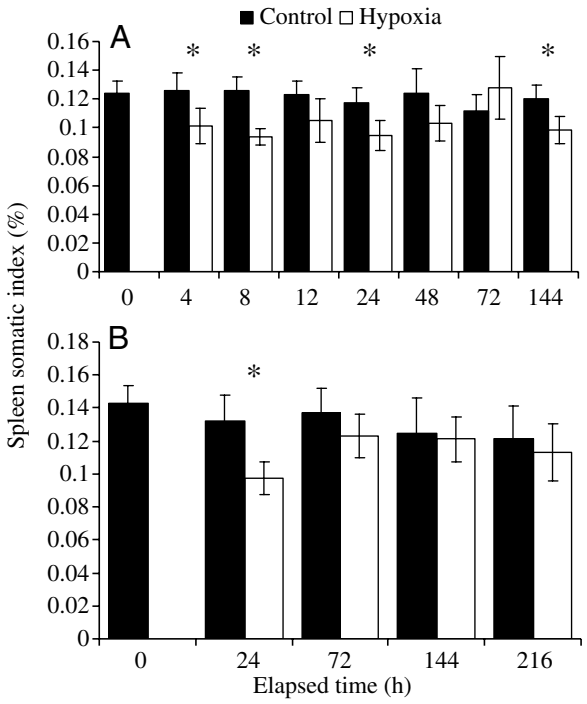

Fig. 4. Spleen somatic index (SSI) for rainbow trout exposed to substantial (A) or moderate hypoxia (B). Values are mean ± s.d., *N*=6. *Significantly different from control (*P*<0.05).

fish and mammals, mechanism behind it may not be exactly the same.

We were able to measure EPO in the kidney and spleen during normoxia, with much higher levels being observed in the kidney. The kidney to spleen EPO ratio (approximately 7) observed under normoxic conditions in trout was much higher than the value (approximately 2.5) reported by Wickramasinghe earlier (Wickramasinhge, 1993). This difference may be due to seasonal variations in baseline renal and spleen EPO levels (I. Kakuta et al., unpublished data).

The initial increase in kidney EPO associated with exposure to either moderate or substantial hypoxia returned to control levels after 2 or 3 days hypoxia exposure. There was a second increase in EPO recorded after 6 days exposure to both moderate and substantial hypoxia. Thus the pattern of EPO increase is similar during both moderate and substantial hypoxia, showing an initial increase during the first day, returning to control levels during the second and third days and then showing a second increase after 6 days. This increase persisted until at least 9 days in the group exposed to moderate hypoxia. It is also possible that the release of red blood cells from the spleen at the onset of hypoxia enhances oxygen delivery to the tissues and this reduces EPO production on the second or third days.

There is no data on renal blood flow and kidney oxygen levels in the fish exposed to hypoxic conditions (Kakuta et al., 1992). In mammals, EPO production by the kidney is inversely related to the oxygen-delivering capacity of the blood perfusing it (Spivak, 1989). Although it is clear that hypoxia results in an increase in EPO levels in fish, we know nothing

of renal blood flow and oxygen levels in the fish kidney and how this is related to tissue EPO levels.

The spleen remained contracted (lower SSI compared to control) throughout substantial hypoxia but returned to normal after a few days exposure to moderate hypoxia. It is possible that between 24 to 72 h exposure to moderate hypoxia, new blood formation is activated by EPO and the blood produced is stored in the spleen therefore SSI returned to the control levels. In contrast, under substantial hypoxia, new blood produced goes into the circulation to increase oxygen capacity and SSI remains at a low level.

EPO levels in the spleen fell during exposure to both moderate and substantial hypoxia and this was associated with a decrease in the spleen-somatic index (SSI). As SSI decreased, Hb concentrations increased (Fig. 6A), presumably due to splenic contraction and subsequent RBC release into the circulation (Yamamoto, 1987; Randall and Perry, 1992). Increases in Hb within the first day exposure are probably too rapid to result from erythropoisis and therefore are likely a result of the release of stored RBCs from the spleen. Subsequent increases in Hb (after days rather than hours), however, could represent EPO-mediated erythropoiesis, since Hb concentration increases are no longer correlated to changes in SSI (Fig. 6B). The time course for erythropoiesis in mammals, as indicated by changes in blood Hb concentrations, is not well established, but it is thought that, following increases in EPO, erythropoiesis occurs within a few days as appears to be the case for fish.

Although EPO protein levels were high, EPO mRNA expression was not detected in trout kidney (J. C. C. Lai, unpublished observations). EPO mRNA levels were high in the heart, and also were detected in the liver and spleen. EPO mRNA expression increased during hypoxia, particularly in the heart. The above findings indicate that EPO in fish is produced mainly in the heart and is then transported in the blood to the kidney, the primary erythropoietic organ in fish.

To investigate the stress to rainbow trout caused by moderate and substantial hypoxic condition exposure, cortisol levels were recorded. From our findings, 30% oxygen saturation is a very stressful condition, causing a tenfold increase in cortisol level, while 55% oxygen saturation created a much lower stressful condition, causing only a twofold induction to cortisol level.

Our results show that there are high levels of erythropoietin in the kidney and spleen of rainbow trout, with much higher levels in the kidney than the spleen. Hypoxia is associated with an increase in kidney EPO, but a decrease in spleen EPO related to a decrease in spleen-somatic index caused by splenic contraction, indicating that EPO is bound to red blood cells. The initial increases in hemoglobin levels in the blood during hypoxia reflect the release of red blood cells from the spleen, whereas subsequent increases in hemoglobin concentration are probably related to EPO induced erythropoiesis.

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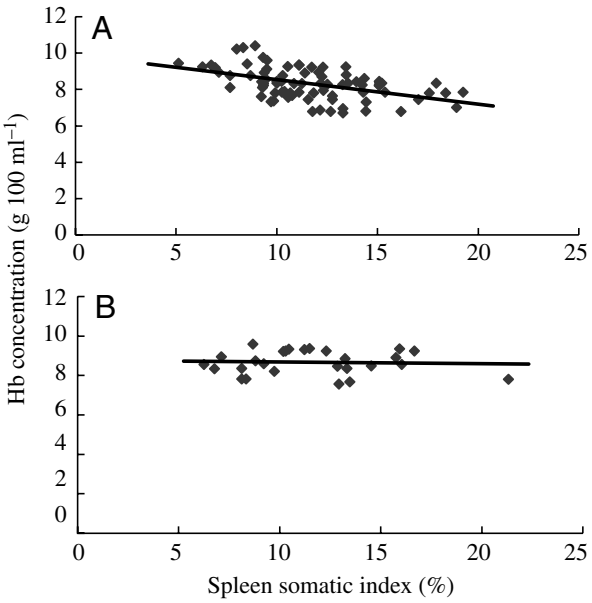

Fig. 6. Plots of spleen somatic index against Hb concentration for rainbow trout exposed to short-term hypoxia (within 24 h) with the controls (A) and prolonged hypoxia (144–216 h) (B).

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