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Blood sampling techniques and storage duration: Effects on the presence and magnitude of the red blood cell β-adrenergic response in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Many teleostean fish, including rainbow trout, regulate red blood cell (RBC) pH (pH_i) in the presence of a stress-induced acidosis such as hypoxia, hypercapnia, or exhaustive exercise. This is accomplished through activation of RBC Na^+/H^+ exchange (β-NHE), ultimately minimizing impairment to oxygen transport. Presence and characterization of the RBC β-NHE in fish is best tested in blood from cannulated, resting animals; however, several studies have used blood from stressed animals drawn from the caudal vein and stored prior to use. The effects of sampling procedures and storage on the β-NHE response is not known and is the focus of this study. Whole blood drawn from cannulated, resting rainbow trout was compared with RBCs obtained from the caudal vein rinsed and stored at 4 °C for 0, 6, 24, 48, 96 or 144 h. Isoproterenol (10^{-5} M), a β-adrenergic agonist, was added to hypoxia/hypercapnia incubated RBCs in vitro. In all treatments, isoproterenol induced a large β-NHE response, and storage duration (≤96 h) had a minimal affect, indicating that rinsing and storing is an easy and viable means by which to obtain RBCs and investigate function. Storage for 144 h still resulted in a significant RBC β-NHE response; however, viability of RBCs may be compromised.

Keywords: β-NHE; Catecholamine; Rainbow trout; Blood storage; β-adrenergic response; Caudal puncture; Cannulation; Red blood cells

1. Introduction

Some teleosts secrete catecholamines shortly following about a 50% decrease in arterial oxygen (O_2) content (Nikinmaa et al., 1984; Primmett et al., 1986; Perry and Kinkead, 1989; Perry et al., 1989; Fievet et al., 1990; Cossins and Kilbey, 1991; Berenbrink and Bridges, 1994). Noradrenaline binds to β-adrenergic receptors on the red blood cell (RBC) membrane, stimulating G-proteins, which in turn signal adenylate cyclase to synthesize adenosine 3′, 5′-cyclic monophosphate (cAMP) subsequently activating the Na^+/H^+ exchanger (β-NHE) on the RBC surface (Nikinmaa and Huestis, 1984; Cossins and Richardson, 1985; Perry and Gilmour, 1996; Val et al., 1998). β-NHEs serve to rapidly transport Na^+ ions into, and H^+ out of the RBC (Nikinmaa et al., 1990; Nickerson et al., 2001), producing a state of disequilibrium across the cell membrane, ultimately elevating intracellular pH (pH_i) (Powers et al., 1979; Nikinmaa and Huestis, 1984; Salama and Nikinmaa, 1988; Tufts and Randall, 1989; Lessard et al., 1995). As pH_i increases, intracellular bicarbonate (HCO_3^-) increases and is exported in exchange for chloride (Cl^-). The increase in intracellular Na^+ and Cl^- draws water osmotically into the cell causing RBC swelling and an increase in hematocrit (Hct) (Forster and Steen, 1969; Borgese et al., 1987; Nikinmaa et al., 1990; Guizouarn et al., 1993). Maintaining pH_i during a generalized acidosis is important in safeguarding O_2 transport because the Bohr effect, and to a greater extent the Root effect – a phenomenon restricted to only some teleosts where a decrease in pH promotes O_2 release from Hb – prevents Hb from becoming completely saturated with O_2, even at high O_2 partial pressures (PO_2), and can compromise O_2 uptake at the respiratory surface (Bohr et al.,

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1904; Root, 1931; Root and Irving, 1943; Nikinmaa and Soivio, 1979; Pelster and Weber, 1991 Nikinmaa, 1997).

Thorough studies conducted on fish RBC function and specifically, the β-adrenergic response at the RBC, to date, are limited to a few species that are readily available in large numbers and that can be held under stable laboratory conditions. However, investigating the ubiquity and evolution of the functional properties of fish RBCs necessitates the use of phylogenetically-diverse fish species that are often only available in the field or difficult to hold in captivity. In addition to obtaining and maintaining live animals, blood must be drawn from an unstressed animal and tested within a short period of time. Several studies have used whole blood taken from cannulated fish after a 48-h, post-surgery recovery period, which has been found sufficient time for stressed fish to naturally eliminate plasma catecholamines (Bourne and Cossins, 1982). Other studies have characterized the β-NHE in RBCs after whole blood, taken from the caudal vein, was rinsed and refrigerated (Motais et al., 1989; Val et al., 1998); however it is not known how this procedure affects red blood cell function. The primary purpose of this study was to determine whether differences could be detected in rainbow trout red blood cell function in blood obtained via caudal puncture as opposed to cannulation, as seen by changes in hematocrit, hemoglobin, pH_i, pH_e, intracellar Na^+ and H_2O, and total NTP following β-adrenergic stimulation in vitro. Secondly, we aimed to determine whether rainbow trout RBCs, rinsed and resuspended in physiological saline, are significantly affected in their response to β-adrenergic stimulation when stored at 4 °C for various durations (up to 144 h). Development of methods to acquire RBCs from live fish allowing characterization of RBC function over days to weeks will be important for field-based studies to investigate fish red blood cell function.

2. Materials and methods

2.1. Animal care

Live rainbow trout (*Oncorhynchus mykiss*) (wet mass = 350–500 g) were obtained from Whitewater Trout Farm (Riverside, CA, USA) between June and October and fed a maintenance diet consisting of commercial trout pellets obtained from the hatchery. Fish were held in a 4000-L recirculating fresh water system maintained at 15 °C. Prior to each treatment, a benzocaine solution (2×10^{-4} M *p*-aminobenzoate) was used to anaesthetize live fish.

2.2. Experimental protocol

To investigate the effect of sampling technique on the RBC β-adrenergic response, blood was obtained via (1) caudal puncture and (2) cannulae. For the first technique ($N=6$), fish were removed from the water upon anaesthetization, and approximately 5 mL of blood was removed from the caudal vein into a heparinized syringe. For the second technique ($N=6$), fish, once anaesthetized, were placed on a surgery table, gills intubated and continuously irrigated with water containing anaesthetic (2×10^{-5} M *p*-aminobenzoate), and an indwelling catheter (PE50) was surgically implanted into the dorsal aorta according to (Soivio et al., 1975). Following surgery, fish were left to recover in a Perspex box supplied with aerated 15 °C fresh water for 48 h during which time cannulae were flushed twice daily with heparinized physiological saline. Prior to experimentation, blood was removed from the catheter into a heparinized syringe, but at the first sign of struggling, no further blood was removed to ensure plasma catecholamine levels remained low.

For the second component of the study, RBCs obtained via caudal puncture were rinsed twice with ice-cold Cortland's saline, and stored at 4 °C for 0, 6, 24, 48, 96, or 144 h, before being tested at 15 °C, the temperature to which trout were acclimated ($N=6$ for each storage duration).

For both components of the project, either 5 mL (experimental) or 3.5 mL (control) of resuspended RBCs or whole blood adjusted to a hematocrit of 25% was placed into an Eschweiler tonometer and equilibrated for one hour with a humidified gas mixture of 1.6% CO_2, 2% O_2, balance N_2 before any experimentation was initiated. These gas tensions have been found to potentiate the β-NHE response of RBCs in rainbow trout (Brauner et al., 2002). In control replicates, nothing was added to the blood, but for experimental replicates, either isoproterenol (a β-agonist), or a combination of isoproterenol and propranolol (a β-antagonist) was added (10 μL per 1 mL blood) to achieve a final concentration of 10^{-5} M for isoproterenol and 2×10^{-5} M propranolol (Fuchs and Albers, 1988; Tetens et al., 1988). Isoproterenol is preferred over naturally-occurring catecholamines, because it is more stable (Fuchs and Albers, 1988). Propranolol is a β-adrenergic antagonist which has been shown to limit cAMP, therefore blocking the resulting effects of the β-NHE (Fuchs and Albers, 1988; Motais et al., 1989). Addition of propranolol was initiated to determine whether or not the β-adrenergic response was solely responsible for the changes measured, or whether other pathways were involved.

Blood samples (0.8 mL) at 0, 5, 15, 30, 60, and 120 min intervals were removed from each tonometer for measurement of hematocrit, hemoglobin, pH_i, pH_e, intracellar $[Na^+]$ and H_2O content, in the presence and absence of isoproterenol, and in the isoproterenol + propranolol treatment ($n=2$ for each parameter). Additional blood was removed for total NTP analysis at the first and last time interval and in resuspended RBCs following 0 and 144 h storage in the presence and absence of isoproterenol, and in the isoproterenol + propranolol treatment ($n=2$).

2.3. Analytical techniques

Hematocrit was measured in duplicate after centrifugation of microcapillary tubes of whole blood at 3000 revs min^{-1} for 5 min. Time to half-maximal response ($t_{1/2}$) and maximum percent increase in hematocrit (Hct_{max}) were both calculated by likening the parameters to K_m and V_{max}, respectively, taken from Michaelis–Menton enzyme kinetics and using a Lineweaver–Burke plot. Hemoglobin was measured in duplicate spectrophotometrically (Beckman 640) using a hemoglobin test

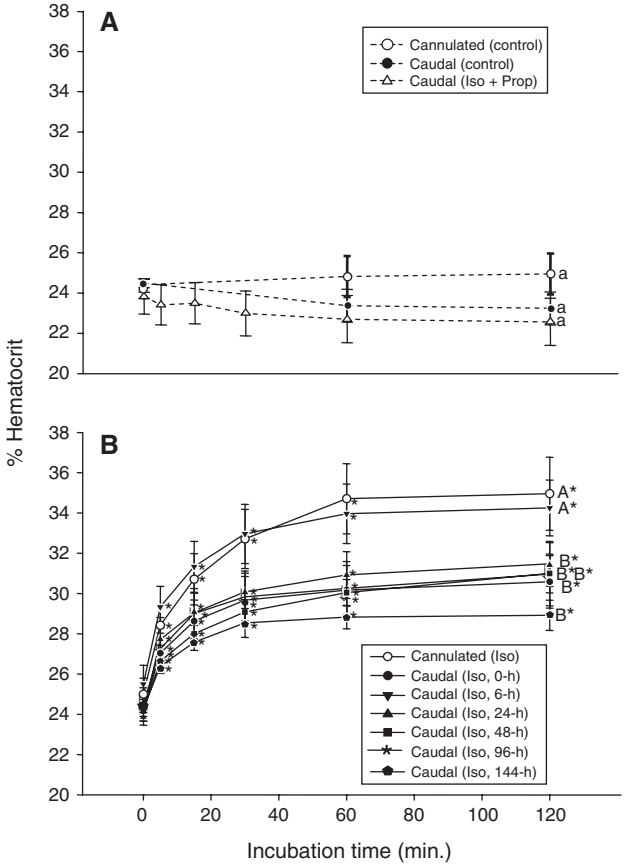

Fig. 1. Data (means±standard error, $N=6$) are presented to illustrate the change in hematocrit (%) with tonometer incubation time (min) for cannulated control (--○--), caudal puncture control (--●--), and caudal puncture isoproterenol +propranolol (--△--), all displayed in A, and cannulated isoproterenol (–○–), caudal puncture 0-h storage isoproterenol (–●–),caudal puncture 6-h storage isoproterenol (–▼–), caudal puncture 24-h storage isoproterenol (–▲–), caudal puncture 48-h storage isoproterenol (–■–), caudal puncture 96-h storage isoproterenol (–✱–), and caudal puncture 144-h storage isoproterenol (–⬟–), all displayed in B. An asterisk (*) demarcates significant differences from the time-0 value within a group, and different letters indicate statistically homogeneous groups but only at 120 min incubation time.

kit (Sigma Diagnostics Total Hemoglobin 525-A). The freeze–thaw method of (Zeidler and Kim, 1977) was used to prepare isolated RBCs, and pH_i and pH_e were measured in duplicate using a BMS 3 Mk2 Blood Microsystem (Radiometer, Copenhagen). NTP concentration was analyzed in duplicate using an NTP analysis kit (Sigma Diagnostics Adenosine 5′-Triphosphate 366). RBC water content was determined in duplicate by calculating the difference between RBC wet weight and dry weight (following drying at 60 ° C for 48 h). RBC Na^+ concentrations were determined in duplicate using flame atomic absorption spectrophotometry.

2.4. Statistical analyses

Significant differences between mean values were determined using a one-way repeated measures ANOVA test followed by a post hoc Student–Newman Keuls' test. A probability of $p<0.05$ was used to assess statistically significant differences.

3. Results

3.1. Effects on hematocrit

Following addition of isoproterenol, hematocrit significantly increased in blood samples collected via (1) caudal puncture (after rinse and resuspension) and (2) cannulae (post 48-h recovery period) (Fig. 1A), and over the duration of the tonometer incubation period (up to 120 min) relative to time zero (Fig. 1B). The magnitude of increase was not as great in the caudal blood sample, approximately 26%, when compared to the 40% increase in hematocrit in blood samples drawn from cannulae (Fig. 2). Significant changes in hematocrit were not observed in control groups (no isoproterenol) from either sampling protocol, or in the sample treated with isoproterenol+propranolol (Fig. 1A). Hematocrit was significantly elevated, at the maximal response (Hct_{max}) by at least 19 but up to 34.5% beyond control values over the duration of the incubation period in all caudal blood samples from all storage durations; although the response was not as substantial in samples that had been stored for 144 h (an increase

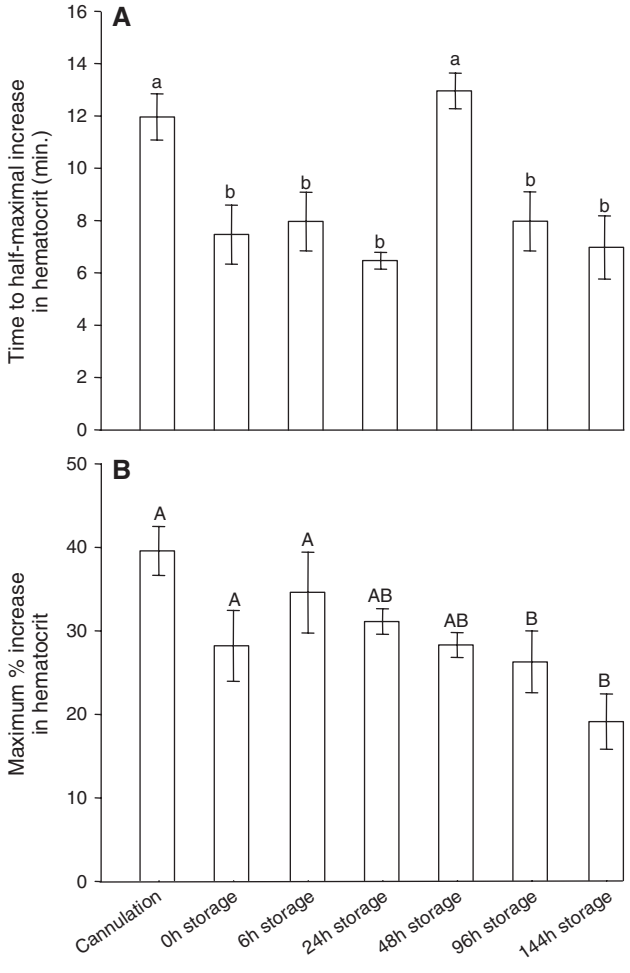

Fig. 2. Data (means±standard error, $N=6$) are presented to compare time (min) to half-maximal increase in hematocrit (A) and maximum % increase in hematocrit (B) between experimental groups (i.e. the cannulation isoproterenol group and caudal puncture isoproterenol for all storage durations). Different letters indicate statistically homogeneous groups.

of 19.07±3.299%) (Fig. 2). Interestingly, rinsed, resuspended caudal blood stored for 6 h exhibited the most pronounced change in hematocrit upon exposure to isoproterenol when compared to samples from other storage durations; the response was most similar to that of whole blood from cannulated fish (Figs. 1 and 2). Ultimately, the magnitude of the response was not significantly different from that which was seen in whole blood from cannulated fish until 96 h storage and beyond. Another point to consider was the time to half-maximal response ($t_{1/2}$) in each storage group (Fig. 2). Interestingly, samples from the 48-h storage group demonstrated $t_{1/2}$ values most similar to samples taken from cannulated fish, approximately 12–13 min, whereas all other storage groups, including 0 h, exhibited $t_{1/2}$ values ranging 6.5–8 min, significantly lower than the aforementioned ($p<0.001$) but not significantly different from one another (Fig. 2).

3.2. Effects on mean cell hemoglobin concentration

Mean cell hemoglobin concentration (MCHC) decreased significantly in both caudal and cannulae blood samples (3.19±0.172 to 2.29±0.043 mM and 3.96±0.065 to 3.00±0.131 mM respectively) following addition of isoproterenol over the tonometer incubation period ($p<0.001$) (Fig. 3). Control samples did not exhibit a significant change in MCHC over the tonometer incubation period and neither did the sample treated with isoproterenol+propranolol ($p>0.05$) (Fig. 3). Samples exposed to isoproterenol from all storage durations exhibited a significant decrease in MCHC; although values for samples taken via cannulation and values reported for caudal samples stored for 144 h were not statistically distinct from one another. All other storage durations exhibited MCHC values significantly lower ($p<0.001$) but not different from one another (Fig. 3).

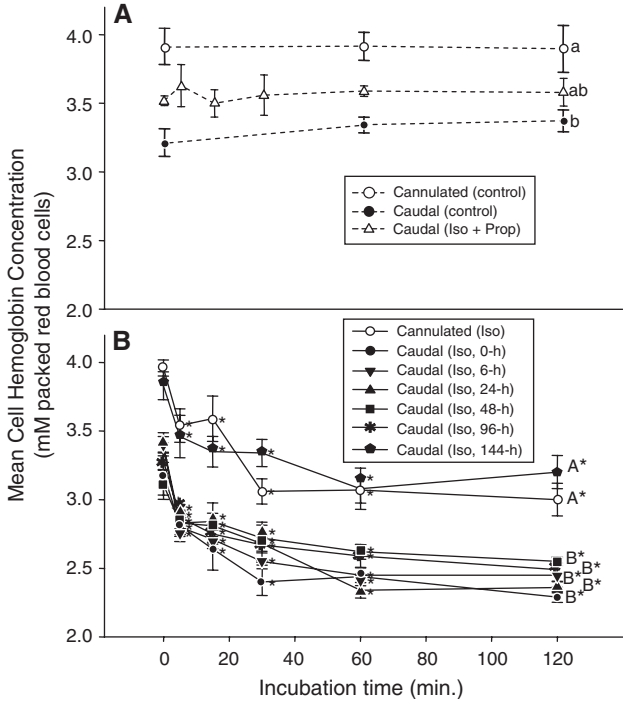

Fig. 3. Data (means±standard error, $N=6$) are presented to illustrate the change in mean cell hemoglobin concentration (MCHC) (mM packed red blood cells) with tonometer incubation time (min) for all control and experimental groups. See caption for Fig. 1 for further details. An asterisk (*) demarcates significant differences from the time-0 value within a group, and different letters indicate statistically homogeneous groups only at 120 min incubation time.

3.3. Effects on RBC water content

Both resuspended caudal blood samples and blood samples drawn from cannulae treated with isoproterenol exhibited an increase in RBC H_2O content, albeit an increase of only 3% and 6% respectively with the former not proving statistically significant (Table 1). Control samples taken via cannulation exhibited a slight and significant increase in RBC H_2O content (Table), but control samples taken via caudal puncture and resuspended did not exhibit a significant increase in RBC H_2O content and neither did the sample treated with isoproterenol+propranolol. In all storage groups (except 48 h), RBC H_2O content increased significantly (from approximately 76% up to nearly 83%), but no storage group was statistically distinct from the others ($p>0.050$) (Table 1).

3.4. Effects on pH gradient

The intra- to extra-cellular pH gradient (pH_e–pH_i), which was found to typically be approximately 0.30 units in unstimulated blood samples, decreased significantly to 0.19 and 0.14 in both sampling groups (caudal and cannulae, respectively) over the tonometer incubation period following addition of isoproterenol. Control samples did not exhibit significant change in the pH_e–pH_i gradient over the duration of tonometry and neither did the sample treated with isoproterenol+propranolol (Table 1). The pH_e–pH_i gradient decreased significantly in all storage groups down to as low as 0.1 units, but was significant only after 30 min incubation in the 0 h storage group, not until 120 min incubation in the 6 h storage group, by 5 min incubation in the 24 h storage group (most similar to the immediate response seen in the sample drawn via cannulation), at 60 min incubation in the 48 h storage group, by 15 min incubation in the 96 h storage group, and only after 30 and 60 min incubation in the 144 h storage group (Table 1). There was no significant difference between storage durations ($p>0.050$).

3.5. Effects on RBC Na^+ concentration

Resuspended caudal blood samples and samples from cannulated fish treated with isoproterenol exhibited a qualitative but not significant increase in RBC (intracellular) Na^+ concentration, from 16.40±1.791 to, at most, 20.49±1.119 mM and from 15.68±2.446 to, at most, 18.23±2.584 mM respectively (Table 1). Control samples and the sample treated with isoproterenol+propranolol exhibited no observable increase in RBC Na^+. Caudal blood samples (at all storage durations) treated with isoproterenol exhibited a significant increase in RBC Na^+ concentration, by 15 min incubation in samples stored for 6 h, as soon as 5 min in samples stored for 24 h, after 60 min incubation in samples stored for 48 h, by 30 min in samples stored for 96 h, and only at the 15 min incubation time interval in samples stored

Table 1
Means (±standard error, $N=6$) for red blood cell (RBC) water %, the change in pH between the extracellular (pH_e) and intracellular (pH_i) environments, RBC sodium (Na^+), and the nucleoside triphosphate concentration [NTP] are presented for each sampling protocol and storage duration

Sampling protocol / Incubation (min)	RBC water (%)	ΔpH ($pH_e - pH_i$)	RBC Na^+ (mM)	[NTP] (mM)
Cannulation (control)				
0	74.25±1.475	0.25±0.029	15.56±2.551	0.69±0.044
60	75.85±1.462	0.27±0.025	15.17±1.325	n/a
120	77.34±0.607*	0.26±0.058	13.89±1.545	0.70±0.023
Caudal puncture (control)				
0	77.75±1.889	0.22±0.031	15.69±3.704	0.62±0.026
60	77.35±0.973	0.28±0.045	16.51±1.592	n/a
120	78.18±1.228	0.25±0.040	16.15±1.289	0.53±0.077
Caudal puncture (isoproterenol+propranolol)				
0	70.34±7.463	0.30±0.025	12.45±0.653	0.49±0.030
5	74.61±1.842	0.30±0.020	12.14±0.839	n/a
15	76.76±0.881	0.30±0.012	12.91±1.053	n/a
30	75.75±1.192	0.30±0.018	13.73±1.638	n/a
60	76.70±1.461	0.31±0.013	11.69±1.486	n/a
120	75.96±0.368	0.25±0.021	12.06±0.724	0.48±0.056
Cannulation (isoproterenol)				
0	76.21±0.713	0.32±0.031	15.68±2.446	0.67±0.042
5	77.15±0.693	0.15±0.039*	17.76±1.516	n/a
15	77.85±1.003	0.17±0.033*	17.48±2.311	n/a
30	78.57±0.590	0.16±0.025*	18.23±2.584	n/a
60	79.70±0.698*	0.18±0.027*	17.05±1.004	n/a
120	81.26±0.983*	0.14±0.026*	15.34±1.139	0.47±0.100*
Caudal puncture (isoproterenol, 0 h storage)				
0	79.96±1.524	0.30±0.059	16.40±1.791	0.55±0.071
5	83.06±2.324	0.25±0.062	17.79±2.645	n/a
15	81.07±1.005	0.19±0.048	19.55±1.105	n/a
30	82.37±1.799	0.20±0.045*	20.49±1.119	n/a
60	81.94±0.826	0.16±0.041*	20.02±0.759	n/a
120	81.58±1.997	0.19±0.041*	19.40±0.948	0.42±0.058
Caudal puncture (isoproterenol, 6 h storage)				
0	76.50±1.510	0.22±0.032	15.13±0.669	0.55±0.027
5	80.04±1.292*	0.20±0.024	18.55±2.192	n/a
15	79.07±2.351	0.20±0.038	19.60±1.564*	n/a
30	80.17±0.689	0.15±0.025	19.55±1.759	n/a
60	80.69±0.638*	0.15±0.032	22.79±4.164	n/a
120	80.86±1.305*	0.10±0.022*	19.88±2.035	0.33±0.093
Caudal puncture (isoproterenol, 24 h storage)				
0	77.22±0.942	0.31±0.023	15.55±0.651	n/a
5	80.47±1.167*	0.24±0.021*	17.74±0.927*	n/a
15	80.71±1.534	0.23±0.039*	18.46±0.951*	n/a
30	80.90±0.756*	0.25±0.030*	18.92±0.992*	n/a
60	81.01±0.731*	0.21±0.030*	19.08±0.729*	n/a
120	82.60±1.450*	0.19±0.024*	16.53±1.438	n/a
Caudal puncture (isoproterenol, 48 h storage)				
0	81.91±2.367	0.24±0.045	15.68±0.826	n/a
5	80.50±1.152	0.20±0.024	16.10±0.708	n/a
15	81.21±0.690	0.18±0.028	17.38±0.274	n/a
30	82.49±0.749	0.17±0.042	17.04±1.484	n/a
60	81.70±1.049	0.13±0.030*	20.14±1.601*	n/a
120	83.27±0.864	0.10±0.037*	16.52±0.846	n/a
Caudal puncture (isoproterenol, 96 h storage)				
0	77.71±1.644	0.25±0.020	13.51±1.138	n/a
5	79.13±0.587	0.25±0.030	15.17±0.648	n/a

Table 1 (*continued*)

Sampling protocol Incubation (min)	RBC water (%)	ΔpH ($pH_e - pH_i$)	RBC Na^+ (mM)	[NTP] (mM)
15	82.03±0.877*	0.21±0.024*	16.33±1.016	n/a
30	80.24±1.723	0.18±0.026*	17.27±0.645*	n/a
60	81.68±1.357*	0.19±0.020*	17.54±0.572*	n/a
120	82.06±0.874*	0.17±0.030*	14.47±3.488	n/a
Caudal puncture (isoproterenol, 144 h storage)				
0	76.14±0.819	0.26±0.032	14.36±1.614	0.32±0.043
5	78.16±0.735*	0.23±0.021	15.05±1.294	n/a
15	78.72±0.757*	0.19±0.026	16.21±1.283*	n/a
30	79.40±1.119*	0.20±0.016*	16.05±1.527	n/a
60	79.22±0.961*	0.18±0.022*	15.96±1.691	n/a
120	79.38±1.175*	0.15±0.042	13.20±0.905	0.31±0.079

Asterisks (*) are used to demarcate significant differences from the time-0 incubation value within each group.

for 144 h. Values ranged from a starting concentration as low as 13 mM in some cases, up to a final concentration – upon exposure to isoproterenol – of nearly 23 mM. There were no significant differences in the magnitude of change between storage duration groups ($p > 0.050$) (Table 1).

3.6. Effects on NTP concentration

NTP concentrations were only measured at the beginning and end of tonometer incubation periods (time 0 and 120 min) in samples taken from cannulae and only following 0, 6, and 144 h storage in resuspended caudal blood samples. Addition of isoproterenol resulted in a significant reduction in NTP concentration (from 0.67±0.042 to 0.47±0.100 mM) over the 120-min incubation period in blood drawn from cannulae and in resuspended caudal blood (from 0.55±0.071 to 0.42±0.058 mM); a decrease was not observed in either control group nor was a decrease observed in samples treated with isoproterenol+propranolol (Table 1). Initial NTP concentration was lowest for resuspended caudal blood stored for 144 h (0.30–0.40 mM), in comparison with time 0 whole blood drawn from cannulated fish (0.65–0.70 mM). Addition of isoproterenol to whole blood drawn from cannulae resulted in a significant reduction (by approximately 30%) in NTP concentration over 120 min. Resuspended caudal blood stored for 0 h also exhibited a decrease in NTP levels, approximately 23%, although the change was not noted statistically significant. Samples stored for 6 h also showed a qualitative decrease in NTP concentration, although not statistically significant, and samples stored for 144 h did not exhibit any characteristic decrease in NTP following addition of isoproterenol (Table 1).

4. Discussion

Upon β-adrenergic stimulation with the β-agonist, isoproterenol, rainbow trout RBCs exposed to hypoxic and hypercapnic conditions undergo a series of physiological changes, most of which are easily characterized (Nikinmaa et al., 1984; Primmett et al., 1986; Perry and Kinkead, 1989; Perry et al., 1989; Fievet et al., 1990; Cossins and Kilbey, 1991; Berenbrink and Bridges, 1994). RBC swelling, as quantified by increase in hematocrit, was the most obvious sign of β-adrenergic stimulation. Increases in MCHC and RBC H_2O content are also directly related to RBC swelling and quantified as part of the response. Additionally, an increase in pH_i and decrease in pH_e were also expected and characterized by the overall decrease in the intra- to extra-cellular RBC pH gradient (pH_e–pH_i). Intra-cellular Na^+ was expected to increase in response to β-adrenergic stimulation due to exchange with H^+ as a result of β-NHE activation. Finally, overall NTP concentration was predicted to decrease in the advent of β-adrenergic stimulation. To ensure isoproterenol was provoking the cascade of events associated with β-adrenergic stimulation, a treatment with propranolol in addition to isoproterenol was conducted, and results matched those of unstimulated control samples.

Of all parameters measured for this study, most notable and consistent were changes in hematocrit. Interestingly, the response most similar to that of whole blood drawn from resting, undisturbed, cannulated fish was the response demonstrated in samples drawn via caudal puncture, resuspended, rinsed, and stored for 6 h. The finding that RBCs stored for 6 h exhibited a more pronounced β-adrenergic response than those stored 0 h is probably related to elevated catecholamines associated with caudal puncture. Soivio and colleagues demonstrated measurable RBC swelling within 20 s of initial exposure to a stressor, which, in that study was exposure to air (Soivio et al., 1975). While the inactivation half-time for epinephrine is approximately 10 min, catecholamine levels still remained within the in vivo stress range 90 min following air-exposure. Despite the time to rinse and resuspend RBCs, standardize samples to a hematocrit of 25% (about one hour), and 1 h incubation in tonometers prior to experimentation, it is possible that, in this study, time 0 h RBCs were still slightly stimulated when isoproterenol was added, therefore explaining the apparent reduction in RBC sensitivity to isoproterenol relative to samples stored for 6 h. Conceivably, catecholamines associated with caudal sampling would have been metabolized by 6 h (Bourne and Cossins, 1982). Resuspended RBCs stored for 24, 48 and 96 h responded similarly, but after 144 h storage, the response, though significant, was less than what was observed in samples stored for a shorter duration. Erythrocyte handling (centrifugation and resuspension) and/or endogenous

catecholamine levels may affect β-adrenoreceptor density or affinity (Reid et al., 1991), instigating a less than maximal response in all treatment groups. Reid and colleagues found that, for isoproterenol and propranolol binding sites, increases in the dissociation constant (30–77%) and decreases in receptor density (11–25%) could be observed when RBCs were rinsed and resuspended in Cortland's saline and that RBCs may require plasma-borne factors to maintain structural and functional integrity of β-adrenoreceptors to elicit the maximal β-adrenergic response (Reid et al., 1991). In this study, however, we demonstrated that a significant response can still be generated in RBCs rinsed, resuspended, and stored for up to 144 h.

Additional parameters measured to demonstrate the presence and magnitude of the β-adrenergic response in rainbow trout RBCs also followed predicted patterns, although not as clearly as the response observed with hematocrit. MCHC in blood treated with isoproterenol was expected to decrease following addition of propranolol. Samples obtained via cannulation possessed the highest initial MCHC, and the response upon β-adrenergic stimulation followed a similar pattern to resuspended RBCs stored for 0, 6, 24, 48, and 96 h. Again, samples drawn via caudal puncture and stored for 144 h demonstrated a significant response, but it was reduced in comparison to samples stored for shorter durations. RBC swelling can also be quantified by determining changes in H_2O content. Activation of β-NHE results in a net removal of H^+ from the intracellular environment, resulting in pH_e reduction and an increase in pH_i, reducing the pH_e–pH_i gradient. This is consistent with what was observed in all samples treated with isoproterenol. There was a trend toward the expected increase in RBC Na^+ due to activation of the β-NHE and resulting influx of Na^+ from outside the cells in exchange for H^+ where isoproterenol was added. NTP content was highest in whole blood, although the concentrations for resuspended RBCs stored for 0 h were not significantly lower. Most interesting was the blood sample stored for 144 h, which possessed NTP concentrations of approximately 50% of the initial concentrations observed in fresh blood collected via cannulae. This finding suggests that, although RBCs stored for 144 h may show a significant adrenergic response, the metabolic state of the cells may be compromised.

It is evident that trout RBCs obtained by caudal puncture, rinsed, and resuspended in Cortland's saline exhibit a significant β-adrenergic response following addition of β-adrenergic agonist, isoproterenol (10^{-5} M), even when stored for up to 96 h. Blood stored for 144 h demonstrated a significant, but reduced response, and viability of RBCs stored for longer than 96 h requires further investigation. In addition, the physiological properties measured in this study of RBCs collected from rainbow trout via caudal puncture are not significantly different from those of blood taken via cannulae following 48 h post-surgery recovery. These data support the use of washing and storing red blood cells to assess physiological status of red cells as has been used in the past (Motais et al., 1989; Val et al., 1998) and simplifies the methodology required to assess metabolic and physiological function of nucleated red blood cells of fishes, a fascinating topic that has recently been a focus of renewed investigation (Berenbrink et al., 2005).

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References

Berenbrink, M., Bridges, C., 1994. Catecholamine-activated sodium/proton exchange in the red blood cells of the marine teleost *Gadus morhua*. J. Exp. Biol. 192, 253–267.

Berenbrink, M., Koldkjaer, P., Kepp, O., Cossins, A.R., 2005. Evolution of oxygen secretion in fishes and the emergence of a complex physiological system. Science 307, 1752–1757.

Bohr, C., Hasselbalch, K., Krogh, A., 1904. Ueber einen in biologischer Beziehung wichtigen Einfluss, den die Kohlensaeurspannung des Blutes auf dessen Sauerstoffbindung uebt. Skand. Arch. Physiol. 16, 402–412.

Borgese, F., Garcia-Romeu, F., Motais, R., 1987. Control of cell volume and ion transport by beta-adrenergic catecholamines in erythrocytes of rainbow trout, *Salmo gairdneri*. J. Physiol. 382, 123–144.

Bourne, P.K., Cossins, A., 1982. On the instability of K^+ influx in erythrocytes of the rainbow trout, *Salmo gairdneri*, and the role of catecholamine hormones in maintaining in vivo influx activity. J. Exp. Biol. 101, 93–104.

Brauner, C.J., Wang, T., Jensen, F.B., 2002. Influence of hyperosmotic shrinkage and b-adrenergic stimulation on red blood cell volume regulation and oxygen binding properties in rainbow trout and carp. J. Comp. Physiol., B 172, 251–262.

Cossins, A.R., Kilbey, R.V., 1991. Adrenergic responses and the root effect in erythrocytes of freshwater fish. J. Fish Biol. 38, 421–429.

Cossins, A.R., Richardson, P.A., 1985. Adrenaline-induced Na^+/H^+ exchange in trout erythrocytes and its effects upon oxygen-carrying capacity. J. Exp. Biol. 118, 229–246.

Fievet, B., Caroff, J., Motais, R., 1990. Catecholamine release controlled by blood oxygen tension during deep hypoxia in trout: effect on red blood cell Na^+/H^+ exchanger activity. Respir. Physiol. 79, 81–90.

Forster, R.E., Steen, J.B., 1969. The rate of the 'Root shift' in eel red cells and eel haemoglobin solutions. J. Physiol. 204, 259–282.

Fuchs, D.A., Albers, C., 1988. Effect of adrenaline and blood gas conditions on red cell volume and intra-erythrocytic electrolytes in the carp, *Cyprinus carpio*. J. Exp. Biol. 137, 457–477.

Guizouarn, H., Harvey, B., Borgese, F., Gabillat, N., Garcia-Romeu, F., Motais, R., 1993. Volume-activated Cl^--independent and Cl^--dependent K^+ pathways in trout red blood cells. J. Physiol. 462, 609–626.

Lessard, J., Val, A.L., Aota, A., Randall, D.J., 1995. Why is there no carbonic anhydrase activity available to fish plasma? J. Exp. Biol. 198, 31–38.

Motais, R., Fievet, B., Garcia-Romeu, F., Thomas, S., 1989. Na^+/H^+ exchange and pH regulation in red blood cells: role of uncatalyzed H_2CO_3 dehydration. Am. J. Physiol. 256, C728–C735.

Nickerson, J., Gugan, S., Drouin, G., Moon, T.W., 2001. A putative β_2-adrenoceptor from the rainbow trout (*Oncorhynchus mykiss*). Eur. J. Biochem. 268, 6465–6472.

Nikinmaa, M., 1997. Oxygen and carbon dioxide transport in vertebrate erythrocytes: an evolutionary change in the role of membrane transport. J. Exp. Biol. 200, 369–380.

Nikinmaa, M., Cech, J.J., McEnroe, M., 1984. Blood oxygen transport in stressed striped bass (*Morone saxatilis*): role of β-adrenergic responses. J. Comp. Physiol. 154, 365–369.

Nikinmaa, M., Huestis, W.H., 1984. Shape changes in goose erythrocytes. Biomembranes 773, 317–320.

Nikinmaa, M., Soivio, A., 1979. Oxygen dissociation curves and oxygen capacities of blood of a freshwater fish, *Salmo gairdneri*. Ann. Zool. Fenn. 16, 217–221.

Nikinmaa, M., Tiihonen, K., Paajaste, M., 1990. Adrenergic control of red cell pH in salmonid fish: roles of the sodium/proton exchange, Jacobs–Stewart cycle and membrane potential. J. Exp. Biol. 154, 257–271.

Pelster, B., Weber, R.E., 1991. The physiology of the root effect. Advances in Comparative and Environmental Physiology, vol. 8. Springer-Verlag, Berlin, pp. 51–77.

Perry, S.F., Gilmour, K.M., 1996. Consequences of catecholamine release on ventilation and blood oxygen transport during hypoxia and hypercapnia in an elasmobranch (*Squalus acanthias*) and a teleost (*Oncorhynchus mykiss*). J. Exp. Biol. 199, 2105–2118.

Perry, S.F., Kinkead, R., 1989. The role of catecholamines in regulating arterial oxygen content during acute hypercapnic acidosis in rainbow trout (*Salmo gairdneri*). Respir. Physiol. 77, 365–377.

Perry, S.F., Kinkead, R., Gallaugher, P., Randall, D.J., 1989. Evidence that hypoxemia promotes catecholamine release during hypercapnic acidosis in rainbow trout (*Salmo gairdneri*). Respir. Physiol. 77, 351–364.

Powers, D.A., Fyhn, H.J., Fyhn, U.E.H., Martin, J.P., Garlick, R.L., Wood, S.C., 1979. A comparative study of the oxygen equilibria of blood from 40 genera of Amazonian fishes. Comp. Biochem. Physiol., A 62, 67–85.

Primmett, D., Randall, D., Mazeaud, M., Boutilier, R., 1986. The role of catecholamines in erythrocyte pH regulation and oxygen transport in rainbow trout (*Salmo gairdneri*) during exercise. J. Exp. Biol. 122, 139–148.

Reid, S., Moon, T.W., Perry, S., 1991. Characterization of beta-adrenoreceptors of rainbow trout (*Oncorhynchus mykiss*) erythrocytes. J. Exp. Biol. 158, 199–216.

Root, R.W., 1931. The respiratory function of the blood of marine fishes. Biol. Bull. Mar. Biol. Lab. Woods Hole 61, 427–456.

Root, R.W., Irving, L., 1943. The effect of carbon dioxide and lactic acid on the oxygen-combining power of whole and hemolyzed blood of the marine fish *Tautoga onitis* (Linn.). Biol. Bull. Mar. Biol. Lab. Woods Hole 84, 207–242.

Salama, A., Nikinmaa, M., 1988. The adrenergic responses of carp (*Cyprinus carpio*) red cells: effects of PO_2 and pH. J. Exp. Biol. 136, 405–416.

Soivio, A., Nyholm, K., Westman, K., 1975. A technique for repeated sampling of the blood of individual resting fish. J. Exp. Biol. 63, 207–217.

Tetens, V., Lykkeboe, G., Christensen, N.J., 1988. Potency of adrenaline and noradrenaline for β-adrenergic proton extrusion from red cells of rainbow trout, *Salmo gairdneri*. J. Exp. Biol. 134, 267–280.

Tufts, B.L., Randall, D.J., 1989. The functional significance of adrenergic pH regulation in fish erythrocytes. Can. J. Zool. 67, 235–238.

Val, A.L., de Menezes, G.C., Wood, C.M., 1998. Red blood cell adrenergic responses in Amazonian teleosts. J. Fish Biol. 52, 83–93.

Zeidler, R., Kim, D.H., 1977. Preferential hemolysis of postnatal calf red cells induced by internal alkalinization. J. Gen. Physiol. 70, 385–401.