

THE EFFECTS OF CELL AGEING ON PROTEIN SYNTHESIS IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) RED BLOOD CELLS

SUSAN G. LUND*, MATTHEW C. L. PHILLIPS, CHRISTOPHER D. MOYES AND BRUCE L. TUFTS

Department of Biology, Queen's University, Kingston, Ontario, Canada K7L 3N6

*e-mail: lunds@biology.queensu.ca

Accepted 25 April; published on WWW 22 June 2000

Summary

The effects of cell age on protein synthesis were examined in the nucleated red blood cells of rainbow trout (*Oncorhynchus mykiss*). Total DNA content was unaffected by cell age, whereas total RNA content in young red blood cells was roughly ten times as high as that in old red blood cells. The mRNA levels for haemoglobin, carbonic anhydrase and the chloride/bicarbonate ($\text{Cl}^-/\text{HCO}_3^-$) exchanger were also approximately tenfold higher in young red blood cells. Although young red blood cells synthesized roughly five times more protein under steady-state conditions, total protein concentration was not affected by cell age. Despite large reductions in mRNA levels with red blood cell ageing, the concentrations and/or activities of the

respiratory proteins were largely preserved. In contrast, the ability to mount a heat shock response was greatly reduced in older red blood cells. Young red blood cells produced 13 times more heat shock protein 70 mRNA following heat shock and four times more 70 kDa protein after recovery. They also transcribed much more heat shock cognate 71 and heat shock factor mRNA than did older red blood cells under steady-state conditions.

Key words: band 3, carbonic anhydrase, haemoglobin, heat shock, stress protein, rainbow trout, *Oncorhynchus mykiss*, ageing, red blood cell.

Introduction

In many ways, the red blood cells of non-mammalian vertebrates are very different from those of mammals. One of the most obvious differences is that non-mammalian red blood cells are nucleated whereas mammalian red blood cells extrude their nuclei before entering the circulation. In addition, these nucleated red blood cells retain ribosomes (Sekhon and Beams, 1969; Lane and Tharp, 1980; Lane et al., 1982) which enable them to synthesize proteins such as haemoglobin (Keen et al., 1989; Speckner et al., 1989) and heat shock proteins (Currie and Tufts, 1997). They also retain functional mitochondria and maintain higher rates of metabolism than their mammalian counterparts (Boutilier and Ferguson, 1989). At present, however, it is not known whether cell ageing greatly diminishes these unique properties of nucleated red blood cells or whether they are largely maintained throughout the circulatory life span of the cells.

Fish red blood cells are thought to live in the circulation for as long as 6 months, during which time their main function is the transport of respiratory gases. Previous studies on fish red blood cell morphology have shown that cellular ageing is associated with a loss of ultrastructural features such as ribosomes and mitochondria (Sekhon and Beams, 1969; Lane and Tharp, 1980; Lane et al., 1982; Keen et al., 1989). Recently, it has been shown that the loss of organelles and ribosomes in ageing rainbow trout red blood cells is also accompanied by a substantial reduction in red blood cell

aerobic metabolic rate (Hunter and Hunter, 1957; Lane, 1984; Phillips et al., 2000). However, the impact of these changes on protein synthesis has yet to be determined. Changes in protein synthesis as nucleated red blood cells age may act to alter the concentration and/or activity of important respiratory proteins as well as the ability of red blood cells to respond to stress by synthesizing heat shock proteins. Heat shock proteins are highly conserved and ubiquitous proteins that not only provide increased protection to cells during stressful conditions, but act constitutively as 'molecular chaperones' to guide the proper folding and assembly of nascent polypeptides (Parsell and Lindquist, 1993; Becker and Craig, 1994; Hartl, 1996). Previous studies have shown that the ability to respond to stress, reflected by the increased production of heat shock mRNA and protein, generally becomes attenuated with increasing cell age in several mammalian tissues (Liu et al., 1989; Niedzwiecki and Fleming, 1993; Pahlavani et al., 1995; Lee et al., 1996). Thus, ageing in nucleated red blood cells may have significant implications in terms of the role of red blood cells in gas exchange as well as their ability to maintain homeostasis during periods of stress.

Although there is a paucity of direct evidence in this area, significant differences in the red blood cell age profile *in vivo* are probably relatively common in fish. Studies have shown that the red blood cell population of fish *in vivo* is a mixture of cells of different ages and that juvenile cells make up

5–57% of the circulating red blood cell population (Härdig, 1978; Lane and Tharp, 1980; Keen et al., 1989; Murad et al., 1990; Houston and Murad, 1991). Moreover, numerous factors have been shown to stimulate erythropoiesis and would therefore be expected to increase the relative proportion of juvenile cells. In rainbow trout, for example, erythropoiesis is generally stimulated by a decrease in the tissue oxygen supply-to-demand ratio that may occur as a result of hypoxia, anaemia or temperature increases (Hevesy et al., 1964; Lane et al., 1982; Nikinmaa, 1990). In addition, seasonal release of thyroid hormones can result in changes in the relative proportions of young and old cells observed in salmonids (Denton and Yousef, 1975; Lane, 1979; Härdig and Hoglund, 1984). Thus, age-related differences in red blood cell properties could have important implications *in vivo*. Furthermore, differences in blood properties due to different red blood cell age profiles may also explain some of the variation in results that is commonly observed between individual studies or from different laboratories working in this area.

Against this background, the present study examines how cell ageing affects protein synthesis and the concentration and/or activity of specific respiratory proteins in the nucleated red blood cells of the rainbow trout (*Oncorhynchus mykiss*). It also investigates the effects of ageing on the ability of the red blood cells to respond to stress by examining protein synthesis and mRNA transcription of key stress proteins during heat shock. It is hypothesized that the reduction in aerobic metabolic rate observed during ageing in nucleated red blood cells (Phillips et al., 2000) will be associated with lower rates of protein synthesis during normal conditions and a reduction in the ability to synthesize heat shock proteins in response to temperature stress.

Materials and methods

Blood preparation and separation of age fractions

Freshwater rainbow trout, *Oncorhynchus mykiss* (Walbaum) (1–4 kg), obtained from a commercial hatchery (Pure Springs Trout Farm, Belleville, Ontario, Canada), were held in the Biosciences Aquatic Facility at Queen's University where they were maintained in aerated dechlorinated water at 10–15 °C and fed commercial fish pellets.

Trout were anaesthetized in dechlorinated water containing 250 mg l⁻¹ 3-aminobenzoic acid ethyl ester (MS-222, Sigma) buffered with 500 mg l⁻¹ NaHCO₃. Approximately 15 ml of blood was collected by caudal puncture into a chilled round-bottomed flask containing heparinized (40 i.u. ml⁻¹) saline (in mmol⁻¹: 124 NaCl, 5 KCl, 0.5 MgCl₂, 1.1 CaCl₂, 5.5 glucose and 10 NaHCO₃). Red blood cells were then washed three times with saline. During the washing procedure, white blood cells were removed and discarded with the supernatant.

Red blood cells were separated into different age fractions using the fixed-angle centrifugation method. This technique has been used successfully in a number of previous studies (Murphy, 1973; Cohen et al., 1976; Speckner et al., 1989) and takes advantage of density differences between young and old

cells that largely result from a rise in mean erythrocyte haemoglobin concentration during maturation. It should be noted that some degree of contamination between age fractions is expected when cells are separated using this approach (Tooze and Davies, 1963; Härdig, 1978; Keen et al., 1989). Moreover, the resolution is limited by the trade-off between the number of fractions that can be chosen while still providing enough sample volume for analysis. Nonetheless, this technique does provide valuable insight into the relative effects of age on red blood cell variables. Furthermore, it should be recognized that the magnitude of any observed differences between the youngest and oldest cell fractions are probably conservative estimates that might be even larger if the cells were separated into a greater number of fractions.

In the present study, the methodology for separating the red blood cells *via* fixed-angle centrifugation was identical to that recently described by Phillips et al. (2000), by whom the procedure was optimized for trout blood. Briefly, cells were adjusted to a hematocrit (Hct) of approximately 80% and centrifuged in a narrow centrifuge tube (diameter 13 mm, length 100 mm, volume 6.5 ml) at a fixed angle of 30° to the vertical (10 000 g, 14 °C) for 15 min using a model J2-21M centrifuge (Beckman). This approach creates an internal circulation within the filled centrifuge tube, allowing red blood cells to be separated on the basis of density alone into different age fractions (Murphy, 1973; Cohen et al., 1976; Speckner et al., 1989; Phillips et al., 2000). Following centrifugation, the red blood cells were carefully pipetted from the tube in six equal 1 ml fractions (the youngest cells being in the uppermost fraction, the oldest in the bottom), washed twice in saline, and maintained on ice until subjected to one of the six experimental series described below.

Series 1. Total DNA, RNA and protein: isolation and analyses

Total DNA was extracted as previously described (Leary et al., 1998). Initially, 200 µl of packed red blood cells was added to 1.8 ml of extraction buffer (20 mmol l⁻¹ Hepes at pH 7.0, 1 mmol l⁻¹ EDTA, 0.1% Triton X-100). This extract was treated with 10 µl of sodium dodecyl sulphate (SDS) (0.1%) and DNAase-free RNAase (1 µg µl⁻¹) to degrade RNA. Proteinase K treatment began with the addition of 500 µl of buffer, bringing the final concentrations in the extract to 100 mmol l⁻¹ NaCl, 25 mmol l⁻¹ EDTA, 10 mmol l⁻¹ Tris (pH 8.0), 0.5% SDS and 0.1 mg ml⁻¹ proteinase K, followed by digestion for 20 h at 50 °C. Samples were extracted in an equal volume of phenol/chloroform/isoamyl alcohol (24:25:1 by volume), vortexed for a few seconds, and centrifuged at 25 °C for 10 min at 3000 g. The supernatant was then removed, and the DNA was precipitated overnight in 0.5 volume of 7.5 mol l⁻¹ ammonium acetate and 2 volumes of 100% ethanol. The DNA samples were centrifuged for 5 min at 3000 g, washed in 70% ethanol and dissolved in 1 ml of autoclaved water. Total DNA quantity and purity were assessed by measuring absorbance at 260 and 280 nm with a Spectramax Plus plate spectrophotometer (Molecular Devices) in triplicate.

Total RNA was extracted by the acid/phenol method of Chomczynski and Sacchi (1987), as modified for fish blood by

Currie et al. (1999). Packed red blood cells (300 μ l) from each fraction were added to 20 ml of guanidinium thiocyanate (GTC), shaken vigorously for 5 min and stored at -80°C . Upon thawing, the following were then added with thorough mixing after each step: 2 ml of 2 mol l^{-1} sodium acetate (pH 4.0), 20 ml of buffer-saturated phenol (pH 4.0) and 4 ml of chloroform/isoamyl alcohol (49:1 by volume). Samples were shaken vigorously for 20 s, left on ice for 15 min, and centrifuged for 30 min at 3000 g . The supernatant (20 ml) was collected and added to an equal volume of isopropanol to precipitate the RNA overnight at -20°C . The RNA pellet was collected by centrifugation for 30 min at 3000 g , dissolved in 5 ml of GTC and the phenol extraction/isopropanol precipitation repeated. Following this, the RNA pellet was redissolved in 0.3 ml of GTC and reprecipitated in 0.3 ml of isopropanol, centrifuged (10 min at 3000 g), washed in 75 % ethanol diluted with diethyl pyrocarbonate (DEPC)-treated water, dried and dissolved in 20–60 μ l of DEPC-treated water. Total RNA was quantified using a Spectramax Plate Spectrophotometer (Molecular Devices) in triplicate.

Total protein from each age fraction was assayed in triplicate on the packed red blood cells from each of the six fractions using the bicinchoninic acid (BCA) protein assay reagent method (Pierce).

Series II. Northern blot analyses of β -globin, carbonic anhydrase and the $\text{Cl}^{-}/\text{HCO}_3^{-}$ exchanger

For northern blots, 10 μ g of total RNA was fractionated by glyoxal/dimethyl sulphoxide (DMSO) denaturing electrophoresis on a 1 % agarose gel and transferred to a nylon membrane (Stratagene Duralon) using $20\times$ standard saline citrate (SSC). Membranes were ultraviolet-crosslinked (Fisher UV crosslinker) twice at optimal setting prior to hybridization.

Probes for β -globin (a subunit of haemoglobin), carbonic anhydrase and the $\text{Cl}^{-}/\text{HCO}_3^{-}$ exchanger (AE1) were generated from first-strand cDNA from rainbow trout blood mRNA. The β -globin probe was a 446 base pair (bp) fragment between nucleotides 9 and 455 from the β -globin rainbow trout mRNA sequence (GenBank accession no. D82926) (Yoshikazi et al., 1996). This probe was amplified using the forward primer 5'-ATGGTCGACTGGACAGATCC-3' and the reverse primer 5'-CTAGTGGTACTGTCTGCCAAG-3'. The probe for carbonic anhydrase was a 322 bp fragment between nucleotides 336 and 658 from the zebrafish mRNA sequence (GenBank accession no. U55177) (Peterson et al., 1997). The forward primer, 5'-CAGTTCCATTTCCATTTGGG-3' and the reverse primer 5'-GTGGTCAGAGAGCCCTCA-3' were used to amplify the carbonic anhydrase probe. The probe for AE1 was a 117 bp fragment between amino acids M423 and E461 of the rainbow trout AE1 protein (Hübner et al., 1992). This probe was amplified using the forward primer 5'-GGGAATTCATGGGCGTGTCTGA-3' and the reverse primer 5'-TTTGGATCCCTCAAACA-3'. All amplifications were performed at an annealing temperature of 50°C . Polymerase chain reaction (PCR) products were ligated into plasmids and sequenced.

Probes were labelled using $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ (specific activity $10^9\text{ cts min}^{-1}\mu\text{g}^{-1}\text{DNA}$) and the Ready-To-Go labelling system (Pharmacia). Membranes were prehybridized at 68°C for 30 min with QuickHyb hybridization solution (Stratagene). Blots were hybridized for 2 h in the same solution at 68°C , with approximately 10^9 cts min^{-1} of denatured probe. Blots were then washed twice with a $1\times\text{SSC}/0.1\%$ SDS solution (15 min, 26°C) followed by an additional wash in $0.1\times\text{SSC}/0.1\%$ SDS (30 min, 68°C). Blots were exposed to a phosphor screen (Kodak) and then visualized and quantified using a phosphoimager (Molecular Dynamics) driven by ImageQuant software. Band densities for β -globin, carbonic anhydrase and AE1 were corrected for total RNA and expressed as a ratio of the band density of fraction 1. All membranes were also probed with a human 18S rRNA (Battersby and Moyes, 1998) to correct blots for loading differences.

Series III. Radioisotopic labelling to quantify protein synthesis at 10°C

The amount of protein synthesized by 50 μ l of red blood cells (adjusted to a Hct of 20 %) over a 2 h interval was also determined. Samples of red blood cells were incubated for 30 min at 10°C before labelling with 10 μ l of $[\text{S}^{35}]\text{methionine}$ (4000 kBq ml^{-1} ; NEN Life Science). In preparation for fluorographic analysis, each fraction was washed twice for 2 min at 2000 g and sonicated for several seconds at a low setting. Nuclei were removed from the suspension by centrifugation for 10 min at 1000 g , and the supernatant was then frozen at -80°C . Following thawing, proteins were denatured by adding 100 μ l from each sample to 300 μ l of a 1 % SDS/10 mmol Tris (pH 8) solution and boiling for 5 min. After boiling, 20 μ l of a $6\times\text{SDS}$ loading dye was added to a 100 μ l subsample of the 400 μ l denatured solution. Samples of 10 μ l were then loaded on a one-dimensional SDS polyacrylamide (15 %) gel and subjected to electrophoresis (Laemmli, 1970). Following this, gels were stained for 1 h with 0.1 % Coomassie Blue R250 and destained overnight in 40 % methanol, 10 % acetic acid, 50 % water. Gels were placed on a phosphor screen to be imaged and quantified for all resulting band densities.

Series IV. Protein concentrations/activities

Mean erythrocyte haemoglobin (Hb) concentration (MEHC) was measured using the Hb/cyanide method (Betke and Savelsberg, 1950; van Kampen and Zijlstra, 1961) and a DU 640 spectrophotometer (Beckman). The Hct of each fraction was determined following centrifugation of the samples for 4 min in an IEC MB microhaematocrit centrifuge (Damon/IEC Division). The MEHC for each fraction was calculated as $[\text{Hb}]/\text{Hct}$.

Carbonic anhydrase activity was assayed using the electrometric pH method (Henry, 1991; Henry et al., 1993). Packed red blood cells from each fraction were diluted 1:500 (by volume) in distilled water to form lysates which were frozen at -80°C until the day of the assay. Following thawing,

50 µl of lysate was added to 10 ml of reaction buffer (in mmol⁻¹: 225 mannitol, 75 sucrose and 10 Tris base adjusted to pH 7.4 with 1.0 mol⁻¹ HCl) and held at 4 °C. To start the reaction, 400 µl of CO₂-saturated distilled water was added to the mixture using a 1000 µl gas-tight Hamilton syringe. The reaction was then measured over a change of 0.15 pH units using a GK2401 C combined electrode (Radiometer) attached to a PHM64 research pH meter (Radiometer). The true catalyzed rate was calculated by subtracting the uncatalyzed rate from the observed rate and measuring buffer capacity to convert from pH units min⁻¹ to mol H⁺ min⁻¹.

The rate of Cl⁻/HCO₃⁻ exchange across the red blood cell membrane was measured according to the method of Lambert and Lowe (1978) with minor modifications (Stabeneau et al., 1991; Tufts et al., 1999). Red blood cells from each age fraction were washed three times in HCO₃⁻-free, Cl⁻-rich saline (in mmol⁻¹: 124 NaCl, 6 KCl, 1.5 CaCl₂, 5 glucose, 1 Hepes, 5 EDTA, adjusted to pH 7.8). After the final wash, the supernatant was removed and 100 µl of red blood cell pellet was injected into 10 ml of a rapidly stirred HCO₃⁻-rich, Cl⁻-free solution (in mmol⁻¹: 320 sucrose, 2 NaHCO₃, 1 Hepes, 0.005 carbonic anhydrase, adjusted to pH 7.8) in a thermostatted (10 °C) vessel. The pH change was then monitored for 10 s using a GK2401 C combined electrode (Radiometer) attached to a PHM64 research pH meter (Radiometer). The initial rate of Cl⁻/HCO₃⁻ exchange (θ_{uni}) was then calculated according to the following equation:

$$\theta_{uni} = (dpHe/dt)(\beta_e)(1/Hct)(1/V),$$

where pHe is extracellular pH, t is time, β_e is the extracellular non-HCO₃⁻ buffer capacity at the initial pHe, Hct is the pellet haematocrit and V is the volume of pellet added (in µl).

Series V. Northern blot analyses of heat shock factor, heat shock cognate 71 and stress-inducible heat shock protein 70

With the exception of heat shock protein 70 (Hsp 70) experiments, total RNA was extracted from each of six fractions immediately following the separation process. For Hsp 70 measurements, red blood cells from the youngest (fraction 1) and oldest (fraction 6) fractions were resuspended in heparinized saline (20% Hct) and incubated in rotating aerated tonometers (1% CO₂) at 10 °C for 30 min. A 500 µl control sample was taken and homogenized by shaking in 10 ml of GTC. The temperature was then raised to 25 °C (the temperature of peak Hsp 70 production; Currie and Tufts, 1997), and 500 µl samples were taken after 0.5, 1, 1.5, 2, 3 and 4 h. Samples were frozen at -80 °C until analyzed. Northern blots of Hsp 70 mRNA levels were then performed using the methods described for series II. The heat shock factor (HSF) probe was a 468 bp fragment between nucleotides 46 and 513 generated by PCR amplification of cDNA from reverse transcription of RNA from C212 immortalized mouse myoblasts. Primers were designed for heat shock transcription factor from a human HSF1 mRNA coding sequence reported by Rabindran et al. (1991) (GenBank accession no. M64673). This probe was amplified with the forward primer 5'-

CCGGCCTTCCTRACCAAGCTGTGG-3' and the reverse primer 5'-CTCCCGCCACAGRGCCTCRTTSTC-3', in which R can be A or G and S can be G or C. An annealing temperature of 55 °C was used, and the identity of the product was confirmed by sequencing. Probes for Hsp 70 and heat shock cognate 71 (Hsc 71) were as described previously (Currie et al., 1999).

All probes were labelled as in series II and, with the exception of HSF, were prehybridized and hybridized in QuickHyb hybridization solution (Stratagene), and washed, under the same conditions as in series II. Membranes to be probed for HSF were prehybridized for 6 h in a 6×SSC/0.5% SDS/5×Denhardt's solution, followed by an 18 h hybridization with the probe in the same solution, both at 42 °C. Blots probed for HSF were washed twice in 1×SSC/0.1% SDS (15 min, 42 °C) and once in 0.25×SSC/0.1% SDS (15 min, 42 °C). As in series II, the blots for Hsc 71 and HSF were exposed to a phosphor screen (Kodak), visualized and quantified by phosphoimager (Molecular Dynamics), corrected for total RNA and expressed as a ratio of the band with the highest density. Blots for Hsp 70 were visualized using Kodak X-Omat Blue XB-1 film and quantified using a flatbed scanner (Canon IX-4015) with associated UN-SCAN-IT software. Band densities were corrected for total RNA and were expressed relative to the period of greatest induction. As in series II, all membranes were also probed with human 18S rRNA to determine whether there were any significant differences in loading.

Series VI. Radioisotopic labelling to quantify protein synthesis following heat shock

Following age separation by centrifugation, two 50 µl samples of packed red blood cells from fractions 1 and 6 were resuspended in heparinized saline (adjusted to a Hct of 20%) and incubated at 10 °C for 30 min. Each fraction was heat-shocked for 2 h at 25 °C and then returned to 10 °C and labelled with 4000 kBq ml⁻¹ of [³⁵S]methionine (NEN Life Science) for 2 h. One set was incubated with 20 µg ml⁻¹ actinomycin D for the 2 h period at 10 °C to prevent transcription of new mRNA. In preparation for fluorographic analysis, samples were prepared using the same methods described for series III. Samples of 15 µl were then loaded onto a one-dimensional SDS polyacrylamide (10%) gel and subjected to electrophoresis (Laemmli, 1970). Gels were then stained, imaged and quantified using the methods described in series III.

Statistical analyses

Significant differences ($P \leq 0.05$) between fraction means were detected using one-way analyses of variance (ANOVAs) and identified using the Student–Newman–Keuls test.

Results

DNA and RNA

Total red blood cell DNA content was unaffected by cell

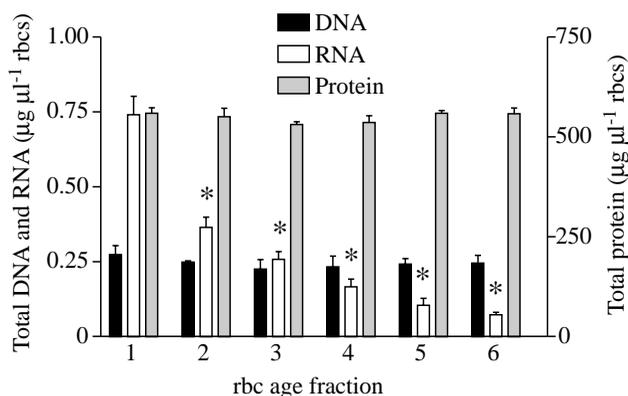


Fig. 1. Total DNA ($N=4$), RNA ($N=6$) and protein ($N=6$) content in six age fractions of rainbow trout red blood cells (rbc). The youngest red blood cells are in fraction 1 and the oldest in fraction 6. Asterisks denote a significant difference from the total RNA in fraction 1 ($P \leq 0.05$). All values are expressed as mean \pm 1 S.E.M.

age, whereas total red blood cell RNA content decreased across the six age fractions (Fig. 1). Total red blood cell RNA content peaked at $0.74 \mu\text{g} \mu\text{l}^{-1}$ red blood cells in fraction 1, ten times higher than that in fraction 6. Total RNA content decreased sharply between fractions 1 and 2 and then declined more gradually across the older fractions. This relationship between age and RNA levels influences all the subsequent data expressing $\text{mRNA} \mu\text{l}^{-1}$ blood. Although samples were electrophoresed with a constant amount of RNA per lane, subsequent results involving quantitative representations are corrected for $\text{RNA} \mu\text{l}^{-1}$ on the basis of these mean values.

Representative northern blots are shown for β -globin, carbonic anhydrase and AE1 (Fig. 2B). After correcting for total RNA (Fig. 1) and loading differences, analysis of band densities revealed that the mRNA content for β -globin, carbonic anhydrase and AE1 was 8–10 times higher in fraction 1 than in fraction 6 (Fig. 2A). The decline in mRNA content for these respiratory proteins was therefore similar to the decline in total RNA content.

Protein synthesis and total protein content under normal conditions

The representative fluorogram from experiments involving [^{35}S]methionine radioisotopic labelling (Fig. 3A) shows that protein synthesis in fraction 1 red blood cells was roughly five times higher than that in fraction 6 red blood cells (Fig. 3B). However, total protein concentration did not change significantly between any of the six fractions (Fig. 1).

Individual protein concentrations/activities

Unique trends were seen for each protein with regards to concentration/activity. Mean erythrocyte haemoglobin concentration (MEHC) increased with red blood cell fraction age (Fig. 4A). The MEHC for fraction 6 of $242 \mu\text{g} \mu\text{l}^{-1}$ red blood cells was 122% of the MEHC of fraction 1 of $198 \mu\text{g} \mu\text{l}^{-1}$ red blood cells. The magnitude of this difference in MEHC across the six fractions in the present

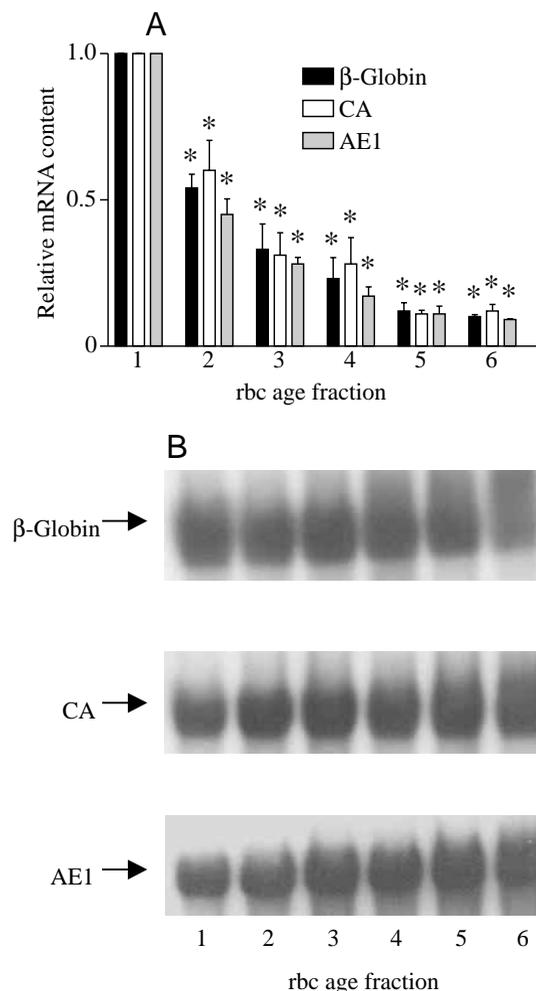


Fig. 2. (A) Relative mRNA concentrations for northern blots of β -globin, carbonic anhydrase (CA) and the $\text{Cl}^-/\text{HCO}_3^-$ exchanger (AE1) in ageing rainbow trout red blood cells (rbc; $N=3$ for each). The youngest red blood cells are in fraction 1 and the oldest in fraction 6. Band densities from northern blots were corrected for total RNA (per μl of red blood cells) as well as loading differences using 18S rRNA and are expressed relative to the age fraction with the highest band density. Asterisks denote a significant difference from the respective band density in fraction 1. All values are expressed as mean \pm 1 S.E.M. (B) Representative northern blots for β -globin, carbonic anhydrase and AE1 in ageing rainbow trout red blood cells.

study is very similar to that observed in previous studies using fish red blood cells (Speckner et al., 1989; Phillips et al., 2000). These results therefore confirm that the methodology used in the present study was effective in separating trout red blood cells into fractions with different mean ages. Unlike MEHC, carbonic anhydrase activity decreased with red blood cell age (Fig. 4B). The mean fraction 1 carbonic anhydrase activity of $67 \text{mmol min}^{-1} \mu\text{l}^{-1}$ red blood cells was nearly 160% of that in fraction 6. In contrast to both MEHC and carbonic anhydrase activity, the rate of $\text{Cl}^-/\text{HCO}_3^-$ exchange across the cell membrane was not significantly affected by the age of the red blood cell (Fig. 4C).

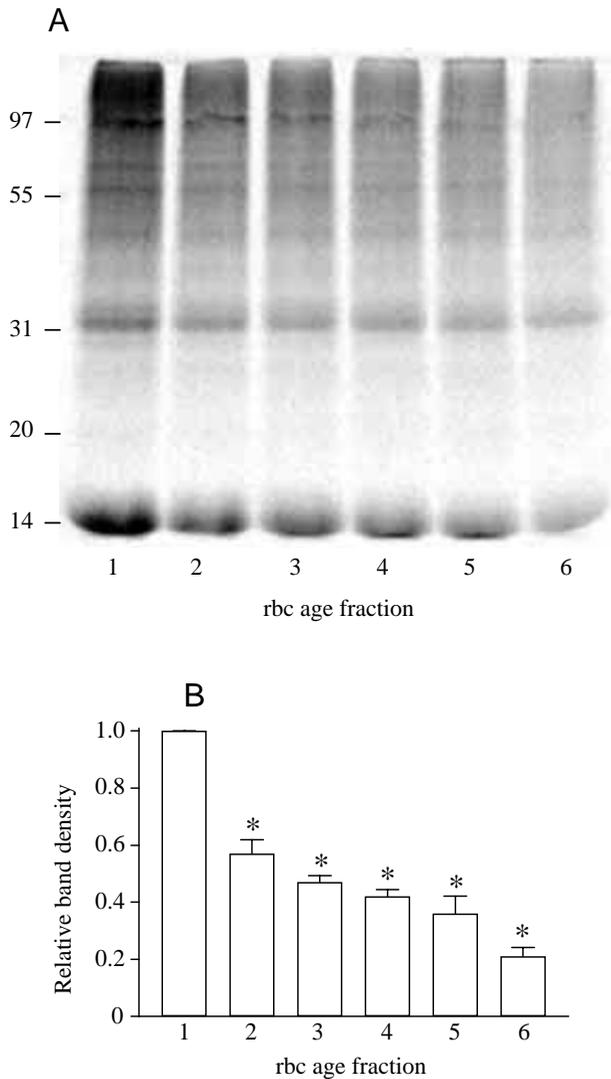


Fig. 3. Representative fluorogram of protein synthesis (A) and relative band densities (B) in ageing rainbow trout red blood cells labelled with [35 S]methionine. The results represent the total amount of protein synthesized by 250 μ l of red blood cells (haematocrit 20%) over 2 h ($N=3$) relative to that synthesized by the youngest cells. Molecular masses of proteins are indicated in kDa. The youngest red blood cells are in fraction 1 and the oldest in fraction 6. Asterisks indicate a significant difference from fraction 1. All band density values are expressed as mean \pm 1 S.E.M.

Stress-associated mRNA concentrations

Upon examination of a representative Hsc 71 blot (Fig. 5B), mRNA levels appear to increase with increasing red blood cell age. Correcting for total RNA differences (Fig. 1), however, reverses this relationship and results in Hsc 71 mRNA levels in fraction 1 being five times greater than those in fraction 6 and in HSF mRNA levels in fraction 1 being 7.7 times greater than those in fraction 6 (Fig. 5A). When comparing Hsp 70 mRNA induction between red blood cells of fraction 1 and fraction 6 over a 4 h time course at 25 $^{\circ}$ C, it is apparent from both the representative blot (Fig. 6B) and the corrected band densities that younger cells produce more Hsp 70 at each

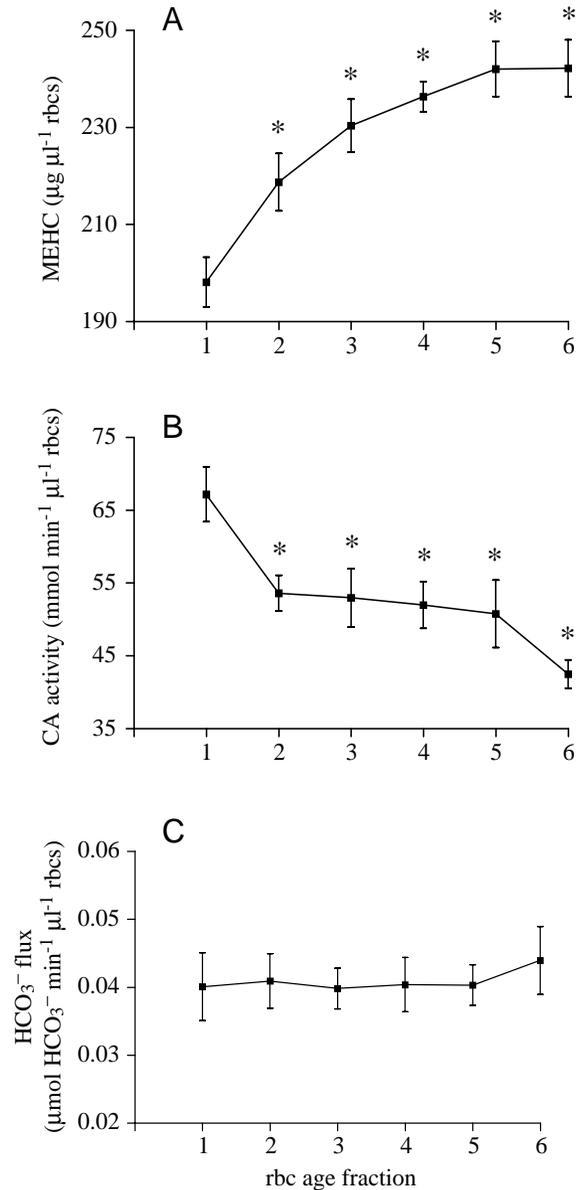


Fig. 4. Concentrations/activities for haemoglobin (measured as MEHC) (A), carbonic anhydrase (CA) (B) and the Cl $^-$ /HCO $_3^-$ exchanger (AE1) (C) in ageing rainbow trout red blood cells ($N=6$ for each). The youngest red blood cells are in fraction 1 and the oldest in fraction 6. Asterisks denote a significant difference from fraction 1 ($P \leq 0.05$). All values are expressed as mean \pm 1 S.E.M. MEHC, mean erythrocyte haemoglobin concentration.

sampling period (Fig. 6A). The Hsp 70 mRNA response reaches a plateau after 1 h of heat shock for both fractions 1 and 6, from which point the younger cells maintain an average 13-fold greater response than the older cells (Fig. 6A).

Protein synthesis following heat shock

Fig. 7 illustrates the effect of a 2 h, 25 $^{\circ}$ C, heat shock on protein synthesis during a subsequent 2 h recovery period at 10 $^{\circ}$ C. Red blood cells from both fractions 1 and 6 produce more of a protein with an apparent molecular mass of 70 kDa

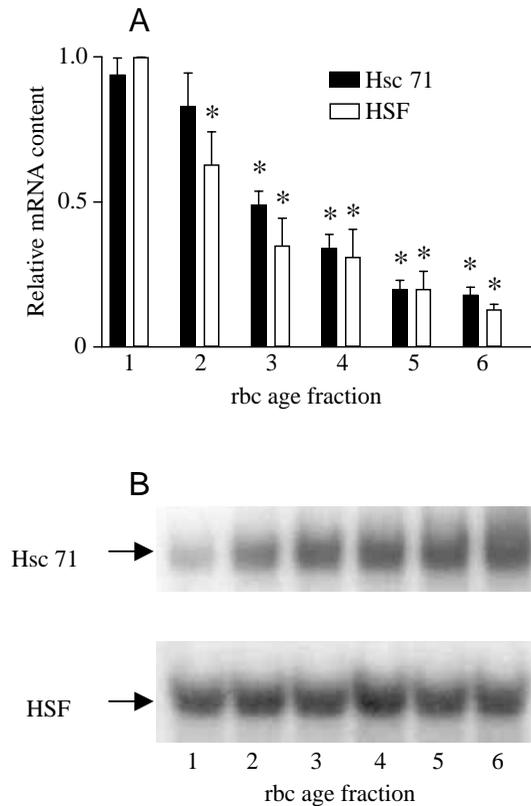


Fig. 5. (A) Relative mRNA concentrations for northern blots of heat shock cognate 71 (Hsc 71) and heat shock factor (HSF) in ageing rainbow trout red blood cells (rbcs; $N=3$ for each). The youngest red blood cells are in fraction 1 and the oldest in fraction 6. Band densities from northern blots were corrected for total RNA (per μl of red blood cells) as well as loading differences using 18S rRNA and are expressed relative to the age fraction with the highest band density. Asterisks denote a significant difference from the respective band density in fraction 1 ($P \leq 0.05$). All values are expressed as mean ± 1 S.E.M. (B) Representative northern blots for Hsc 71 and HSF in ageing rainbow trout red blood cells.

than any other protein both in the presence and in the absence of actinomycin D. Red blood cells from fraction 1 also synthesize a large amount of a protein with an apparent molecular mass of 90 kDa, although this response is attenuated in the presence of actinomycin D.

Discussion

It has been documented that ageing in rainbow trout red blood cells is accompanied by a significant reduction in aerobic metabolic rate (Hunter and Hunter, 1957; Lane, 1984; Phillips et al., 2000). However, the effects of a decline in red blood cell metabolic status on the energetically expensive process of protein synthesis are unknown. The rate of protein synthesis in the present study was found to decline in ageing nucleated red blood cells by roughly 80% (Fig. 3). Since total DNA content remains constant in each age fraction, the decrease in the rate of protein synthesis is probably a reflection of the observed

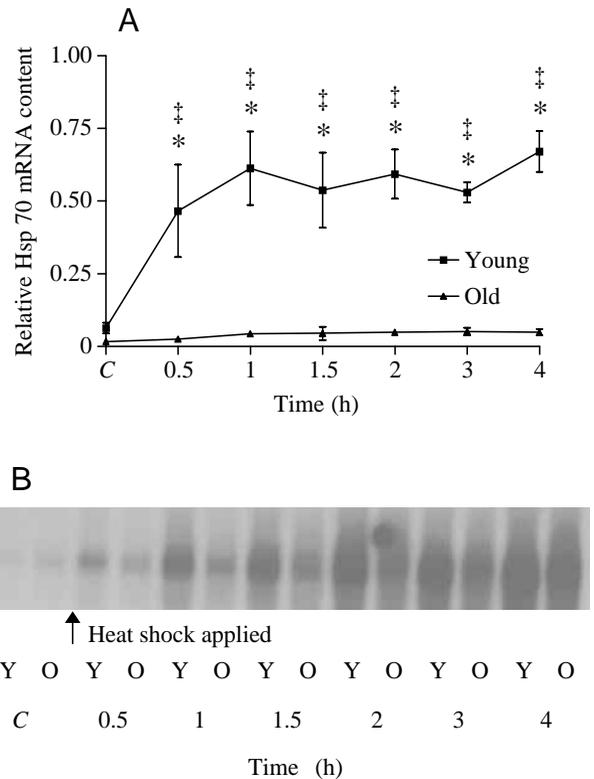


Fig. 6. (A) Relative mRNA concentrations for northern blots of heat shock protein 70 (Hsp 70) in young and old red blood cells (rbcs; $N=3$). Band densities from northern blots were corrected for total RNA (per μl of red blood cells, using total RNA concentrations from Fig. 1) as well as loading differences using 18S rRNA and are expressed relative to the period at which Hsp 70 mRNA level was highest for fraction 1. Asterisks denote a significant difference from the respective band density in fraction 1 at each sampling period ($P \leq 0.05$), and double daggers indicate a significant difference from the control value within the respective age group ($P \leq 0.05$). All values are expressed as mean ± 1 S.E.M. (B) Representative northern blot for Hsp 70 induced during a 4 h time course at 25 °C (starting at 10 °C indicated by C) in both young (Y, fraction 1) and old (O, fraction 6) rainbow trout red blood cells.

90% decrease in total RNA content that occurs as the cell ages (Fig. 1). The disappearance of ribosomal RNA, which constitutes the majority of red blood cell RNA, severely restricts the translation potential of the cell. Northern blots for β -globin, carbonic anhydrase and AE1 show that levels of mRNA for these specific proteins also fall by nearly 90% (Fig. 2). The observed reduction in mRNA for these respiratory proteins (as well as the heat shock associated proteins below) suggests either (i) that transcription declines with increasing red blood cell age, or (ii) that transcription ceases, but because of the differential stabilities of the mRNAs measured, some still remains even in the oldest cells. Further studies would be necessary, however, to distinguish between these two possibilities.

Despite the fact that mRNA levels for the essential respiratory proteins decline with increasing red blood cell age,

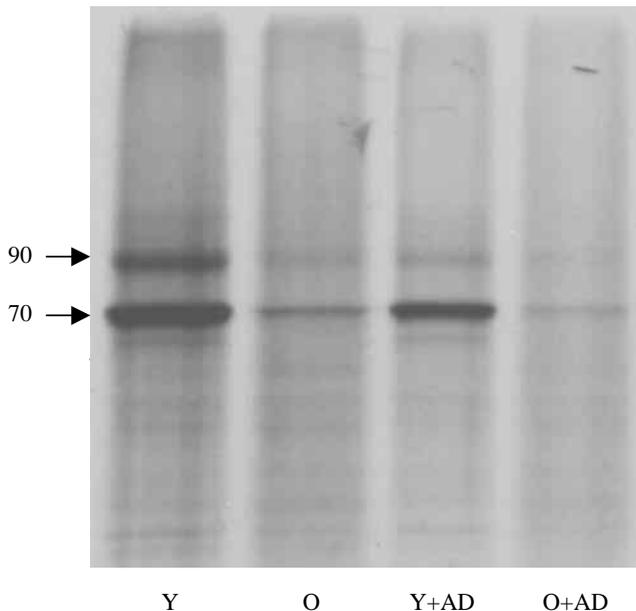


Fig. 7. Representative fluorogram of protein synthesis in both young (Y, fraction 1) and old (O, fraction 6) rainbow trout red blood cells labelled with [35 S]methionine. The results represent the total amount of protein synthesized by 250 μ l of red blood cells (haematocrit 20%) over 2 h at 10 $^{\circ}$ C following a 2 h heat shock at 25 $^{\circ}$ C, both in the presence and in the absence of actinomycin D (AD). Molecular masses of proteins are indicated in kDa. This fluorogram was representative of three separate experiments.

the respiratory functions of the red blood cells seem to be largely preserved throughout the lifetime of the cell. Although results from this study show that the carbonic anhydrase activity in young red blood cells is 160% of that in old red blood cells (Fig. 4B), MEHC actually increases with red blood cell age (Fig. 4A) and the rate of $\text{Cl}^-/\text{HCO}_3^-$ exchange across the cell membrane is unaltered during red blood cell ageing (Fig. 4C). This is interesting because the rate of $\text{Cl}^-/\text{HCO}_3^-$ exchange across vertebrate red blood cell membranes is thought to be the rate-limiting factor in blood CO_2 transport (Perry, 1986; Perry and Gilmour, 1993). Thus, while trout red blood cells appear to lose nearly half their carbonic anhydrase activity during ageing, it is unlikely that these changes will have a negative impact on the contribution of the cell to the process of blood CO_2 transport. Preserved functional capacity, despite low rates of synthesis, suggests that enzyme and transporter levels are maintained by low rates of protein turnover or degradation. Since total RNA measurements and northern blots represent the balance between RNA synthesis and degradation, it is not possible to distinguish between these two possibilities. It is also interesting that, while protein synthesis in rainbow trout red blood cells declines by 90% during ageing (Fig. 3), the total protein content of the cell remains relatively constant (Fig. 1). These results demonstrate that proteins with relatively long half-lives, such as haemoglobin and the anion exchanger AE1, probably exert a greater influence on total protein concentration than proteins

such as metabolic enzymes that have relatively rapid turnover rates (Phillips et al., 2000). It should be noted, however, that the relative importance of other membrane transport proteins or cytoskeletal proteins in this regard is unknown.

Although their basic respiratory function seems to be maintained, older red blood cells display a greatly reduced capacity to mount a heat shock response. Levels of Hsp 70 mRNA following 1 h of heat shock are over 13 times higher in younger cells (Fig. 6) and result in the translation of four times as much 70 kDa protein, 2 h after the heat stress (Fig. 7), in comparison with older red blood cells. The observed 80% decline in the amount of steady-state Hsc 71 mRNA and 87% decline in HSF mRNA with increasing red blood cell age (Fig. 5) also suggest that ageing may be associated with a decreased ability to 'sense' stress or a deficit in the capacity to 'chaperone' the synthesis of new proteins. This is based on the assumption that a reduction in Hsc 71 and HSF mRNA leads to a decrease in the amount of their respective proteins being synthesized. Because the heat shock response is believed to be initiated by the trimerization of HSF and its subsequent binding to the heat shock element on the heat shock gene (Morimoto, 1993; Kroeger and Morimoto, 1995; Mestrlil and Dillman, 1995), one would hypothesize that a reduction in HSF mRNA with increasing red blood cell age might lead to a reduction in the amount of HSF protein available for binding and, thus, to a possible decrease in the heat shock response. Again, the declines in Hsc 71 and HSF mRNA with age directly parallel the 90% decrease in total RNA (Fig. 1) that occurs as the red blood cells age. This study focuses mainly on Hsp 70, but it is interesting to note that there was also a reduction in the amount of a 90 kDa protein in response to heat shock (Fig. 7). In mammals, Hsp 90 has been shown to be necessary for the function and stability of many signalling proteins and transcription factors, including HSF-1 (Nadeau et al., 1993).

In the presence of actinomycin D, there is an obvious reduction in the amount of the 70 and 90 kDa proteins synthesized during the 2 h recovery period following the 2 h heat shock in both young and old red blood cells. This indicates that a significant amount of the mRNA response to heat stress occurs during the recovery period in both young and old red blood cells, not solely during the heat stress (Fig. 7). From the present mRNA and protein data, it is impossible to deduce whether the decreased Hsp 70 response in older cells occurred as a result of a general decline in the ability of the majority of older cells to respond to temperature stress or whether there is a subpopulation of older cells that eventually are unable to respond to the temperature stress. A significant challenge for future studies in this area may, therefore, be to determine how the characteristics of individual red blood cells change with age.

In conclusion, the present study indicates that the activity of the major respiratory proteins, and therefore the basic respiratory function, of trout red blood cells is largely preserved throughout their lifespan, despite large declines in mRNA for β -globin, carbonic anhydrase and AE1 and a general reduction in protein synthesis with increasing red blood cell age. The

preserved functional capacity of these proteins, despite low levels of synthesis, suggests that enzyme and transporter levels are maintained because of low rates of protein turnover or degradation. While the lower level of transcription is adequate to maintain respiratory function in older cells, they have a severely reduced capacity to mount a heat shock response. Not only do older red blood cells produce significantly less Hsp 70 mRNA and 70 kDa protein in comparison with younger red blood cells upon heat shock, but they also have much lower steady-state levels of Hsc 71 and HSF mRNA. This indicates that the ability of trout red blood cells to sense and respond to stress is reduced with increasing age. Although nucleated red blood cells differ from the enucleated red blood cells of mammals in terms of their metabolic capacity (Phillips et al., 2000), protein synthetic ability (Speckner et al., 1989) and heat shock protein response (Currie and Tufts, 1997; Currie et al., 1999), the present study shows that these differences may be largely diminished with increasing cell age, at least in the nucleated red blood cells of the rainbow trout.

Special thanks to M. Fortner for her technical assistance. This work was supported by a Queen's Graduate Fellowship to S.L. and Natural Sciences and Engineering Research Council Grants to B.L.T. and C.D.M.

References

- Battersby, B. J. and Moyes, C. D.** (1998). Influence of acclimation temperature on mitochondrial DNA, RNA and enzymes in skeletal muscle. *Am. J. Physiol.* **275**, R905–R912.
- Becker, J. and Craig, E. A.** (1994). Heat-shock proteins as molecular chaperones. *Eur. J. Biochem.* **219**, 11–23.
- Betke, K. and Savelsberg, W.** (1950). Stufenphotometrische hämoglobinstimmung mittels cyanhäoglobin. *Biochem. Z.* **320**, 431–439.
- Boutilier, R. G. and Ferguson, R. A.** (1989). Nucleated red cell function: metabolism and pH regulation. *Can. J. Zool.* **67**, 2986–2993.
- Chomczynski, P. and Sacchi, N.** (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Analyt. Biochem.* **162**, 156–159.
- Cohen, N. S., Ekholm, J. E., Luthra, M. G. and Hanahan, D. J.** (1976). Biochemical characterization of density-separated human erythrocytes. *Biochim. Biophys. Acta* **419**, 229–242.
- Currie, S. and Tufts, B. L.** (1997). Synthesis of stress protein 70 (Hsp70) in rainbow trout (*Oncorhynchus mykiss*) red blood cells. *J. Exp. Biol.* **200**, 607–614.
- Currie, S., Tufts, B. L. and Moyes, C. D.** (1999). Influence of bioenergetic stress on heat shock protein gene expression in nucleated red blood cells of fish. *Am. J. Physiol.* **276**, R990–R996.
- Denton, J. E. and Yousef, M. K.** (1975). Seasonal changes in hematology of rainbow trout, *Salmo gairdneri*. *Comp. Biochem. Physiol.* **51A**, 151–153.
- Härdig, J.** (1978). Maturation of circulating red blood cells in young Baltic salmon (*Salmo salar* L.). *Acta Physiol. Scand.* **102**, 290–300.
- Härdig, J. and Hoglund, L. B.** (1984). Seasonal variation in blood components of reared Baltic salmon, *Salmo salar* L. *J. Fish Biol.* **24**, 565–579.
- Hartl, F. U.** (1996). Molecular chaperones in cellular protein folding. *Nature* **381**, 571–580.
- Henry, R. P.** (1991). Techniques for measuring carbonic anhydrase activity *in vitro*. In *The Carbonic Anhydrases* (ed. S. J. Dodgson, R. E. Tashian, G. Gros and N. D. Carter), pp. 119–131. New York: Plenum Press.
- Henry, R. P., Tufts, B. L. and Boutilier, R. G.** (1993). The distribution of carbonic anhydrase type I and II isozymes in lamprey and trout: possible co-evolution with erythrocyte chloride/bicarbonate exchange. *J. Comp. Physiol.* **163**, 380–388.
- Hevesy, G., Lockner, D. and Sletten, K.** (1964). Iron metabolism and erythrocyte formation in fish. *Acta Physiol. Scand.* **60**, 256–266.
- Houston, A. H. and Murad, A.** (1991). Hematological characterization of goldfish, *Carassius auratus* L., by image analysis: effects of thermal acclimation and heat shock. *Can. J. Zool.* **69**, 2041–2047.
- Hübner, S., Michel, F., Rudloff, V. and Appelhans, H.** (1992). Amino acid sequence of band-3 protein from rainbow trout erythrocytes derived from cDNA. *Biochem. J.* **285**, 17–23.
- Hunter, A. S. and Hunter, F. R.** (1957). A comparative study of erythrocyte metabolism. *J. Cell. Comp. Physiol.* **49**, 479–502.
- Keen, A. M., Steele, C. and Houston, A. H.** (1989). The circulating erythrocytes of rainbow trout. *Comp. Biochem. Physiol.* **94A**, 699–711.
- Kroeger, P. E. and Morimoto, R. I.** (1995). The heat shock transcriptional response. In *Inducible Gene Expression*, vol. 1, *Environmental Stresses and Nutrients* (ed. P. A. Baeuerle), pp. 25–61. Boston: Birkhauser.
- Laemmli, E. K.** (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lambert, A. and Lowe, A. G.** (1978). Chloride/bicarbonate exchange in human erythrocytes. *J. Physiol., Lond.* **275**, 51–63.
- Lane, H. C.** (1979). The response of the haemoglobin system of fed and starved rainbow trout, *Salmo gairdneri* Richardson, to bleeding. *J. Fish Biol.* **16**, 405–411.
- Lane, H.** (1984). Nucleoside triphosphate changes during the peripheral lifespan of erythrocytes of rainbow trout (*Salmo gairdneri*). *J. Exp. Zool.* **231**, 57–62.
- Lane, H. C. and Tharp, T. P.** (1980). Changes in the population of ribosomal red cells of peripheral blood of rainbow trout, *Salmo gairdneri* Richardson, following starvation and bleeding. *J. Fish Biol.* **17**, 75–81.
- Lane, H. C., Weaver, J. W., Benson, J. A. and Nichols, H. A.** (1982). Some age related changes of adult rainbow trout, *Salmo gairdneri* Rich., peripheral erythrocytes separated by velocity sedimentation at unit gravity. *J. Fish Biol.* **21**, 1–13.
- Leary, S. C., Battersby, B. J., Hansford, R. G. and Moyes, C. D.** (1998). Interactions between bioenergetics and mitochondrial biogenesis. *Biochim. Biophys. Acta* **1365**, 522–530.
- Lee, Y.-K., Manalo, D. and Liu, A. Y.-C.** (1996). Heat shock response, heat shock transcription factor and cell ageing. *Biol. Signals* **5**, 180–191.
- Liu, A. Y.-C., Lin, Z., Choi, H.-S., Sorhage, F. and Li, B.** (1989). Attenuated induction of heat shock gene expression in ageing diploid fibroblasts. *J. Biol. Chem.* **264**, 12037–12045.
- Mestrlil, R. and Dillman, W. H.** (1995). Heat shock proteins and protection against myocardial ischemia. *J. Mol. Cell. Cardiol.* **27**, 45–52.
- Morimoto, R. I.** (1993). Cells in stress: transcriptional activation of heat shock genes. *Science* **259**, 1409.

- Murad, A., Houston, A. H. and Samson, L.** (1990). Hematological response to reduced to reduced oxygen-carrying capacity, increased temperature and hypoxia in goldfish, *Carassius auratus* L. *J. Fish Biol.* **36**, 289–305.
- Murphy, J.** (1973). Influence of temperature and method of centrifugation on the separation of erythrocytes. *J. Lab. Clin. Med.* **82**, 334–341.
- Nadeau, K., Das, A. and Walsh, C. T.** (1993). Hsp 90 chaperonins possess ATPase activity and bind heat shock transcription factors and peptidyl prolyl isomerases. *J. Biol. Chem.* **268**, 1479–1487.
- Niedzwiecki, A. and Fleming, J. E.** (1993). Heat shock induces changes in the expression and binding of ubiquitin in senescent *Drosophila melanogaster*. *Devl. Genet.* **14**, 78–86.
- Nikinmaa, M.** (1990). *Vertebrate Red Blood Cells*. Berlin: Springer-Verlag.
- Pahlavani, M. A., Harris, M. D., Moore, S. A., Weindruch, R. and Richardson, A.** (1995). The expression of heat shock protein 70 decreases with age in lymphocytes from rats and rhesus monkeys. *Exp. Cell Res.* **218**, 310–318.
- Parsell, D. A. and Lindquist, S.** (1993). The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet.* **27**, 437–496.
- Perry, S. F.** (1986). Carbon dioxide excretion in fish. *Can. J. Zool.* **64**, 565–572.
- Perry, S. F. and Gilmour, K.** (1993). An evaluation of factors limiting carbon dioxide excretion by trout red blood cells *in vitro*. *J. Exp. Biol.* **180**, 39–54.
- Peterson, R. E., Tu, C. and Linser, P. J.** (1997). Isolation and characterization of a carbonic anhydrase homologue from the zebrafish (*Danio rerio*). *J. Mol. Evol.* **44**, 432–439.
- Phillips, M. C. L., Moyes, C. D. and Tufts, B. L.** (2000). The effects of cell ageing on metabolism in rainbow trout (*Oncorhynchus mykiss*) red blood cells. *J. Exp. Biol.* **203**, 1039–1045.
- Rabindran, S. H., Giorgi, G., Clos, J. and Wu, C.** (1991). Molecular cloning and expression of a human heat shock factor, HSF 1. *Proc. Natl. Acad. Sci. USA* **88**, 6906–6910.
- Sekhon, S. S. and Beams, H. W.** (1969). Fine structure of the developing trout erythrocytes and thrombocytes with special reference to the marginal band and the cytoplasmic organelles. *Am. J. Anat.* **125**, 353–374.
- Speckner, W., Schindler, J. F. and Albers, C.** (1989). Age-dependent changes in volume and haemoglobin content of erythrocytes in the carp (*Cyprinus carpio* L.). *J. Exp. Biol.* **141**, 133–149.
- Stabeneau, E. K., Vanoye, C. G. and Heming, T. A.** (1991). Characteristics of the anion transport system in sea turtle erythrocytes. *Am. J. Physiol.* **261**, R1218–1225.
- Tooze, J. and Davies, H. G.** (1963). The occurrence and possible significance of haemoglobin in the chromosomal regions of mature erythrocyte nuclei of the newt *Triturus cristatus cristatus*. *J. Exp. Biol.* **141**, 133–149.
- Tufts, B. L., Gervais, M. R., Moss, A. G. and Henry, R. P.** (1999). Carbonic anhydrase and red blood cell anion exchange in the neotenic aquatic salamander, *Necturus maculosus*. *Physiol. Biochem. Zool.* **72**, 317–327.
- van Kampen, E. J. and Zijlstra, W. G.** (1961). Standardization of hemoglobinometry. II. The hemoglobincyanide method. *Clin. Chim. Acta* **6**, 538–544.
- Yoshikazi, G., Kang, J., Sakuma, K., Aoki, T. and Takashima, F.** (1996). Cloning and sequencing of rainbow trout beta-globin cDNA. *Fish.-Sci.-Tokyo* **62**, 723–726.