The Journal of Experimental Biology 203, 3025–3031 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
JEB3046

THE ADRENERGIC VOLUME CHANGES OF IMMATURE AND MATURE RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) ERYTHROCYTES

TIINA LECKLIN[1,2,*], ANTTI TUOMINEN[1] AND MIKKO NIKINMAA[1]

[1]*Laboratory of Animal Physiology, Department of Biology, University of Turku, FIN-20014 Turku, Finland* and [2]*Finnish Game and Fisheries Research Institute, Saimaa Fisheries and Aquaculture, Laasalantie 9, FIN-58175 Enonkoski, Finland*

*Author for correspondence (e-mail: tiilec@utu.fi)

Accepted 10 July; published on WWW 7 September 2000

Summary

In this study, we examined whether the adrenergic volume response of teleost erythrocytes is related to cell maturity. Rainbow trout (*Oncorhynchus mykiss*) were made anaemic by reducing their haematocrit to approximately 50 % of the original value. After 3–4 weeks, small, young erythrocytes were seen in the circulation. By measuring the volume distribution of blood samples from anaemic fish before and after noradrenaline stimulation (10 min, 10^{-5} mol l^{-1} final concentration), we were able to show that the volume response of young, immature erythrocytes to catecholamine stimulation was greater than that of mature erythrocytes. In addition, the membrane fluidity, measured using the steady-state fluorescence polarisation method, was greater in anaemic fish after 24 days of recovery from bleeding than in control fish. Since blood from anaemic fish contained a large fraction of immature erythrocytes, this result indicates that the fluidity of the membrane of immature erythrocytes is greater than that of mature erythrocytes.

Key words: Na^+/H^+ exchanger, catecholamine, cell volume, membrane fluidity, erythropoiesis, cell age, rainbow trout, *Oncorhynchus mykiss*.

Introduction

A seasonal variation in adrenergic response of salmonid erythrocytes has been illustrated in some studies. Nikinmaa and Jensen (1986) observed a complete lack of β-adrenergic responses in erythrocytes of rainbow trout acclimated to winter conditions. Cossins and Kilbey (1989) observed that the adrenergic response of rainbow trout erythrocytes was reduced in winter fish. Similarly, our recent studies have shown that adrenergic activation of the Na^+/H^+ exchanger in Arctic charr (*Salvelinus alpinus*) erythrocytes is greatest in spring (Lecklin and Nikinmaa, 1999). Cossins and Kilbey (1989) suggested that the annual variations in adrenergic responsiveness could be caused by the liberation of young erythrocytes into the circulation if the activity of the Na^+/H^+ exchanger was elevated in young erythrocytes. In many salmonid species, e.g. Baltic salmon *Salmo salar* (Härdig and Höglund, 1984), brown trout *Salmo trutta* (Álvarez et al., 1994) and rainbow trout *Oncorhynchus mykiss* (Houston et al., 1996), the proportion of immature erythrocytes in circulation increases in spring. However, the adrenergic responsiveness of erythrocytes at different maturational state has not been investigated in detail.

In the present study, we have manipulated the age of erythrocytes in circulation by making the fish anaemic and following the production of new erythrocytes. When immature erythrocytes emerged into the circulation, noradrenaline-induced erythrocytic swelling was studied in the newly synthetized and old erythrocytes. In addition, we have investigated whether the newly synthetized and old erythrocytes have different membrane fluidities.

Materials and methods

Experiments were carried out at Saimaa Fisheries Research and Aquaculture at Enonkoski in April 1998. Rainbow trout *Oncorhynchus mykiss* (Walbaum) of both sexes, weighing 350–550 g, were transferred to 1500 l acclimation tanks supplied with a constant flow of lake water adjusted to 1 or 8 °C. During the experimental period, water temperature and oxygen saturation were recorded daily; values were 1.5±0.2 °C and 84.0±0.9 % for the 1 °C tank and 8.3±0.1 °C and 80.3±1.1 % for the 8 °C tank, respectively. The animals were subjected to a natural photoperiod and fed regularly to satiation with commercial pellets (TESS VITAL 5, Raisio, Finland).

Fish were cannulated *via* the dorsal aorta (Soivio et al., 1975). After cannulation, the fish were allowed to recover for at least 5 days prior to the experiments. Trout were made anaemic by bleeding them *via* the cannula. The volume of blood removed was estimated to cause a 50–60 % reduction in haematocrit on the basis of the total blood volume of rainbow

trout (Nikinmaa et al., 1981). The recovery of individual fish was followed by taking 100 µl blood samples 0, 2, 4, 8, 12, 16, 20 and 24 days after bleeding. Control fish were sampled at similar intervals but were not made anaemic. Immediately after sampling, red blood cell number and volume distribution were determined using a Coulter counter (sampling stand IIA and multisizer II, Coulter Electronics Ltd, Luton, UK) on blood samples diluted 1:5000 in fish saline. The composition of the saline used (in mmol l^{-1}) was 128 NaCl, 3 KCl, 1.5 $MgCl_2$, 1.5 $CaCl_2$, 5 glucose, 3 pyruvate and 10 Hepes, pH 7.6 at 20 °C (unless stated otherwise). The haematocrit (Hct) was determined by centrifugation (3 min, 12 500 ***g***). Blood smears were prepared and stained according to the May–Grünwald–Giemsa technique and examined using a Leitz Laborlux microscope (1250× magnification).

When small, immature red blood cells emerged into the circulation, the effect of adrenergic stimulation was determined using volume change as a measure of adrenergic response. Blood sample was diluted 1:5000 with fish saline (composition as above, pH 7.1 at 20 °C). The adrenergic responsiveness was measured at pH 7.1 and at 20 °C, because earlier experiments on rainbow trout had indicated that the volume response to catecholamines is maximal in these conditions (Salama and Nikinmaa, 1989). Erythrocytes were allowed to equilibrate for 30 min before the volume distribution was measured using a Coulter counter. Noradrenaline (10^{-5} mol l^{-1} final concentration) was added, and the volume distribution was measured again after a 10 min incubation. Coulter counter profiles after the fish had been made anaemic were bimodal for all the blood samples studied. However, in some cases, the first peak containing cells with a smaller volume could only be seen as a shoulder on the left-hand side of the second peak and, therefore, could not always be clearly separated from the second peak after noradrenaline stimulation. Therefore, the noradrenaline-induced increase in cell volume was calculated for cells at the 10 % (immature red blood cells) and 90 % (mature red blood cells) points of the volume distribution (Fig. 1).

The erythrocyte membrane fluidities from untreated and bled rainbow trout were measured after a 24-day recovery period. Plasma and erythrocytes were separated by centrifugation, and the erythrocytes were washed twice with the saline. The washed cells were lysed by freezing and thawing. The nuclei and intact cells were removed by low-speed centrifugation (500 ***g***, 10 min). The plasma membranes were then separated from the supernatant by centrifugation (20 000 ***g***, 10 min). The pellet containing cell membranes was washed three times to remove haemoglobin. The membranes were resuspended in a small volume of saline and stored in liquid nitrogen until fluidity measurements. The optical density of the membrane suspension was adjusted to 0.08 absorbance units at 500 nm to standardize the light scattering of the membranes. The fluidity of erythrocyte plasma membranes was measured using a steady-state fluorescence polarization method, as described by Lehti-Koivunen and Kivivuori (1998). The measurements were

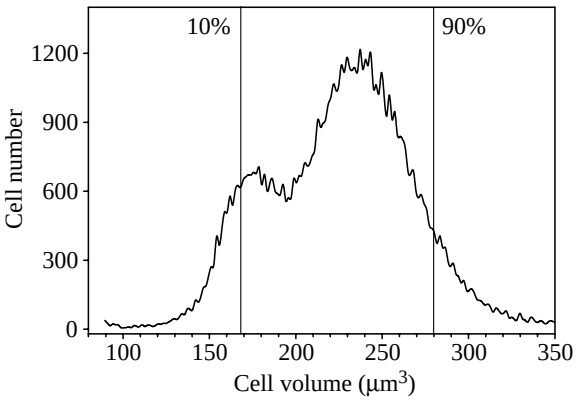

Fig. 1. A Coulter counter profile showing a typical frequency distribution of red blood cell volumes in bled fish 24 days after decreasing their haematocrit. Vertical lines indicate the 10 % and 90 % points of the volume distribution from which the noradrenaline-induced increase in cell volume for erythrocytes was calculated.

carried out using a thermostatted Perkin Elmer LS50 luminescence fluorometer. The sample was excited with vertically polarized light at 355 nm, and emitted light was read at 440 nm. For membrane labelling, 2 µl of 2 mmol l^{-1} 1,6-diphenyl-1,3,5-hexatriene (DPH) in tetrahydrofuran was added to the 3 ml membrane suspensions in phosphate buffer at pH 7.1 and 20 °C in a quartz cell. The suspensions were labelled for 30 min before measurements. The effect of temperature was determined from 1 to 30 °C at intervals of approximately 5 °C.

Statistical analyses

Student's *t*-test (SPSS) was used to analyze whether the magnitude of the adrenergic response differed between immature and mature red blood cells. Analysis of variance (ANOVA) (with repeated measures) was used to determine the differences in the erythrocyte numbers between anaemic and control fish at different time points, and analysis of covariance (ANCOVA; SPSS) was used to determine whether the temperature-dependency of the polarization of the erythrocyte membrane differed between treatments (control or anaemic). In all cases, $P<0.01$ was taken as the significance level. All values are given as means ± S.E.M.

Results

In rainbow trout acclimated to 8 °C, a reduction in haematocrit from 29.8 to 12.4 % induced the production of new erythrocytes. As seen in Fig. 2, there was a slight decrease in the erythrocyte number of control fish over time, resulting from the serial blood sampling. In contrast, the erythrocyte number of fish made anaemic remained constant after the initial marked reduction and showed a tendency to increase between 16 and 24 days, such that the statistically significant difference between control and anaemic fish had disappeared by 24 days of recovery. Simultaneously with the tendency of red blood cell number to increase between 16 and 24 days of recovery,

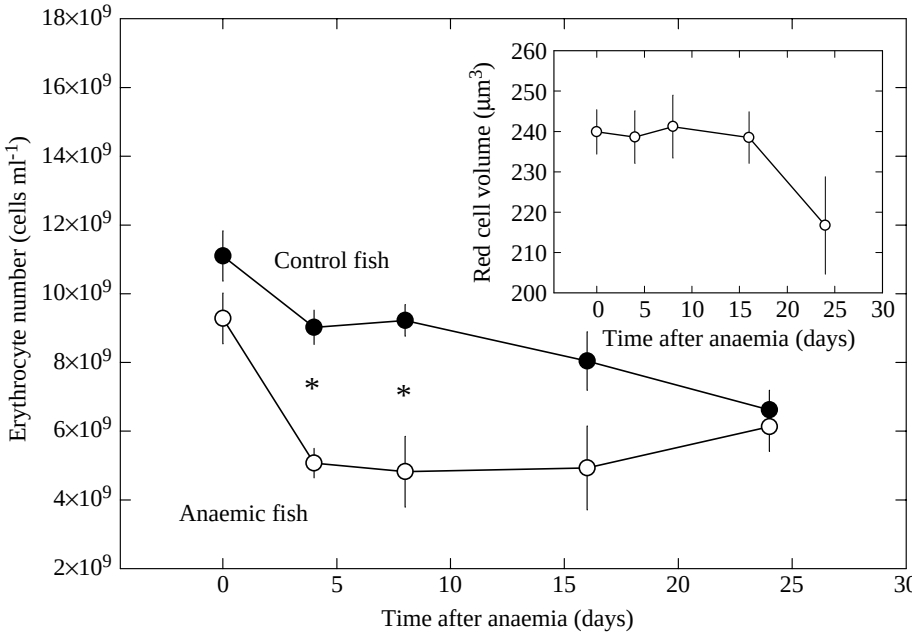

Fig. 2. The erythrocyte number in control fish (filled circles; *N*=5) and in anaemic fish (open circles; *N*=4) before (time zero) and after the haematocrit in the treated group had been reduced by approximately 50 %. Asterisks indicate a statistically significant difference (P<0.05) between the erythrocyte number in control and anaemic fish. The inset shows the erythrocyte volume of anaemic fish as a function of time after bleeding (*N*=4). The mean red cell volume of anaemic fish was significantly (P<0.05) smaller at day 24 than before they were made anaemic. Values are means ± S.E.M.

the mean erythrocyte volume decreased significantly below the initial value, from approximately 240 μm^3 to approximately 220 μm^3. This decrease was due to a population of small cells emerging into the circulation, as shown by a new peak in the Coulter profile (Fig. 3). The immature red blood cells could also be recognized in blood smears on the basis of their rounded appearance and basophilic cytoplasm (Fig. 4B). These findings indicate that, at 8 °C, a major surge in the number of erythrocytes in circulation occurs between 16 and 24 days after the initial reduction in oxygen-carrying capacity. The effect is clearly temperature-dependent, since making the fish anaemic did not induce erythropoiesis within 30 days in trout acclimated to 1 °C (results not shown).

There was a clear difference in the magnitude of the adrenergic response between the newly synthetized and the old erythrocytes, as shown by the Coulter counter profile (Fig. 5A). After the addition of 10^{-5} mol l^{-1} noradrenaline (final concentration) to the cell suspension, the peak representing smaller cells was shifted more to the right than the peak representing larger cells. Fig. 5B shows the change in the erythrocyte volume as a proportion of the initial volume. The noradrenaline-induced increase in cell volume was significantly (P<0.001) greater among the small, immature (10.53±1.04 %) red blood cells than among the mature red blood cells (2.81±0.85 %).

In the present study, the temperature-dependency of erythrocyte membrane fluidity was measured using a steady-state fluorescence polarization technique. There was no statistically significant difference in the DPH fluorescence polarization between erythrocyte membranes from control trout and anaemic trout acclimated to 1 °C (results not shown), corresponding to the observation that anaemia did not induce erythropoiesis at this temperature. In contrast, there was a significant difference (P<0.001) between red cell

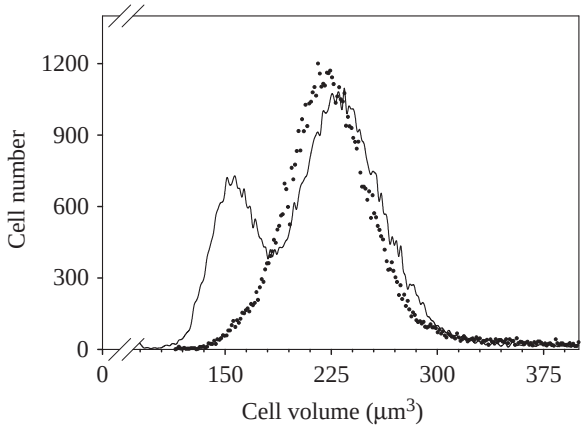

Fig. 3. Representative Coulter counter profiles showing the frequency distributions of red blood cell volumes from rainbow trout acclimated to 8 °C sampled before decreasing the haematocrit to 50 % by bleeding (dotted line) and 24 days after the haematocrit had been reduced (solid line).

membrane fluidities in control and anaemic fish 24 days after bleeding (Fig. 6). The DPH fluorescence polarization of erythrocyte membranes from anaemic trout was lower and, thus, the membrane was more fluid than that from control trout, suggesting that the immature erythrocytes have more fluid membranes than old erythrocytes. In addition, the temperature-dependency of membrane fluidity was greater in the anaemic fish than in the control group: regression equations for the temperature-dependency of red blood cell membrane fluidities were $p=0.315-3.453\times10^{-3}t$ (r=0.989, P<0.0001) for control fish and $p=0.297-4.057\times10^{-3}t$ (r=0.997, P<0.0001) for anaemic fish, where p is membrane fluidity and t is temperature.

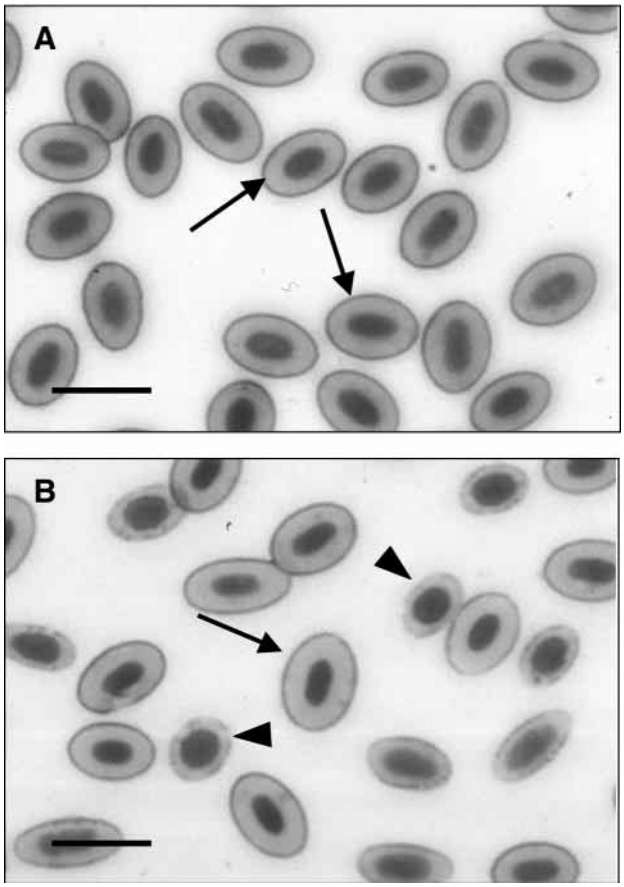

Fig. 4. Blood smears from a rainbow trout acclimated to 8 °C (A) before the haematocrit had been reduced to 50 % by bleeding, in which all the mature erythrocytes (arrows) were more-or-less similar in size, and (B) 24 days after bleeding, in which the presence of immature erythrocytes (arrowheads) is evident. Scale bars, 10 μm.

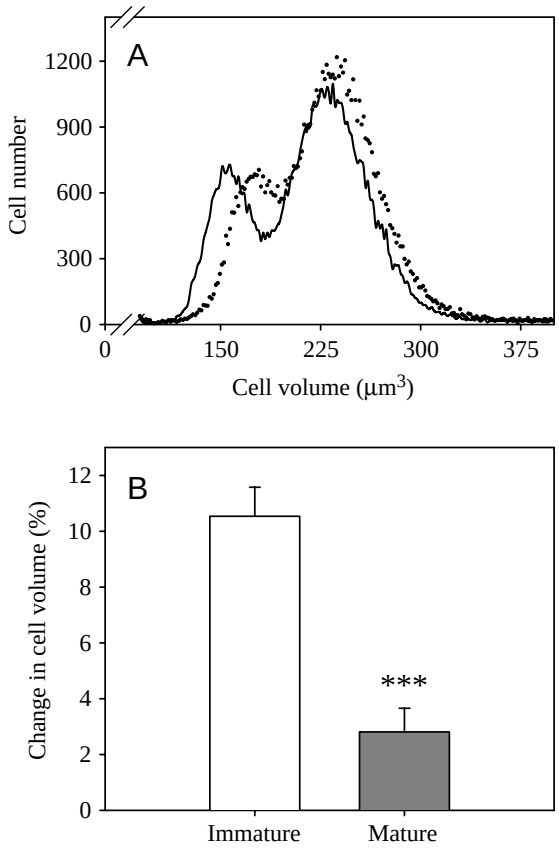

Fig. 5. (A) Representative Coulter counter profiles showing the volume distributions of erythrocytes from rainbow trout acclimated to 8 °C. The measurements were made after a 24-day recovery period from bleeding to reduce the haematocrit by 50 % before (solid line) and after (dotted line) a 10 min stimulation with noradrenaline (final concentration 10^{-5} mol l^{-1}). (B) The change in cell volume during a 10 min incubation of rainbow trout erythrocytes with 10^{-5} mol l^{-1} noradrenaline for immature and mature erythrocytes. Measurements were made at 20 °C, and values are means + S.E.M from five experiments. The increase in size was significantly greater for immature than for mature erythrocytes (***P<0.001; Student's t-test).

Discussion

As observed previously, experimental anaemia induces erythropoiesis in rainbow trout (Lane, 1979), carp *Cyprinus carpio* (Schindler and de Vries, 1986), goldfish *Carassius auratus* (Houston and Murad, 1995) and gilthead sea bream *Sparus aurata* (Montero et al., 1995). In the present study, 24 days after bleeding, two fractions were present in Coulter counter profiles; a first peak at approximately 150 μm^3, representing immature erythrocytes, and a second peak at 240 μm^3, representing mature erythrocytes. The present data clearly show that young and old erythrocytes can be separated on the basis of Coulter counter volume distribution profiles. Furthermore, the data show that the response is relatively sluggish at 8 °C, since significant numbers of immature erythrocytes appeared in the circulation between 16 and 24 days after the fish had been made anaemic. At a very low temperature, 1 °C, new erythrocytes were not formed even 30 days after the induction of anaemia.

The adrenergic volume changes, which give a picture of the magnitude of the adrenergic response, can be reliably estimated using a Coulter counter (Nikinmaa, 1982). Although we did not inhibit the swelling response with amiloride in the present study, earlier data on rainbow trout erythrocytes using a similar procedure (Nikinmaa, 1982) and on other cell types (Grinstein et al., 1984) indicate that cell swelling initiated by the activation of Na^+/H^+ exchange and measured using a Coulter counter can be fully inhibited by amiloride.

It is clear from the present results that the volume changes 10 min after adrenergic stimulation were greater in the immature, small erythrocytes than in the mature erythrocytes. The response was determined after this relatively short period of stimulation to ensure that the volume changes were caused mainly by the activation of Na^+/H^+ exchange. An upper steady-state volume of adrenergically stimulated fish erythrocytes is reached within 30–60 min of stimulation (see Borgese et al., 1987). As discussed in detail by, for example, Nikinmaa and

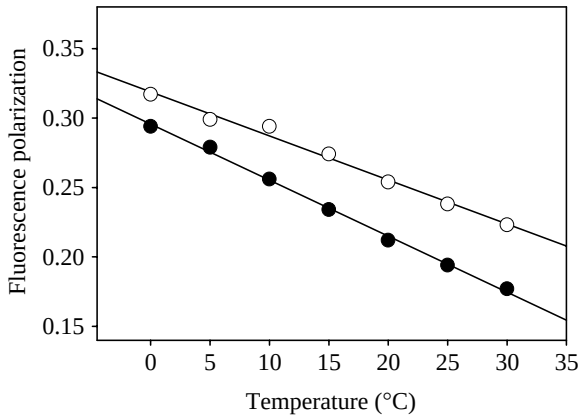

Fig. 6. Dependence of steady-state fluorescence polarization values for the fluorescence probe DPH on temperature in erythrocyte membranes from rainbow trout acclimated to 8 °C. Open circles represent values from control fish and filled circles represent values from fish made anaemic by bleeding. Each symbol represents the mean of 4–5 experiments. Equations for the regression lines are given in the text.

Boutilier (1995), the full adrenergic response depends (i) on the rate of activation of the Na^+/H^+ exchanger, (ii) on the coupling of Na^+ and Cl^- movements as a result of intracellular and extracellular hydration/dehydration reactions between bicarbonate and protons, (iii) on the self-inhibition of the Na^+/H^+ exchanger (Garcia-Romeu et al., 1988), (iv) on the activation of the K^+/Cl^- cotransport pathway as a result of cell swelling (Motais et al., 1991; Guizouarn and Motais, 1999), and (v) on the state of activation of the Na^+ pump (Ferguson et al., 1989). Although we cannot completely exclude the possibility that other factors besides the greater Na^+/H^+ exchange rate in immature than in mature erythrocytes may have influenced the present results, the conclusion about greater adrenergic responsiveness in immature than in mature erythrocytes is not invalidated by this uncertainty. The rate of extracellular hydration/dehydration reactions between bicarbonate and carbon dioxide, which greatly influences the adrenergic response (Motais et al., 1989; Nikinmaa and Boutilier, 1995), was the same for both mature and immature erythrocytes, since they were suspended in the same saline. Intracellular carbonic anhydrase activity and anion exchange activity may be influenced by red cell age but, since both the rate of anion exchange and the rate of intracellular hydration/dehydration reactions are much faster than the rate of Na^+/H^+ exchange and extracellular hydration/dehydration reactions of carbon dioxide/bicarbonate, their influence on the adrenergic response is expected to be small (see Nikinmaa and Boutilier, 1995). Because we determined the volume changes after the initial 10 min of stimulation, the effect of self-inhibition of the Na^+/H^+ exchanger (Garcia-Romeu et al., 1988), even if it differed between young and old erythrocytes, on cell volume was small. Similarly, K^+/Cl^- cotransport, which is activated by cell swelling, the activation being directly proportional to cell volume increase (e.g. Guizouarn and Motais, 1999), will have only a small effect on the cell volume during the first 10 min of stimulation. Furthermore, since the volume changes were larger in immature than in mature erythrocytes, the activation of K^+/Cl^- cotransport, which reduces cell volume, would have reduced any differences in the adrenergic volume response between immature and mature erythrocytes. Similar reasoning applies to the activation of the Na^+ pump. The activity of the Na^+ pump appears to be greater in young than in old erythrocytes, as estimated on the basis of oxygen consumption due to Na^+ pump activity (Phillips et al., 2000). Since the activation of the Na^+ pump will tend to reduce the cell volume, the adrenergic volume response in young cells would probably be affected more than that in mature cells and, again, the difference in the response between young and old erythrocytes would be reduced.

Thus, we have verified experimentally that immature and mature erythrocytes responded differently to catecholamines. Cossins and Kilbey (1989) demonstrated that the adrenergic response of trout erythrocytes changes seasonally and suggested that this variation was due to seasonal variations in mean erythrocyte age. Our present results strengthen this conclusion: young erythrocytes clearly have a larger adrenergic volume response than older ones. Furthermore, it appears that the difference is mainly due to a greater effect of adrenergic stimulation on the Na^+/H^+ exchange rate in immature than in mature erythrocytes.

The present study gives one potential reason for the increased adrenergic response in immature erythrocytes: the fluidity of the membrane was greater in immature erythrocytes than in mature erythrocytes. This result contrasts with the suggestion of Gabbianelli et al. (1996) who, on the basis of studies with density-separated erythrocytes, concluded that the membrane of old erythrocytes would be more fluid than that of young ones. However, in their study, the production of young erythrocytes was not stimulated and, thus, the exact age profile was unknown. The fluidity of the cell membrane may affect the adrenergic response by influencing the lateral movements of membrane proteins. The hormone-receptor complex of the adrenergic system diffuses laterally in the plane of the membrane and collides with the guanyl nucleotide regulatory protein-adenylate cyclase complex (Tolkovsky and Levitzki, 1978), activating the enzyme. Increased lateral mobility as a result of increased membrane fluidity could therefore activate the enzyme more effectively and, thus, increase the production of cyclic AMP. At present, it is not known whether treatments that increase membrane fluidity also affect the formation of cyclic AMP. Furthermore, the effect of red cell age on the accumulation of cyclic AMP in fish is not known.

In addition to membrane fluidity, many other factors may influence the adrenergic volume response. The number of adrenergic receptors and, consequently, the responsiveness to catecholamines may decrease with maturation of trout erythrocytes. Although it is not known whether the number of adrenergic receptors differs between young and old erythrocytes, it has been reported that the receptor number can vary in response to several external and internal stimuli. For

example, in trout cardiac tissue, the density of β-adrenoceptors increases after cold acclimation (Keen et al., 1993). Reid et al. (1993) demonstrated that the exposure of trout erythrocytes to hypoxia resulted in a 1.5-fold increase in the number of surface β-adrenoceptors. Cortisol affects the number of low-affinity receptors in the intracellular pool in trout erythrocytes (Reid and Perry, 1991) and the number of β-adrenoceptors in trout hepatocyte membrane (Reid et al., 1992). In contrast, the continuous presence of catecholamines decreases the number of functional β-receptors (Gilmour et al., 1994; Perry et al., 1996).

The number of Na^+/H^+ exchangers and the turnover of individual Na^+/H^+ exchangers on the cell surface may change during the life span of erythrocytes, being greater in immature than in mature erythrocytes: the occurrence of two pools of exchangers showing different kinetic and regulatory characteristics has been demonstrated (Guizouarn et al., 1995). Reid and Perry (1994) showed that the interspecific differences in adrenergic responsiveness may partly be related to differences in the numbers of antiporters in erythrocyte membrane. They also reported that, under hypoxic conditions, the number of Na^+/H^+ antiporters in trout red blood cell membranes was increased. Raynard and Cossins (1991) and Lee et al. (1991) discussed the possibility that transport systems could be expressed differentially during cell maturation. Direct effects of erythrocyte maturation on transport systems have never been demonstrated for fish erythrocytes, but data on dog erythrocytes show this to be the case for the Na^+ pump. In the red cells of puppies, some ouabain-sensitive K^+ transport is observed (Lee and Miles, 1972), but in the erythrocytes of adult dogs no Na^+/K^+ATPase activity has been demonstrated (Parker, 1977). With regard to fish, the oxygen consumption that can be attributed to the Na^+ pump decreases with increasing erythrocyte age (Phillips et al., 2000).

In conclusion, the present study clearly demonstrates a connection between erythrocyte age and the adrenergic volume response. These data strengthen the suggestion that seasonal variations in the catecholamine response may be caused by erythrocytic patterns. Furthermore, the data demonstrate that the membrane fluidity of immature erythrocytes is greater than that of mature erythrocytes, and this may influence adrenergic responsiveness. However, the results of the present study cannot exclude the possibilities that the number of adrenergic receptors, the number of Na^+/H^+ exchangers or their rate of turnover decrease during erythrocyte maturation, thus influencing the catecholamine response.

This study was supported by grant 40830 from the Academy of Finland. T.L. is a recipient of a Ministry of Education graduate scholarship in the National Graduate Programme on Fish Biology and Fisheries.

References

Álvarez, F., Flaño, E., Villena, A. J., Zapata, A. and Razquin, B. E. (1994). Seasonal intrathymic erythropoietic activity in trout. *Dev. Comp. Immunol.* **18**, 409–420.

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

Cossins, A. R. and Kilbey, R. V. (1989). The seasonal modulation of Na^+/H^+ exchanger activity in trout erythrocytes. *J. Exp. Biol.* **144**, 463–478.

Ferguson, R. A., Tufts, B. L. and Boutilier, R. G. (1989). Energy metabolism in trout red cells: consequences of adrenergic stimulation *in vivo* and *in vitro*. *J. Exp. Biol.* **143**, 133–147.

Gabbianelli, R., Santroni, A. M., Falcioni, G., Bertoli, E., Curatola, G. and Zolese, G. (1996). Physicochemical characterization of plasma membranes from density-separated trout erythrocytes. *Arch. Biochem. Biophys.* **336**, 157–162.

Garcia-Romeu, F., Motais, R. and Borgese, F. (1988). Desensitization by external Na of the cyclic AMP-dependent Na^+/H^+ antiporter in trout red blood cells. *J. Gen. Physiol.* **91**, 529–548.

Gilmour, K. M., Didyk, N. E., Reid, S. G. and Perry, S. F. (1994). Down-regulation of red blood cell β-adrenoceptors in response to chronic elevation of plasma catecholamine levels in the rainbow trout. *J. Exp. Biol.* **186**, 309–314.

Grinstein, S., Goetz, J. D., Furuya, W., Rothstein, A. and Gelfand, E. W. (1984). Amiloride sensitive Na^+–H^+ exchange in platelets and leukocytes: detection by electronic cell sizing. *Am. J. Physiol.* **247**, C293–C298.

Guizouarn, H., Borgese, F., Pellissier, B., Garcia-Romeu, F. and Motais, R. (1995). Regulation of Na^+/H^+ exchange activity by recruitment of new Na^+/H^+ antiporters: Effect of calyculin A. *Am. J. Physiol.* **268**, C434–C441

Guizouarn, H. and Motais, R. (1999). Swelling activation of transport pathways in erythrocytes: effects of Cl^-, ionic strength and volume changes. *Am. J. Physiol.* **276**, C210–C220.

Härdig, J. and Höglund, L.B. (1984). Seasonal variation in blood components of reared Baltic salmon, *Salmo salar* L. *J. Fish Biol.* **24**, 565–579.

Houston, A. H., Dobric, N. and Kahurananga, R. (1996). The nature of hematological response in fish. Studies on rainbow trout *Oncorhynchus mykiss* exposed to simulated winter, spring and summer conditions. *Fish Physiol. Biochem.* **15**, 339–347.

Houston, A. H. and Murad, A. (1995). Erythrodynamics in fish: Recovery of the goldfish *Carassius auratus* from acute anemia. *Can. J. Zool.* **73**, 411–418.

Keen, J. E., Viazon, D.-M., Farrell, A. P. and Tibbits, G. F. (1993). Thermal acclimation alters both adrenergic sensitivity and adrenoceptor density in cardiac tissue of rainbow trout. *J. Exp. Biol.* **181**, 27–47.

Lane, H. C. (1979). Some haematological responses of normal and splenectomized rainbow trout *Salmo gairdneri* to a 12 % blood loss. *J. Fish Biol.* **14**, 159–164.

Lecklin, T. and Nikinmaa, M. (1999). Seasonal and temperature effects on the adrenergic responses of Arctic charr (*Salvelinus alpinus*) erythrocytes. *J. Exp. Biol.* **202**, 2233–2238.

Lee, J. A. C., James, P. S., Smith, M. W. and Cossins, A. R. (1991). Amino acid transport in the intestinal mucosa of temperature-acclimated carp. *J. Therm. Biol.* **16**, 7–11.

Lee, P. and Miles, P. R. (1972). Density distribution and cation composition of red blood cells in newborn puppies. *J. Cell. Physiol.* **79**, 377–388.

Lehti-Koivunen, S. M. and Kivivuori, L. A. (1998). Fluidity of neuronal membranes of crayfish (*Astacus astacus* L.) acclimated to 5 °C and 20 °C. *Comp. Biochem. Physiol.* **119**A, 773–779.

Montero, D., Tort, L. L., Izquierdo, M. S., Socorro, J., Vergara, J. M., Robaina, L. and Fernández-Palacios, H. (1995). Hematological recovery in *Sparus aurata* after bleeding. A time course study. *Rev. Española Fisiol.* **51**, 219–226.

Motais, R., Fievet, B., Garcia-Romeu, F. and 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.

Motais, R., Guizouarn, H. and Garcia-Romeu, F. (1991). Red cell volume regulation: the pivotal role of ionic strength in controlling swelling-dependent transport systems. *Biochim. Biophys. Acta* **1075**, 169–180.

Nikinmaa, M. (1982). Effects of adrenaline on red cell volume and concentration gradient of protons across the red cell membrane in the rainbow trout, *Salmo gairdneri*. *Mol. Physiol.* **2**, 287–297.

Nikinmaa, M. and Boutilier, R. G. (1995). Adrenergic control of red cell pH, organic phosphate concentrations and haemoglobin function in teleost fish. In *Mechanisms of Systemic Regulation: Respiration and Circulation* (ed. N. Heisler), pp. 107–133. Berlin: Springer Verlag.

Nikinmaa, M. and Jensen, F. B. (1986). Blood oxygen transport and acid–base status of stressed trout (*Salmo gairdnerii*): Pre- and postbranchial values in winter fish. *Comp. Biochem. Physiol.* **84**A, 391–396.

Nikinmaa, M., Soivio, A. and Railo, E. (1981). Blood volume of *Salmo gairdneri*: Influence of ambient temperature. *Comp. Biochem. Physiol.* **69**A, 767–769.

Parker, J. C. (1977). Solute and water transport in dog and cat red blood cells. *Membrane Transport in Red Cells* (ed. J. C. Ellory and V. L. Lew), pp. 427–465. London: Academic Press.

Perry, S. F., Reid, S. G. and Salama, A. (1996). The effects of repeated physical stress on the β-adrenergic response of the rainbow trout red blood cell. *J. Exp. Biol.* **199**, 549–562.

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.

Raynard, R. S. and Cossins, A. R. (1991). Homeoviscous adaptation and thermal compensation of sodium pump of trout erythrocytes. *Am. J. Physiol.* **260**, R916–R924.

Reid, S. D., Lebras, Y. and Perry, S. F. (1993). The *in vitro* effect of hypoxia on the trout erythrocyte β-adrenergic signal transduction system. *J. Exp. Biol.* **176**, 103–116.

Reid, S. D., Moon, T. W. and Perry, S. F. (1992). Rainbow trout hepatocytes β-adrenoceptors, catecholamine responsiveness and effects of cortisol. *Am. J. Physiol.* **262**, R794–R799.

Reid, S. D. and Perry, S. F. (1991). The effects and physiological consequences of raised levels of cortisol on rainbow trout (*Onchorhynchus mykiss*) erythrocyte β-adrenoceptors. *J. Exp. Biol.* **158**, 217–240.

Reid, S. D. and Perry, S. F. (1994). Quantification of presumptive Na^+/H^+ antiporters of the erythrocytes of trout and eel. *Fish Physiol. Biochem.* **12**, 455–463.

Salama, A. and Nikinmaa, M. (1989). Species differences in the adrenergic responses of fish red cells: studies on whitefish, pikeperch, trout and carp. *Fish Physiol. Biochem.* **6**, 167–173.

Schindler, J. F. and de Vries, U. (1986). Scanning cytophotometry of carp, *Cyprinus carpio* L., erythrocyte populations: the influence of short-term hypoxic environment and the recovery period following severe bleeding. *J. Fish Biol.* **28**, 741–752.

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

Tolkovsky, A. M. and Levitzki, A. (1978). Mode of coupling between the β-adrenergic receptor and adenylate cyclase in turkey erythrocytes. *Biochemistry* **17**, 3795–3810.