

## THE RESTING MEMBRANE POTENTIAL OF WHITE MUSCLE FROM BROWN TROUT (*SALMO TRUTTA*) EXPOSED TO COPPER IN SOFT, ACIDIC WATER

M. W. BEAUMONT\*, E. W. TAYLOR AND P. J. BUTLER

*School of Biosciences, The University of Birmingham, Edgbaston, Birmingham B15 2TT, UK*

\*e-mail: m.w.beaumont@bham.ac.uk

*Accepted 25 April; published on WWW 22 June 2000*

### Summary

Previously, the distribution of ammonia between the intracellular and extracellular compartments has been used to predict a significant depolarisation of the resting membrane potential ( $E_M$ ) of white muscle from brown trout (*Salmo trutta*) exposed to a sub-lethal combination of copper and low pH. However, this prediction is based upon two assumptions (i) a relatively high membrane permeability for the ammonium ion with respect to that for ammonia gas and (ii) that this is unaltered by exposure to copper and low pH. Since there is conflicting evidence in the literature of the validity of these assumptions, in the present study  $E_M$  was directly measured in white muscle fibres of trout exposed to copper and low pH

( $E_M = -52.2 \pm 4.9$  mV) and compared with that of unexposed, control animals ( $E_M = -86.5 \pm 2.9$  mV) (means  $\pm$  S.E.M.,  $N=6$ ). In confirming the predicted depolarisation, these data support the hypothesis of electrophysiological impairment as a factor in the reduction in the swimming performance of trout exposed to these pollutants. In addition, the results of this study support the role of a significant permeability of the muscle membrane to  $\text{NH}_4^+$  in determining the distribution of ammonia in fish.

Key words: copper, low pH, ammonia, swimming, muscle membrane potential, brown trout, *Salmo trutta*.

### Introduction

Brown trout (*Salmo trutta*) exposed to sub-lethal concentrations of copper and low pH, either alone or in combination, have a reduced ability to swim (Butler et al., 1992; Beaumont et al., 1995a). These pollutants have the potential to affect oxygen uptake by damaging the gill (Wilson and Taylor, 1993a) and perhaps to affect oxygen transfer to the exercising tissues through secondary effects such as haemoconcentration (Randall and Brauner, 1991; Butler et al., 1992). However, at the sub-lethal concentrations used in our studies, there is no evidence for such effects (Beaumont et al., 1995a, 2000). Exposure to copper and/or low pH causes an elevation of plasma ammonia concentration (Wilson and Taylor, 1993a,b; Beaumont et al., 1995b; Day and Butler, 1996), and there are corresponding increases in the ammonia concentration of both red and white muscles (Day and Butler, 1996; Beaumont et al., 2000). In trout exposed to both copper and low pH, there was a significant negative correlation between critical swimming speed ( $U_{\text{crit}}$ : Brett, 1964) and total plasma ammonia concentration ( $[T_{\text{amm}}] = [\text{NH}_3] + [\text{NH}_4^+]$ ), with an  $r^2$  value of almost 0.70 (Beaumont et al., 1995b), and it was hypothesised that ammonia might affect swimming performance through its role as a regulator of certain metabolic pathways or its effect upon neuromuscular function (Beaumont et al., 1995b). The first of these hypotheses was investigated in a recent study (Beaumont et al., 2000) that examined the metabolic status of white and red muscle from

trout exposed to copper and low pH. As a preliminary approach to the second hypothesis, in the same study the measured distribution of ammonia between the tissue and extracellular compartment was used to estimate the resting membrane potential of this tissue. The result indicated that, in comparison with trout from control (pH 7, no copper) conditions, the white muscle fibres of trout exposed to copper and low pH (pH 5,  $0.08 \mu\text{mol l}^{-1} \text{Cu}^{2+}$ ) were depolarised by some 20 mV. Depolarised fibres may become electrically inexcitable (Jenerick, 1959). In frog sartorius muscle, ammonium ions caused membrane depolarisation which led to a reduction in the twitch tension generated (Heald, 1975). Such an effect could account for the absence of white muscle recruitment in trout exposed to sub-lethal low pH (Day and Butler, 1996).

To estimate membrane potential ( $E_{\text{NH}_4}$ ) in this manner, it was assumed that the compartmentalisation of ammonia across the muscle membrane can be predicted by a Nernst distribution (equation 1). This, in turn, assumes that the relative permeability of the muscle membrane for  $\text{NH}_4^+$  is high (for a discussion, see Wood, 1993):

$$E_{\text{NH}_4} = - \frac{RT}{zF} \ln \frac{[\text{NH}_4^+]_i}{[\text{NH}_4^+]_e}, \quad (1)$$

where  $R$  is the gas constant,  $T$  is absolute temperature,  $F$  is the Faraday constant,  $z$  is the valency and the subscripts  $i$

and  $e$  denote intracellular and extracellular concentrations respectively.

This assumption was supported by a measured intracellular/extracellular  $[T_{\text{amm}}]$  ratio ( $[T_{\text{amm}}]_i/[T_{\text{amm}}]_e$ ) of trout in control conditions of  $33.6 \pm 7.8$  (mean  $\pm$  S.E.M.,  $N=6$ ), close to that predicted by a Nernstian distribution, when an  $E_M$  of  $-85$  mV, typical of resting fish muscle (Hidaka and Toida, 1969; Stanfield, 1972; Eugène and Baretts, 1983; Altringham and Johnston, 1988), is applied. In contrast, distribution according to the theory of non-ionic diffusion (Jacobs, 1940; Milne et al., 1958), i.e. one determined by the pH gradient across the membrane (equation 2), which is the case for most mammalian muscle (e.g. Stabenau et al., 1959; Meyer et al., 1980; MacLean et al., 1995; Bangsbo et al., 1996), predicted a  $[T_{\text{amm}}]_i/[T_{\text{amm}}]_e$  ratio of  $5.3 \pm 0.5$  (mean  $\pm$  S.E.M.,  $N=6$ ).

$$\frac{[T_{\text{amm}}]_i}{[T_{\text{amm}}]_e} = \frac{1 + 10^{(\text{pK}-\text{pHi})}}{1 + 10^{(\text{pK}-\text{pHe})}}, \quad (2)$$

where pHe is extracellular pH and pHi is intracellular pH.

A considerable number of other authors (e.g. Wright et al., 1988a,b; Wright and Wood, 1988; Saha and Ratha, 1989; Tang et al., 1992) have observed a high  $[T_{\text{amm}}]_i/[T_{\text{amm}}]_e$  ratio in fish white muscle, suggesting that muscle membrane potential plays a dominant role in the determination of the equilibrium distribution of ammonia in fish at rest. However, in spite of these observations, the hypothesis that ammonia is distributed mainly according to membrane potential has been challenged both on theoretical grounds (Heisler, 1990) and by several studies in which the observed ammonia ratio was not high (e.g. Mommsen and Hochachka, 1988). More recently, studies have suggested a certain degree of plasticity, with the pH gradient having the dominant effect upon ammonia distribution at rest and the effect of muscle membrane potential dominating following exercise (Wang et al., 1994a, 1996). These authors speculated that the relative permeability of the muscle membrane to  $\text{NH}_3$  and  $\text{NH}_4^+$  is altered by exercise-induced acidosis. In view of these questions surrounding the assumptions made in estimating the membrane potential from ammonia distribution and its central importance in the second hypothesis for loss of swimming performance (impaired neuromuscular function), the present study was undertaken to measure directly, using microelectrodes, the membrane potential of white muscle in trout exposed to copper and low pH.

### Materials and methods

Brown trout (*Salmo trutta* L.) were acclimated to artificial soft water (composition range,  $\mu\text{mol l}^{-1}$ :  $\text{Ca}^{2+}$ , 45–56;  $\text{Na}^+$ , 69–77;  $\text{K}^+$ , 4–6;  $\text{Mg}^{2+}$ , 34–48;  $\text{Cl}^-$ , 88–109;  $\text{SO}_4^{2-}$ , 62–68;  $\text{NO}_3^-$ , 4–7) at a temperature of  $10^\circ\text{C}$  for at least 4 weeks. They then each had a catheter implanted into their dorsal aorta (Soivio et al., 1972) and were placed in a Blazka-type swimming flume. Following 2 days of recovery, exposure to the test water was begun. This consisted of either the addition

of a stock solution of  $\text{CuSO}_4$  (to give a final concentration of  $0.08 \mu\text{mol l}^{-1}$   $\text{Cu}^{2+}$ ) and titration with  $\text{H}_2\text{SO}_4$  to pH 5 (CLP,  $N=6$ ) or of no change from the pre-exposure condition of no copper and pH 7 (control,  $N=6$ ). Copper concentration was regularly monitored by anodic stripping voltammetry (using a Radiometer POL150 polarograph with a hanging-drop mercury electrode and Tracemaster 5 software) which, under our conditions, had an experimental detection limit of approximately  $0.01 \mu\text{mol l}^{-1}$ . In the control conditions, copper concentration was always below this detection limit. After 96 h of exposure, blood samples were taken from the catheter and analysed for plasma pH (Cameron BGM200 at  $10^\circ\text{C}$ ) and  $[T_{\text{amm}}]$  (Sigma kit 171). The fish was rapidly killed by an overdose of the anaesthetic Saffan (Alfaxalone/Alfadolone, Mallinckdrodt Veterinary Ltd, Uxbridge, UK) and a blow to the head prior to the dissection. White muscle samples were quickly excised from the left-hand side of the fish and freeze-clamped with pre-cooled aluminium tongs, before being stored in liquid nitrogen for later analysis. Skin was carefully but rapidly removed from the right-hand side of the trout, which was then secured by dissection pins in a bath of ice-cold saline (pH 7.8; composition, in  $\text{mmol l}^{-1}$ :  $\text{NaCl}$ , 133.5; sodium pyruvate, 5;  $\text{KCl}$ , 3;  $\text{MgCl}_2$ , 2;  $\text{NaHCO}_3$ , 8.5;  $\text{NaH}_2\text{PO}_4$ , 3;  $\text{CaCl}_2$ , 3). Membrane potential ( $E_M$ ) was measured in fibres from a location just below and caudal to the dorsal fin. Membrane potential measurements were made using glass micropipettes pulled from 1.0 mm diameter borosilicate capillaries (Clarke Electromedical) with an electrode puller (BioScience). The microelectrodes were filled with  $3 \text{ mol l}^{-1}$   $\text{KCl}$  and connected to a Neurolog headstage and preamplifier (NL102G, Digitimer). Their measured resistance was between 15 and 35  $\text{M}\Omega$ . A  $\text{Ag/AgCl}$  reference electrode was connected to the tissue bath. The signal was analysed *via* a 'software oscilloscope' on a PC using a Lab-PC+ A/D interface board and LabVIEW software (National Instruments). Measurements were made from 8–12 fibres of each fish within 20 min.

The frozen muscle samples were ground to a powder under liquid nitrogen, and ammonia concentration was measured using the glutamate dehydrogenase method of Kun and Kearney (1974). In the same samples, intracellular phosphocreatine (PCr) concentration was measured in the coupled assay system described by Lamprecht et al. (1974). Tissue intracellular pH (pHi) was determined using the metabolic inhibition method of Pörtner et al. (1990) and a Cameron BGM200 blood gas system equilibrated at  $10^\circ\text{C}$ . Intracellular (ICFV) and extracellular (ECFV) fluid volume were measured using tritiated polyethylene glycol ( $^3\text{H}[\text{PEG}]$ , Dupont), which was dissolved in Young's teleost saline (Hale, 1965) and injected *via* the cannula into the dorsal aorta at a rate of  $0.925 \text{ MBq kg}^{-1}$  body mass. On the basis of trials previously conducted in our laboratory of equilibration times for  $^3\text{H}[\text{PEG}]$  in rainbow (*Oncorhynchus mykiss*) and brown trout tissues (S. E. Taylor, J. L. Mair and M. W. Beaumont, unpublished results), these injections were made 18 h prior to the termination of the experiment. Plasma and tissue samples were solubilised in 'Optisolv' (LKB Scintillation Products),

neutralised with glacial acetic acid and added to HiSafe 3 scintillation cocktail (LKB Scintillation Products). Radioactivity was counted using a Beckman LS 1701 counter, against a preprepared quench curve for trout tissue. Samples of tissue were also dried to constant mass at 70 °C to determine water content.

To determine tissue ion concentration, samples were digested in a 1:10 (mass: volume) dilution with 1 mol l<sup>-1</sup> nitric acid for 24 h. Sodium and potassium concentrations were determined in the neutralised supernatants and also in plasma samples using a Pye Unicam SP9 atomic absorption spectrophotometer. Chloride concentration was measured in the samples using an automated chloride titrator (Aminco).

#### Calculations

Extracellular fluid volume (ECFV) was calculated from the ratio of DPM measured in tissue (disints min<sup>-1</sup> g<sup>-1</sup> wet mass) and plasma (disints min<sup>-1</sup> ml<sup>-1</sup>) samples (equation 3) and intracellular fluid volume (ICFV; ml g<sup>-1</sup>) by subtraction from total tissue water (equation 4):

$$\text{ECFV} = \frac{\text{DPM}_{\text{tissue}}}{\text{DPM}_{\text{plasma}}}, \quad (3)$$

$$\text{ICFV} = \text{total tissue water} - \text{ECFV}. \quad (4)$$

#### Statistical analyses

Results are presented as mean  $\pm$  standard error (S.E.M.). Significant differences between treatments (control and CLP) were determined by analysis of variance (ANOVA) performed using Systat software (Statsoft Inc.).

## Results and discussion

### CLP exposure and muscle membrane potential

The results of the plasma and tissue analysis are shown in Table 1. In common with our previous studies (Beaumont et al., 1995b, 2000), total ammonia concentrations of both plasma ( $P < 0.001$ ) and white muscle ( $P < 0.05$ ) were significantly higher in CLP-exposed trout than in trout from control conditions. There were no significant differences in mean plasma pH and white muscle intracellular pH between fish from the two groups. If, as in our earlier study (Beaumont et

al., 2000), it is assumed that membrane potential is equivalent to  $E_{\text{NH}_4}$ , then, in the present study, the  $E_{\text{M}}$  of CLP-exposed trout is apparently depolarised by 38.3 mV in comparison with that of trout in control conditions (Table 2). This is in agreement with our previous study, although the size of the apparent depolarisation is somewhat larger, a consequence of a higher  $E_{\text{NH}_4}$  in the control fish.

However, as pointed out above, this analysis makes a number of assumptions and so, in the present study, direct measurements of white muscle  $E_{\text{M}}$  of trout from both control and CLP conditions have been made. The mean membrane potential recorded in the white muscle of control trout was  $-86.5 \pm 2.9$  mV (Table 2). The white muscle of CLP-exposed trout was significantly depolarised in comparison ( $-52.2 \pm 4.9$  mV,  $P < 0.001$ ). This approach supports the predictions made from the ammonia distribution, that CLP-exposed fish exhibit a depolarisation of the white muscle of 34 mV in comparison with trout from control conditions (Table 2).

The question of the cause of muscle depolarisation in trout exposed to copper and low pH remains. CLP exposure causes an increase in the absolute levels of ammonia, but it does not necessarily follow that this is responsible for the change in electrical status. It is possible that the observed change in ammonia distribution is simply a consequence of a change in  $E_{\text{M}}$  arising from some other factor. In mammalian neurons, ammonia activates *N*-methyl-D-aspartate (NMDA)-type glutamate receptors (Marcaida et al., 1992), and glutamate excitotoxicity is characterised by sustained membrane depolarisation (Rothman and Olney, 1987). Copper and protons themselves inhibit ATPases responsible for the transport of ions across cell membranes (e.g. Lorz and McPherson, 1976), and muscle K<sup>+</sup> and Cl<sup>-</sup> concentrations of CLP-exposed fish are, respectively, lower and higher than those of control trout, although there are no differences in the plasma concentrations of these ions (Fig. 1). However, fish exposed to sub-lethal copper concentrations accumulate little or no copper in the plasma or muscle since most is sequestered in the liver (Grosell et al., 1996, 1998). Moreover, in using these ion concentrations to estimate membrane potential from the Goldman-Hodgkin-Katz equation, we find the  $E_{\text{KNaCl}}$  of CLP-exposed animals to be significantly different from the measured  $E_{\text{M}}$  (Table 2;  $P < 0.05$ ). It seems likely that this

Table 1.  $[T_{\text{amm}}]$  and pH of plasma and white muscle of brown trout at rest following 96 h of exposure to either circum-neutral, copper-free water or to water containing 0.08  $\mu\text{mol l}^{-1}$  Cu<sup>2+</sup> at pH 5

| Treatment   | Plasma  |                 | White muscle   |                 |
|---|---|-----------------|--|-----------------|
|   | $[T_{\text{amm}}]_{\text{e}}$<br>( $\mu\text{mol l}^{-1}$ ) | pHe             | $[T_{\text{amm}}]_{\text{i}}$<br>( $\mu\text{mol l}^{-1}$ ICF) | pHi             |
| No copper, pH 7                                     | 108.7 $\pm$ 21.1  | 7.90 $\pm$ 0.08 | 3168 $\pm$ 223   | 7.07 $\pm$ 0.03 |
| 0.08 $\mu\text{mol l}^{-1}$ Cu <sup>2+</sup> , pH 5 | 651.9 $\pm$ 94.6 $\ddagger\ddagger\ddagger$                 | 7.83 $\pm$ 0.08 | 4255 $\pm$ 617 $\ddagger$                                      | 7.01 $\pm$ 0.03 |

Values are means  $\pm$  S.E.M.,  $N=6$ .

$[T_{\text{amm}}]$ , total ammonia concentration;  $[T_{\text{amm}}]_{\text{e}}$ , plasma  $[T_{\text{amm}}]$ ;  $[T_{\text{amm}}]_{\text{i}}$ , intracellular  $[T_{\text{amm}}]$ ; ICF, intracellular fluid.

A double dagger indicates a significant difference between control trout and trout exposed to copper and low pH.  $\ddagger P < 0.05$ ,  $\ddagger\ddagger\ddagger P < 0.001$ .

Table 2. Resting membrane potential of white muscle from brown trout following 96 h of exposure to either circum-neutral, copper-free water or to water containing 0.08  $\mu\text{mol l}^{-1}$   $\text{Cu}^{2+}$  at pH 5

| Treatment  | Nernst<br>$E_{\text{NH}_4}$                | Goldman–Hodgkin–Katz                       |  | Measured<br>$E_{\text{M}}$                 |
|--|--|--|--|--|
|  |  | $E_{\text{KNaCl}}$                         | $E_{\text{KNaClNH}_4}$                     |  |
| No copper, pH 7.                                     | $-92.8 \pm 3.4$                            | $-82.8 \pm 1.9$                            | $-83.2 \pm 2.3$                            | $-86.5 \pm 2.9$                            |
| 0.08 $\mu\text{mol l}^{-1}$ $\text{Cu}^{2+}$ , pH 5. | $-54.5 \pm 5.1 \ddagger \ddagger \ddagger$ | $-69.8 \pm 3.1 \ddagger \ddagger \ddagger$ | $-50.8 \pm 4.0 \ddagger \ddagger \ddagger$ | $-52.2 \pm 4.9 \ddagger \ddagger \ddagger$ |

Values are means  $\pm$  s.e.m.,  $N=6$  (for measured  $E_{\text{M}}$ , the mean of 20 measurements gave the data point for each individual animal).

A double dagger indicates a significant difference between control trout and trout exposed to copper and low pH.  $P < 0.001$ .

Calculated values were obtained from the following equations:

$$E_{\text{NH}_4} = - \frac{RT}{zF} \ln \frac{[\text{NH}_4^+]_i}{[\text{NH}_4^+]_e},$$

$$E_{\text{KNaCl}} = \frac{RT}{zF} \ln \frac{\text{perK}[\text{K}^+]_e + \text{perNa}[\text{Na}^+]_e + \text{perCl}[\text{Cl}^-]_i}{\text{perK}[\text{K}^+]_i + \text{perNa}[\text{Na}^+]_i + \text{perCl}[\text{Cl}^-]_e},$$

$$E_{\text{KNaClNH}_4} = \frac{RT}{zF} \ln \frac{\text{perK}[\text{K}^+]_e + \text{perNa}[\text{Na}^+]_e + \text{perCl}[\text{Cl}^-]_i + \text{perNH}_4[\text{NH}_4^+]_e}{\text{perK}[\text{K}^+]_i + \text{perNa}[\text{Na}^+]_i + \text{perCl}[\text{Cl}^-]_e + \text{perNH}_4[\text{NH}_4^+]_i}.$$

$R$  is the gas constant,  $T$  is absolute temperature,  $F$  is the Faraday constant,  $z$  is the valency and the subscripts  $i$  and  $e$  denote intracellular and extracellular concentrations, respectively.

$\text{perK}$ ,  $\text{perNa}$ ,  $\text{perCl}$  and  $\text{perNH}_4$  are the relative permeability coefficients of  $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{NH}_4^+$ ; values used were 1, 0.01, 2.5 and 0.26, respectively (Hodgkin and Horowitz, 1959; Binstock and Lecar, 1969).

$E_{\text{M}}$ , membrane potential;  $E_{\text{NH}_4}$ , membrane potential estimated from the Nernst equation for the measured distribution of ammonium ions;  $E_{\text{KNaCl}}$ , membrane potential estimated from the Goldman–Hodgkin–Katz equation from the concentration of  $\text{K}^+$ ,  $\text{Na}^+$  and  $\text{Cl}^-$ ;  $E_{\text{KNaClNH}_4}$ , membrane potential estimated from the concentrations of  $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{NH}_4^+$ .

reflects a significant contribution of ammonium ions, which can substitute for  $\text{K}^+$  in transport mechanisms (Binstock and Lecar, 1969). When ammonium ion distribution is included in the Goldman–Hodgkin–Katz estimate, using a value for the relative permeability of  $\text{NH}_4^+$  with respect to  $\text{K}^+$  of 0.26, obtained from a study of the squid giant axon (Binstock and Lecar, 1969), a much better fit with the observed membrane potential is obtained (Table 2). Whatever the cause, the membrane depolarisation and the consequently lower  $[T_{\text{amm}}]_i/[T_{\text{amm}}]_e$  ratio may perhaps protect the tissue against ammonia intoxication by reducing the concentration of ammonia in the tissue for a given extracellular concentration.

#### Ammonia distribution

While the aim of the present study was the validation of the prediction of muscle membrane depolarisation in trout exposed to copper and low pH, the present data contribute to the debate surrounding the factors affecting ammonia distribution in fish. CLP exposure provides an alternative to exercise, the method by which transmembrane conditions have commonly been manipulated in the study of ammonia distribution. Unlike exercise, no significant change occurs in the pH difference across the muscle membrane following CLP exposure but, as we have now shown, there is a significant depolarisation. Both exercise and CLP exposure elevate the intra- and extracellular total ammonia concentrations. While in the former case this is likely to arise mainly from the deamination of adenylates within the muscle, it is probable that the rise in the latter case is mainly a consequence of stress-related changes in hepatic

metabolism (Wendelaar Bonga, 1997) coupled, perhaps, with a limitation of branchial ammonia excretion. Since exercise protocols are necessarily much more acute than the CLP exposure of the present study, equilibrium conditions are more likely to have been achieved in the latter case.

Table 3 shows the measured  $[T_{\text{amm}}]_i/[T_{\text{amm}}]_e$  ratio and also the ratios predicted by the Nernst distribution (with an  $E_{\text{M}}$  of  $-85$  mV or the mean observed  $E_{\text{M}}$  for each fish) and by the pH gradient. In control fish, the observed value is remarkably close to that predicted by the Nernst distribution but much greater than that predicted by the pH gradient. This seems to confirm the hypothesis that ammonia is distributed according to membrane potential in trout white muscle. The mean observed ratio for CLP-exposed fish in the present experiment of  $7.3 \pm 1.4$  (Table 3) is significantly ( $P < 0.05$ ) lower than the value we found in a previous study ( $10.5 \pm 1.2$ ; Beaumont et al., 2000) but, as in that study, this result predicts either a significant depolarisation or a decrease in the relative permeability of the membrane for  $\text{NH}_4^+$  with respect to that for  $\text{NH}_3$  (i.e. to a distribution determined by pH). In the present study, direct measurement of  $E_{\text{M}}$  shows the former to be the case. The mean membrane potential in white muscle of CLP-exposed trout was  $-52.2 \pm 4.9$  mV. Using the observed ammonia distribution, the Nernst potential for ammonia ( $E_{\text{NH}_4}$ ) is  $-54.5 \pm 5.1$  mV (Table 2).

From these results, we might accept the hypothesis that the distribution of ammonia across the white muscle membrane of brown trout at rest is determined predominantly by membrane potential under normal conditions and following CLP

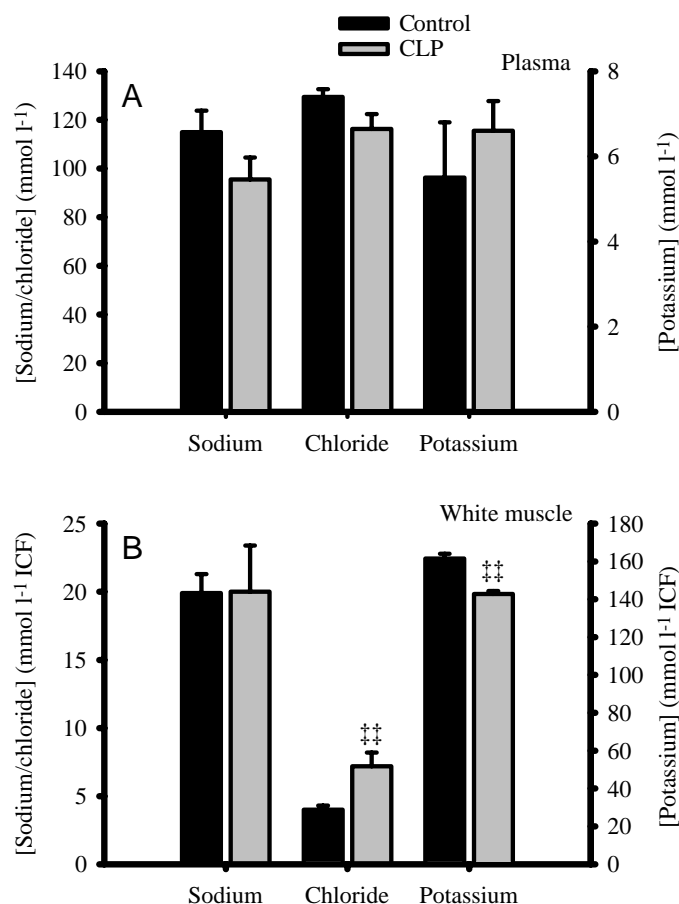


Fig. 1. Concentrations of sodium, chloride and potassium in (A) plasma and (B) white muscle of brown trout exposed either to control conditions (pH 7, no copper) or to copper and low pH (CLP,  $0.08 \mu\text{mol l}^{-1} \text{Cu}^{2+}$ , pH 5) for 96 h. Values are means + S.E.M.,  $N=6$ . †† denotes a significant ( $P < 0.01$ ) difference between treatments. ICF, intracellular fluid.

exposure. However, to explain the much lower  $[T_{\text{amm}}]_{\text{i}}/[T_{\text{amm}}]_{\text{e}}$  ratio in rainbow trout at rest than that found by most previous authors, Wang et al. (1996) suggested that previous studies may have overestimated the  $[T_{\text{amm}}]_{\text{i}}/[T_{\text{amm}}]_{\text{e}}$  ratio. They suggest a number of methodological reasons, particularly the difficulties in measuring tissue  $[T_{\text{amm}}]$  without adenylate

breakdown, which would result in high values for  $[T_{\text{amm}}]_{\text{i}}$ , and inaccuracies in determining  $[T_{\text{amm}}]_{\text{e}}$  using arterial plasma which could result in an underestimation of this variable.

In both the earlier studies (Beaumont et al., 1995b, 2000) and the present study,  $[T_{\text{amm}}]_{\text{i}}$  values of brown trout from control conditions and at rest are indeed somewhat higher than published data for rainbow trout (ranging from  $1.6$  to  $3.2 \mu\text{mol g}^{-1}$  wet mass compared with  $0.8 \mu\text{mol g}^{-1}$  wet mass from Wang et al., 1994b). However, the present study followed a similar procedure (anaesthesia and freeze-clamping) for tissue collection to that recommended by Wang et al. (1994b) except that, rather than using a waterborne anaesthetic such as MS-222 which, in our experience, occasionally leads to some struggling, fish were anaesthetised with Saffan injected *via* the cannula in the dorsal aorta. No struggling occurred, and tissue samples were rapidly freeze-clamped in liquid nitrogen. Both the deproteinisation and neutralisation steps were carried out quickly in the cold to avoid adenylate breakdown during analysis. Indeed, measurement of PCr concentration, a sensitive indicator of metabolic change induced by sampling (Wang et al., 1994b), in the same tissue samples gave a mean value of  $22.4 \pm 1.9 \mu\text{mol g}^{-1}$ , similar to that measured by Wang et al. (1994b). It is possible that the higher tissue ammonia levels in the brown trout of the present study in comparison with those reported in the literature for rainbow trout is simply a species difference; for example white muscle  $[T_{\text{amm}}]_{\text{i}}$  of both cod (*Gadus morhua*) and sole (*Parophrys vetulus*) at rest has been found to be higher even than in brown trout at over  $6 \text{mmol l}^{-1}$  (Fraser et al., 1966; Wright et al., 1988a). Alternatively, the difference may arise as a consequence of the soft water used in the present experiments.

The use of arterial plasma levels to estimate  $[T_{\text{amm}}]_{\text{e}}$  could also introduce error into the analysis of ammonia distribution. Arterial  $[T_{\text{amm}}]$  is likely to be significantly lower than the local  $[T_{\text{amm}}]_{\text{e}}$ . Such a discrepancy would cause the greatest error at the low plasma values of resting, control fish (Wang et al., 1994a). A better estimate of  $[T_{\text{amm}}]_{\text{e}}$  would perhaps come from venous plasma. However, there is a relatively consistent difference between arterial and venous  $[T_{\text{amm}}]$  of brown trout from both control and CLP conditions of approximately  $60 \mu\text{mol l}^{-1}$  (M. W. Beaumont, unpublished results). Application of this factor decreases the  $[T_{\text{amm}}]_{\text{i}}/[T_{\text{amm}}]_{\text{e}}$  ratio,

Table 3. The ratio  $[T_{\text{amm}}]_{\text{i}}/[T_{\text{amm}}]_{\text{e}}$  in white muscle of brown trout at rest following 96 h of exposure to either circum-neutral, copper-free water or to water containing  $0.08 \mu\text{mol l}^{-1} \text{Cu}^{2+}$  at pH 5

| Treatment   | $[T_{\text{amm}}]_{\text{i}}/[T_{\text{amm}}]_{\text{e}}$ |   |   | Measured       |
|---|---|---|---|----------------|
|   | Predicted from non-ionic diffusion (pH)                   | Predicted from Nernst using $E_{\text{M}} = -85 \text{ mV}$ | Predicted from Nernst using measured $E_{\text{M}}$ |                |
| No copper, pH 7                                   | $7.2 \pm 1.2$   | $32.2 \pm 0.1$  | $35.5 \pm 4.4$                                      | $33.0 \pm 4.3$ |
| $0.08 \mu\text{mol l}^{-1} \text{Cu}^{2+}$ , pH 5 | $7.5 \pm 1.9$   | $32.3 \pm 0.1$  | $9.2 \pm 1.8$                                       | $7.3 \pm 1.4$  |

Values are means  $\pm$  S.E.M.,  $N=6$ .

The ratios presented are those calculated from the measured values of  $[T_{\text{amm}}]$ , from the measured pH gradient (equation 2) and from the Nernst equation (equation 1) assuming a muscle membrane potential of  $-85 \text{ mV}$  or using the measured values.

$[T_{\text{amm}}]_{\text{i}}$  and  $[T_{\text{amm}}]_{\text{e}}$  are the internal and external ammonia concentrations, respectively;  $E_{\text{M}}$ , membrane potential.

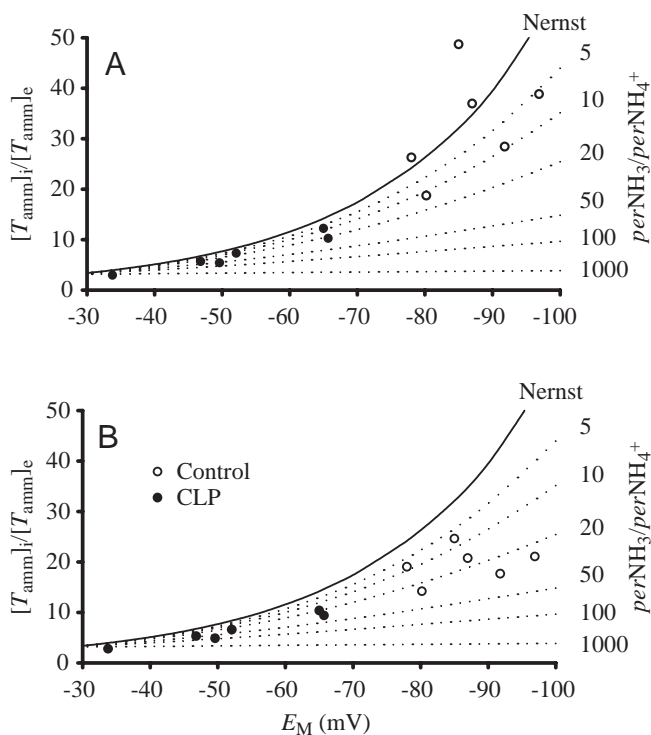


Fig. 2. The relationship between ammonia distribution and muscle membrane potential ( $E_M$ ) for brown trout exposed either to control conditions (pH 7, no copper) or to copper and low pH (CLP,  $0.08 \mu\text{mol l}^{-1} \text{Cu}^{2+}$ , pH 5) for 96 h. (A)  $[T_{\text{amm}}]_i/[T_{\text{amm}}]_e$  for each fish, where  $[T_{\text{amm}}]_e$  is the measured arterial  $[T_{\text{amm}}]$ . (B)  $[T_{\text{amm}}]_i/[T_{\text{amm}}]_e$  for each fish, where  $[T_{\text{amm}}]_e$  is the estimated venous  $[T_{\text{amm}}]$ .  $[T_{\text{amm}}]_i$  and  $[T_{\text{amm}}]_e$  are the internal and external total ammonia concentrations respectively. Also shown in each graph are the curves calculated from the Nernst equation (solid line) and from the Roos and Boron equation (dotted lines) using an extracellular minus intracellular pH difference ( $\Delta\text{pH}$ ) of 0.8 and a range of values for the membrane permeability ratio  $\text{perNH}_3/\text{perNH}_4^+$ .

particularly that of control fish in which  $[T_{\text{amm}}]_e$  is low. The effect of this adjustment is shown in Fig. 2, in which the measured membrane potential is plotted against the measured arterial (Fig. 2A) or adjusted (venous) (Fig. 2B) ammonia distribution for each fish. The two patterns of distribution described so far, i.e. according to  $E_M$  or to pH, represent the two ends of a range of possible states that can be described by the general equation (equation 5) given by Roos and Boron (1981) (see also Wood, 1993):

$$\frac{[T_{\text{amm}}]_i}{[T_{\text{amm}}]_e} = \frac{([\text{H}^+]_i + K)}{([\text{H}^+]_e + K)} \times \frac{(\text{perNH}_3/\text{perNH}_4^+) - [F \times E_M/R \times T(1 - \gamma)] \times ([\text{H}^+]_e/K)}{(\text{perNH}_3/\text{perNH}_4^+) - [F \times E_M \times \gamma/R \times T(1 - \gamma)] \times ([\text{H}^+]_i/K)}, \quad (5)$$

where  $\gamma = \exp(E_M F/RT)$ ,  $K$  is the dissociation constant for  $\text{NH}_3/\text{NH}_4^+$  and  $\text{perNH}_3/\text{perNH}_4^+$  the ratio of membrane permeability to  $\text{NH}_3$  and to  $\text{NH}_4^+$ .

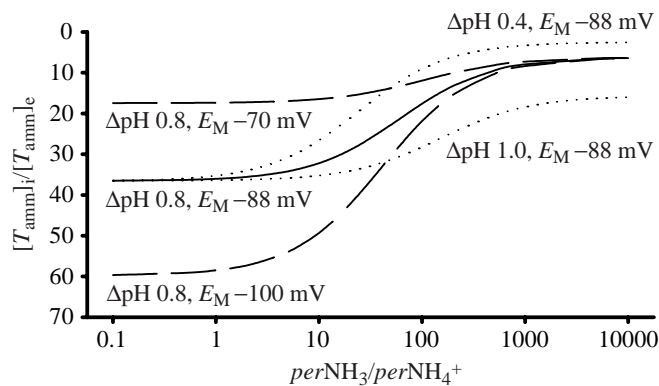


Fig. 3. Relationship between relative membrane permeability to  $\text{NH}_3$  and  $\text{NH}_4^+$  ( $\text{perNH}_3/\text{perNH}_4^+$ ) and ammonia distribution ( $[T_{\text{amm}}]_i/[T_{\text{amm}}]_e$ ), where  $[T_{\text{amm}}]_i$  and  $[T_{\text{amm}}]_e$  are the internal and external total ammonia concentrations, respectively, using the Roos and Boron equation (equation 3) and typical values for membrane potential ( $E_M$ ) and the extracellular minus intracellular pH difference ( $\Delta\text{pH}$ ) in brown trout from control conditions (solid line). Curves are also plotted showing the effects of alterations to membrane potential (broken lines) and to  $\Delta\text{pH}$  (dotted lines). Adapted from Wood (1993).

Also shown in Fig. 2A,B are the relationships between ammonia distribution and membrane potential based upon the Nernst equation (equation 1) and the Roos and Boron equation (equation 5). For the latter, the mean measured  $\text{pH}_e - \text{pH}_i$  of 0.8 (Table 1) was used, and the relationship is plotted for a range of values of  $\text{perNH}_3/\text{perNH}_4^+$ . The  $[T_{\text{amm}}]_i/[T_{\text{amm}}]_e$  ratio is usually lower than that predicted from  $E_M$  alone (i.e. using the Nernst distribution), and the data in Fig. 2B appear to show a reasonable correlation with the predictions of the Roos and Boron equation where the ratio of membrane permeability is approximately 20–50. As Fig. 3 shows, this represents almost the mid-point between dependence solely upon  $E_M$  or solely upon pH. Ammonia distribution in this case could be regulated equally by changes in the pH status or the muscle membrane potential. As Wood et al. (1989) suggest, the significance of a permeability to both  $\text{NH}_3$  and  $\text{NH}_4^+$  in ammoniotelic organisms such as fish may be the requirement for a greater capacity for the movement of ammonia across cell membranes than in their ureotelic counterparts. With a permeability ratio of 20–50, there is perhaps a maximum sensitivity to changes in both  $E_M$  and  $\Delta\text{pH}$  for the regulation of ammonia distribution (Fig. 3).

In summary, the present study has confirmed the prediction that the resting membrane potential of white muscle from trout exposed to copper and low pH is significantly depolarised in comparison with that of control fish. This seems to be a consequence of the elevated ammonia concentrations in these fish. This, in turn, may influence swimming performance by inactivating voltage-gated  $\text{Na}^+$  channels and causing a subsequent loss of electrical excitability in these muscle fibres (Jenerick, 1959). A similar phenomenon could account for the absence of white muscle recruitment in trout exposed to sub-lethal low pH, swimming at speeds at which such recruitment

normally occurs (Day and Butler, 1996), as these fish also accumulate ammonia in their tissues.

This project was supported by the Natural Environment Research Council.

### References

- Altringham, J. D. and Johnston, I. A.** (1988). Activation of multiply innervated fast and slow myotomal muscle fibres of the teleost *Myoxocephalus scorpius*. *J. Exp. Biol.* **140**, 313–324.
- Bangsbo, J., Kiens, B. and Richter, E. A.** (1996). Ammonia uptake in inactive muscles during exercise in humans. *Am. J. Physiol.* **270**, E101–E106.
- Beaumont, M. W., Butler, P. J. and Taylor, E. W.** (1995a). Exposure of brown trout, *Salmo trutta*, to sub-lethal copper concentrations in soft acidic water and its effect upon sustained swimming performance. *Aquat. Toxicol.* **33**, 45–63.
- Beaumont, M. W., Butler, P. J. and Taylor, E. W.** (1995b). Plasma ammonia concentration in brown trout (*Salmo trutta*) exposed to sub-lethal copper concentrations and its relationship to decreased swimming performance. *J. Exp. Biol.* **198**, 2213–2220.
- Beaumont, M. W., Butler, P. J. and Taylor, E. W.** (2000). Exposure of brown trout, *Salmo trutta*, to a sub-lethal concentration of copper in soft acidic water: effects upon muscle metabolism and membrane potential. *Aquat. Toxicol.* (in press).
- Binstock, L. and Lecar, H.** (1969). Ammonium ion currents in the squid giant axon. *J. Gen. Physiol.* **53**, 342–361.
- Brett, J. R.** (1964). The respiratory metabolism and swimming performance of young sockeye salmon. *J. Fish. Res. Bd Can.* **21**, 1183–1226.
- Butler, P. J., Day, N. and Namba, K.** (1992). Interactive effects of seasonal temperature and low pH on resting oxygen uptake and swimming performance of adult brown trout (*Salmo trutta*). *J. Exp. Biol.* **165**, 195–212.
- Day, N. and Butler, P. J.** (1996). Environmental acidity and white muscle recruitment during swimming in the brown trout (*Salmo trutta*). *J. Exp. Biol.* **199**, 1947–1959.
- Eugène, D. and Baretts, A.** (1983). Electrical activity of trout skeletal muscle fibers. *J. Physiol., Paris* **78**, 814–820.
- Fraser, D. I., Dyer, W. J., Weinstein, H. M., Dingle, J. R. and Hines, J. A.** (1966). Glycolytic metabolites and their distribution at death in the white and red muscle of cod following various degrees of antemortem muscular activity. *Can. J. Biochem.* **44**, 1015–1033.
- Grosell, M., Boetius, I., Hansen, H. J. M. and Rosenkilde, P.** (1996). Influence of preexposure to sublethal levels of copper on <sup>64</sup>Cu uptake and distribution among tissues of the European eel (*Anguilla anguilla*). *Comp. Biochem. Physiol.* **114C**, 229–235.
- Grosell, M. H., Hogstrand, C. and Wood, C. M.** (1998). Renal Cu and Na excretion and hepatic Cu metabolism in both Cu acclimated and non acclimated rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* **40**, 275–291.
- Hale, L. J.** (1965). *Biological Laboratory Data*. London: Methuen & Co. Ltd.
- Heald, D. E.** (1975). Influence of ammonium ions on mechanical and electrophysiological responses of skeletal muscle. *Am. J. Physiol.* **229**, 1174–1179.
- Heisler, N.** (1990). Mechanisms of ammonia elimination in fishes. In *Animal Nutrition and Transport Processes 2. Transport, Respiration and Excretion: Comparative and Environmental Aspects* (ed. J. P. Truchot and B. Lahlou), pp. 137–157. Karger: Basel.
- Hidaka, T. and Toida, N.** (1969). Biophysical and mechanical properties of red and white muscle fibres in fish. *J. Physiol., Lond.* **201**, 49–59.
- Hodgkin, A. L. and Horowitz, P.** (1959). The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol., Lond.* **148**, 127–160.
- Jacobs, M. H.** (1940). Some aspects of cell permeability to weak electrolytes. *Cold Spring Harbour Symp. Quant. Biol.* **8**, 30–39.
- Jenerick, H. P.** (1959). The control of membrane ionic currents by the membrane potential of muscle. *J. Gen. Physiol.* **42**, 923–930.
- Kun, E. and Kearney, E. B.** (1974). Ammonia. In *Methods of Enzymatic Analysis*, vol. 4 (ed. H. U. Bergmeyer), pp. 1802–1806. London: Verlag Chemie Academic Press.
- Lamprecht, W., Stein, P., Heinz, F. and Weisser, H.** (1974). Creatine phosphate. In *Methods of Enzymatic Analysis*, vol. 4 (ed. H. U. Bergmeyer), pp. 1777–1785. London: Verlag Chemie Academic Press.
- Lorz, H. W. and McPherson, B. P.** (1976). Effects of copper or zinc in fresh water on the adaptation to sea water and ATPase activity and the effects of copper on migratory disposition of coho salmon (*Oncorhynchus kisutch*). *J. Fish. Res. Bd Can.* **33**, 2023–2030.
- MacLean, D. A., Barclay, J. K. and Graham, T. E.** (1995). Electrical stimulation and amino acid and ammonia metabolism in the canine gastrocnemius muscle. *Am. J. Physiol.* **268**, R759–R770.
- Marcáida, G., Felipo, V., Hermenegildo, C., Miñana, M.-D. and Grisolia, S.** (1992). Acute ammonia toxicity is mediated by the NMDA type of glutamate receptors. *FEBS Lett.* **296**, 67–68.
- Meyer, R. A., Dudley, G. A. and Terjung, R. L.** (1980). Ammonia and IMP in different skeletal muscle fibres after exercise in rats. *J. Appl. Physiol.* **49**, 1037–1041.
- Milne, M. D., Scribner, B. H. and Crawford, M. A.** (1958). Non-ionic diffusion and the excretion of weak acids and bases. *Am. J. Med.* **24**, 709–729.
- Mommsen, T. P. and Hochachka, P. W.** (1988). The purine nucleotide cycle as two temporally separated metabolic units: a study on trout muscle. *Metabolism* **37**, 552–556.
- Pörtner, H. O., Boutillier, R. G., Tang, Y. and Toews, D. P.** (1990). Determination of intracellular pH and P<sub>CO<sub>2</sub></sub> after metabolic inhibition by fluoride and nitrilotriacetic acid. *Respir. Physiol.* **81**, 255–274.
- Randall, D. J. and Brauner, C.** (1991). Effects of environmental factors on exercise in fish. *J. Exp. Biol.* **160**, 113–126.
- Roos, A. and Boron, W. F.** (1981). Intracellular pH. *Physiol. Rev.* **61**, 296–434.
- Rothman, S. M. and Olney, J. W.** (1987). Excitotoxicity and the NMDA receptor. *Trends Neurosci.* **10**, 299–302.
- Saha, N. and Ratha, B. K.** (1989). Comparative study of ureogenesis in freshwater, air-breathing teleosts. *J. Exp. Zool.* **252**, 1–8.
- Soivio, A., Westman, K. and Nyholm, K.** (1972). Improved method of dorsal aorta catheterization: haematological effects followed for three weeks in rainbow trout (*Salmo gairdneri*). *Finn. Fish Res.* **1**, 11–21.
- Stabenau, J. R., Warren, K. S. and Rall, D. P.** (1959). The role of pH gradient in the distribution of ammonia between blood and cerebrospinal fluid. *J. Clin. Invest.* **38**, 373–383.
- Stanfield, P. R.** (1972). Electrical properties of white and red muscle fibres of the elasmobranch fish *Scyliorhinus canicula*. *J. Physiol., Lond.* **222**, 161–186.
- Tang, Y., Lin, H. and Randall, D. J.** (1992). Compartmental

- distributions of carbon dioxide and ammonia in rainbow trout at rest and following exercise and the effect of bicarbonate infusion. *J. Exp. Biol.* **169**, 235–249.
- Wang, Y. X., Heigenhauser, G. J. F. and Wood, C. M.** (1994a). Integrated responses to exhaustive exercise and recovery in rainbow trout white muscle: acid–base, phosphogen, carbohydrate, lipid, ammonia, fluid volume and electrolyte metabolism. *J. Exp. Biol.* **195**, 227–258.
- Wang, Y. X., Heigenhauser, G. J. F. and Wood, C. M.** (1996). Ammonia movement and distribution after exercise across white muscle cell membranes in rainbow trout. *Am. J. Physiol.* **40**, R738–R750.
- Wang, Y. X., Wilkie, M. P., Heigenhauser, G. J. F. and Wood, C. M.** (1994b). The analysis of metabolites in rainbow trout white muscle: a comparison of different sampling and processing methods. *J. Fish Biol.* **45**, 855–873.
- Wendelaar Bonga, S. E.** (1997). The stress response in fish. *Physiol. Rev.* **77**, 591–625.
- Wilson, R. W. and Taylor, E. W.** (1993a). The physiological responses of freshwater rainbow trout, *Oncorhynchus mykiss*, during acutely lethal copper exposure. *J. Comp Physiol. B* **163**, 38–47.
- Wilson, R. W. and Taylor, E. W.** (1993b). Differential responses to copper in rainbow trout (*Oncorhynchus mykiss*) acclimated to sea water and brackish water. *J. Comp Physiol. B* **163**, 239–246.
- Wood, C. M.** (1993). Ammonia and urea metabolism and excretion. In *The Physiology of Fishes* (ed. D. H. Evans), pp. 379–425. Boca Raton, FL: CRC Press.
- Wood, C. M., Munger, R. S. and Toews, D. P.** (1989). Ammonia, urea and H<sup>+</sup> distribution and the evolution of ureotelism in amphibians. *J. Exp. Biol.* **144**, 215–233.
- Wright, P. A., Randall, D. J. and Wood, C. M.** (1988a). The distribution of ammonia and H<sup>+</sup> between tissue compartments in lemon sole (*Parophrys vetulus*) at rest, during hypercapnia and following exercise. *J. Exp. Biol.* **136**, 149–175.
- Wright, P. A. and Wood, C. M.** (1988). Muscle ammonia stores are not determined by pH gradients. *Fish Physiol. Biochem.* **5**, 159–162.
- Wright, P. A., Wood, C. M. and Randall, D. J.** (1988b). An *in vitro* and *in vivo* study of the distribution of ammonia between plasma and red cells of rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.* **134**, 423–428.