



Estimation of intracellular pH in muscle of fishes from different thermal environments

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Abstract

A technique based on homogenisation of rapidly frozen tissue was used to investigate the regulation of intracellular pH (pH_i) in freshwater and marine fish from diverse environmental temperatures. The following species were held at ambient temperatures of ca. 1°C (*Notothenia coriiceps*; Antarctica), 5°C (*Pleuronectes platessa*, *Myoxocephalus scorpius*; North Sea), and 26°C (*Oreochromis niloticus*; African lakes). The effects of seasonal acclimatisation to 4, 11 and 18°C were also examined in rainbow trout in the winter, autumn and summer, respectively. Extracellular (whole blood) pH (pH_e) did not follow the constant relative alkalinity relationship, where $\text{pH}^+ = \text{pOH}^-$ for any particular temperature, over a range of 1–26°C (overall $\delta\text{pH}_e/\delta T = 0.009 \pm 0.002 \text{ U } ^\circ\text{C}^{-1}$; $P < 0.001$), apparently being regulated by ionic fluxes and ventilation. Intracellular pH (pH_i) was also regulated independently of pN (=0.5 pK water) in all species of fish examined. The inverse relationship between pH_i and environmental temperature gave an overall $\delta\text{pH}_i/\delta T$ of $-0.010 \pm 0.001 \text{ U } ^\circ\text{C}^{-1}$ (for both white and red muscle) and $-0.004 \pm 0.003 \text{ U } ^\circ\text{C}^{-1}$ (cardiac muscle). However, between 1 and 11°C $\delta\text{pH}_i/\delta T$ was much higher ($P < 0.001$), $-0.022 \pm 0.003 \text{ U } ^\circ\text{C}^{-1}$ (white muscle) and $-0.022 \pm 0.004 \text{ U } ^\circ\text{C}^{-1}$ (red muscle). The possible adaptive roles for these different acid–base responses to environmental temperature variation among tissues and species, and the potential difficulties of estimating pH_i , are discussed. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Most fish species are ectothermic, with cell temperature closely reflecting that of the environment. Temperature directly influences the rate of enzyme

reactions, diffusion, and binding of ligands to proteins and proteins to membrane receptors (Somero, 1995). This results in Q_{10} values of 2–3 for the rise in metabolic rate with acute temperature change. Strategies for the thermal compensation of biological processes are widespread in the animal kingdom, requiring a period of acclimation to the new condition (Cossins and Bowler, 1987). An important modulator of cellular metabolism is pH which, due to the inherent thermal sensitivity of physico–chemical processes, usually varies inversely with temperature; the relationship of extracellular pH (pH_e) versus temperature has been typified

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as having a slope varying from -0.016 to -0.019 U $^{\circ}\text{C}^{-1}$ (Dejours and Armand, 1983; Truchot, 1987). This parallels the change in the pH of neutrality, which follows the dissociation of water and is defined as the point where $\text{pH}^+ = \text{pOH}^-$ for any particular temperature (the concept of constant relative alkalinity; Rahn, 1967). pH_i was considered to parallel changes in the neutral point of water ($\text{pN} = 0.5 \text{ pK}_w$) which would tend to maintain a constant net charge on histidine residues, and hence maintain the structural integrity and biological activity of proteins (the alpha-stat hypothesis; Reeves, 1972, 1976). Although many authors have demonstrated this relationship in a variety of species, few studies have measured pH_e or pH_i below 10°C (Moerland and Egginton, 1998). Interestingly, some seasonally acclimatised fish have been shown to regulate pH_i independently of environmental temperature (Heisler et al., 1976; Walsh and Moon, 1982; Butler and Day, 1993; Taylor, 1995), with relatively acidotic pH values observed at low temperatures. Similar data have been described for crustaceans, with a relatively acidotic pH_e between 1 – 11°C and very different values for $\delta\text{pH}_i/\delta T$ among tissues (Whiteley and Taylor, 1993; Whiteley et al., 1995). The accumulation of protons at low temperatures may reduce metabolic rate by inactivation of enzymes during the winter, which may be beneficial in allowing energy stores to be conserved when resources are low (Walsh and Moon, 1982).

However, comparisons among different studies are complicated by the variety of methods used to measure pH. Various studies have compared the different techniques available to measure pH_i (Waddell and Bates, 1969; Hinke and Menard, 1976; Roos and Boron, 1981; Wood and Cameron, 1985; Pörtner, 1989). Analysis of published data suggests that the slope of the pH_i –temperature relationship depends on the methods used, with microelectrodes significantly underestimating the value of $\delta\text{pH}_i/\delta T$ relative to other accepted methods (Moerland and Egginton, 1998). The most common approaches include distribution of a weak acid (Waddell and Bates, 1969), and tissue homogenisation in conjunction with metabolic inhibitors (Costhill et al., 1982; Pörtner et al., 1990). Both techniques involve a number of assumptions, and absolute cytosolic pH values are difficult to obtain due to the differential distribution of protons among heterogeneous intracellular compartments (e.g. mitochondria).

The primary aim of the present study was to examine the regulation of pH_i in a variety of species from a range of latitudes either acclimatised seasonally, or experimentally acclimated to a wide range of temperatures, to determine whether there are particular trends in pH_i regulation among fish from different thermal habits. This allowed us to further examine the

utility of the homogenising technique for use with fish tissue.

2. Material and methods

2.1. Experimental animals

Adult rainbow trout, *Oncorhynchus mykiss* Walbaum, mass 600–1500 g, were obtained from Leadmill trout farm, Hathersage, Derbyshire, when ambient and consequent experimental temperatures were 18°C (late summer), 11°C (autumn) and 4°C (winter). The fish were held indoors for a period of up to 28 days in large circular tanks holding 600 l of continuously flowing aerated, dechlorinated tap water, maintained at the appropriate experimental temperature ($\pm 0.5^{\circ}\text{C}$). Throughout this period, the fish were exposed to the natural photoperiod and fed a maintenance diet of commercial trout pellets. Adult tilapia, *Oreochromis niloticus*, mass 168–754 g, were kept in recirculating filtered freshwater at $26 \pm 1^{\circ}\text{C}$, fed trout pellets (EWOS) and held at 12:12 L:D photoperiod. Adult plaice, *Pleuronectes platessa*, mass 98–146 g, and sculpins, *Myoxocephalus scorpius*, mass 125–452 g, were obtained by trawl from the North Sea (St. Andrews Bay; $56^{\circ} 25' \text{N}$, $3^{\circ} 20' \text{W}$) at depths of 10–20 m, fed on chopped fish and shrimps, held in tanks of seawater at the ambient temperature of $5 \pm 1^{\circ}\text{C}$ and 12:12 L:D. Adult *Notothenia coriiceps* Richardson, mass 182–1050 g, were caught by trammel nets at depths of 10–50 m around Signy Island, South Orkney Islands ($60^{\circ} 43' \text{S}$ $45^{\circ} 36' \text{W}$) by the British Antarctic Survey, and transported back to the UK. Fish were kept in recirculated, filtered seawater at ca. 1°C and fed on chopped squid. In all cases, feeding was stopped at least 2 days prior to tissue sampling to avoid any post-prandial elevation in metabolism.

2.2. Measurement of pH

A blood sample (ca. 500 μl) was taken for measurement of the pH of whole blood (i.e. extracellular pH, pH_e) for reference purposes, using a glass capillary electrode (Radiometer G279/G2) coupled with a calomel reference electrode (K497), both thermostatted to the experimental temperature and used in conjunction with a pH/blood gas monitor (Radiometer PHM73). A two point calibration was performed before and a single point check after each sample, using precision buffers (BDH, Poole). For most samples chronically indwelling catheters were used, following surgery under MS222 anaesthesia and full recovery (Taylor et al., 1996), with others taken by rapid caudal artery puncture.

In order to avoid disturbance of intracellular pH

(pH_i) in skeletal muscle due to struggling, the fish were first injected via an arterial cannula with dilute anaesthetic (MS222, 1:1000,000) prior to being killed by a cephalic blow, and subsequent pithing. Tissue samples were quickly excised (usually within 2 min), blotted, wrapped in aluminium foil and frozen in liquid nitrogen. Intracellular pH determination involved the inhibition of metabolic activity within the crude homogenates by addition of potassium fluoride (KF) and nitrilotriacetic acid (NTA). The method used was similar to that described by Pörtner et al. (1990), with preliminary experiments being performed to ascertain the optimal concentration of each inhibitor and their interaction (Taylor, 1995). Briefly, frozen tissue was ground under liquid nitrogen, using a pre-cooled pestle and mortar, to a fine powder. Aliquots of the powder (100–100 mg) were added to 0.2 ml of ice-cold medium (KF, 150 mmol l⁻¹; NTA, 6 mmol l⁻¹; adjusted to pH 7.0 using potassium hydroxide) in pre-weighed tubes. After reweighing, the medium was stirred briefly with a mounted needle to release bubbles, then capped. The contents were well mixed on a vortex mixer and centrifuged for about 15 s. Aliquots of the supernatant were taken for repeated measurement of pH. Variation in pH readings between replicates was usually below ± 0.01 pH units among samples from the same tissue powder. Measured values for tissue pH, from the rainbow trout, were corrected for the influence exerted by the medium (buffering and dilution effects) according to the equation of Pörtner et al. (1990), using values of extracellular fluid volume and tissue water content determined previously (Taylor, 1995).

2.3. Assay of muscle metabolites

Tissue lactate assay was based on a modification of a standard kit (Sigma No. 735). The powdered tissue samples were freeze-dried for 48 h and stored with silica gel in an air-tight container at -20°C for a maximum of 10 days. Samples were homogenised for 5–10 min with 50–100 vol 0.3 M perchloric acid in a Duall 1 ml glass homogeniser held in an ice slurry. The homogenate was centrifuged for 5 min and a sample of the supernatant taken and neutralised with K₂HPO₄. Fifty microlitres of neutralised samples were analysed using a Dynatech MR5000 plate spectrophotometer, enabling 96 samples to be read and analysed within 3 min. Duplicates were routinely run, and where there was a >10% difference a third sample was run. Assays were calibrated and validated with the appropriate standards and controls.

Samples were analysed for concentrations of creatine (Cr), phosphocreatine (PCr) and the adenosine compounds, AMP, ADP and ATP by high performance liquid chromatography (HPLC) analysis. Following homogenisation (above) metabolites were extracted for

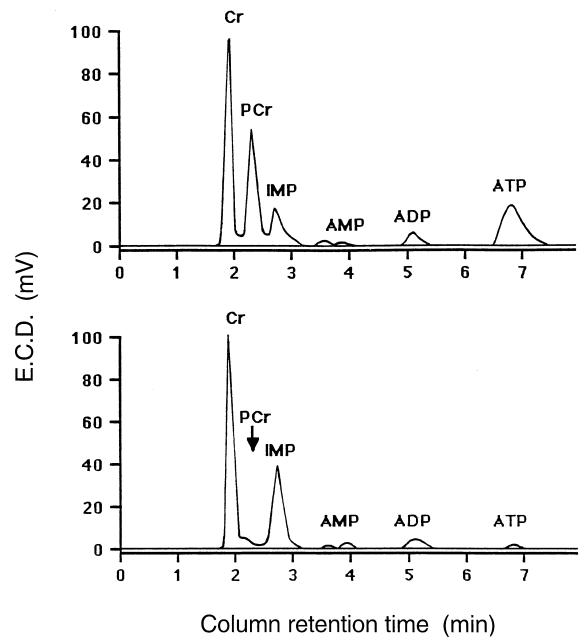


Fig. 1. HPLC chromatograms illustrating the variability of sample composition in *Notothenia* white muscle between individual fish that were sampled without noticeable trauma, and struggled during biopsy. (A) high PCr and ATP peaks with low IMP and ADP peaks, representative of glycolytic muscle in a resting state. (B) muscle with similar pH_i showing the inverse situation, indicative of tissue having undergone significant post-sampling degradation of intracellular stores.

10–15 min on ice, then centrifuged at 13,000 g for 3 min. The pH of the supernatant was increased to 6.3 with a measured aliquot of K₂HPO₄ and the precipitated perchlorate removed by centrifugation. The supernatant was immediately assayed for muscle metabolites by HPLC, using a modification of the protocols described by Moon et al. (1991). Metabolites were eluted isocratically with a mobile phase containing 215 mM KH₂PO₄, 2.3 mM tetrabutyl-ammonium hydrogen sulphate and 3.5% acetonitrile, adjusted to pH 6.25 with KOH. The solution was filtered through a 2 μm cellulose acetate filter and degassed under helium for 20 min. The flow rate was set at 1 ml min⁻¹ and elution typically required a 15 min run time. Each column was calibrated with a range of authentic standards, and standard curves based upon peak areas were established. Each peak was baseline-integrated using appropriate software (Systems Manager 704; Gilson). Standards were run daily, but column deterioration was slight until just before column collapse (300–500 injections). Both pyruvate and lactate were eluted using this system but detector sensitivity for these compounds was low relative to that for the nucleotides, and they could thus be ignored. Each

Table 1
Extracellular pH of fishes from a range of thermal habitats. Values are mean \pm SEM (*n*)

| Species | Habitat temperature ($^{\circ}$ C) | Cannulated pH_e | Caudal puncture pH_e |
|-------------------|-------------------------------------|--------------------------|------------------------------------|
| <i>Notothenia</i> | 1 | 7.863 \pm 0.138 (7) | ^b 7.493 \pm 0.059 (4) |
| Sculpin | 5 | – | 7.614 \pm 0.049 (7) |
| Plaice | 5 | – | 7.499 \pm 0.021 (6) |
| Trout | 4 | 7.912 \pm 0.018 (6) | – |
| Trout | 11 | 7.847 \pm 0.028 (8) | – |
| Trout | 18 | 7.757 \pm 0.008 (7) | – |
| Tilapia | 26 | 7.636 \pm 0.061 (4) | ^a 7.473 \pm 0.030 (8) |

^a $P < 0.05$, ^b $P < 0.005$ versus cannulated.

muscle sample was run in duplicate, peak areas were compared to the appropriate standard curves, and the separate values were then averaged (variation did not

exceed 10%) to give concentration in $\mu\text{mol g}^{-1}$ dry muscle mass. The sensitivity of this method was adequate to reveal inter-animal variability in sampling stress (Fig. 1).

2.4. Statistical analysis

Differences in the values of pH and tissue metabolites were examined using analysis of variance (ANOVA) and Scheffe's *F*-test for multiple comparisons. Differences were considered significant at the 95% level of confidence, i.e. when $P \leq 0.05$. Data are presented as mean \pm S.E.M.

3. Results

3.1. pH measurements

When compared in tilapia, values for whole blood pH, (pH_e), from either cardiac or caudal artery puncture (taken during tissue sampling) were on average 0.16 pH units lower than values obtained from blood withdrawn via an arterial cannula (paired *t*-test, $P < 0.01$), probably due to handling stress. This difference was larger in *Notothenia* at 0.37 pH units ($P < 0.01$), possibly due to the added stress of removal from water at 1°C (Table 1). Although both forms of sampling produced the expected inverse relationship with environmental temperature, the slope of $\delta\text{pH}_e/\delta T$ for cannulated fish whole blood pH (-0.009 ± 0.002) was significantly greater than by caudal artery puncture obtained just prior to tissue sampling (-0.003 ± 0.002 ; $P < 0.05$). Both slopes were less steep than that for the neutral point of water, pN (Fig. 2). However, there was no significant difference between the 26 and 18°C values, while the respective $\delta\text{pH}_e/\delta T$ values for rainbow trout between 4 and 11°C was $-0.010 \text{ U } ^{\circ}\text{C}^{-1}$, and between 11 and 18°C was $-0.013 \text{ U } ^{\circ}\text{C}^{-1}$. The difference between pH_e and pH_i for oxidative muscle and individual species was maximally 0.27 and 0.82 for uncannulated and cannulated fish, respectively.

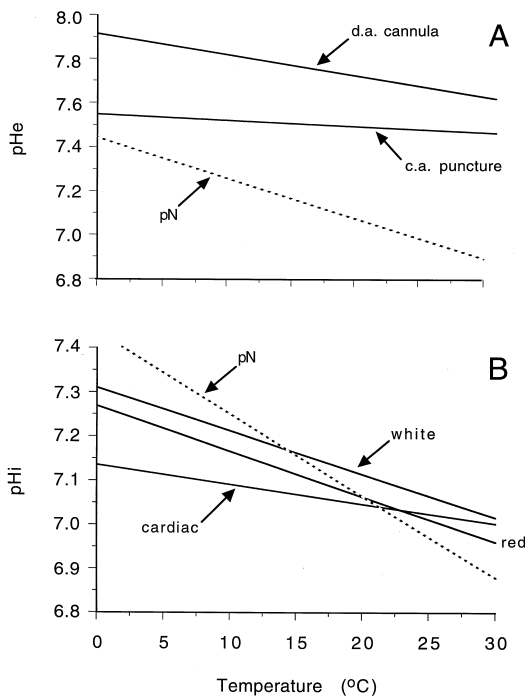


Fig. 2. (A) Extracellular pH (pH_e) as a function of environmental temperature. The whole blood pH obtained via caudal artery puncture underestimated the $\delta\text{pH}_e/\delta T$ (regression = $7.553 - 0.003 \times T$; $R^2 = 0.08$) compared with values using cannulae ($7.919 - 0.009 \times T$; $R^2 = 0.44$). Data for individual species are given in Table 1; the line for the neutral point of water is shown for reference (ionic strength, $I = 0$; at physiological salt concentrations the line will have the same slope but a lower intercept). (B) Intracellular pH (pH_i) as a function of temperature in white, red and cardiac muscle of all species studied. The regression equation are $\text{pH}_i = 7.313 - 0.01 \times T$, $R^2 = 0.60$ (WM), $\text{pH}_i = 7.270 - 0.01 \times T$, $R^2 = 0.64$ (RM) and $\text{pH}_i = 7.137 - 0.004 \times T$, $R^2 = 0.15$ (CM). Data for individual species are given in Table 2; the line for the neutral point of water is shown for reference.

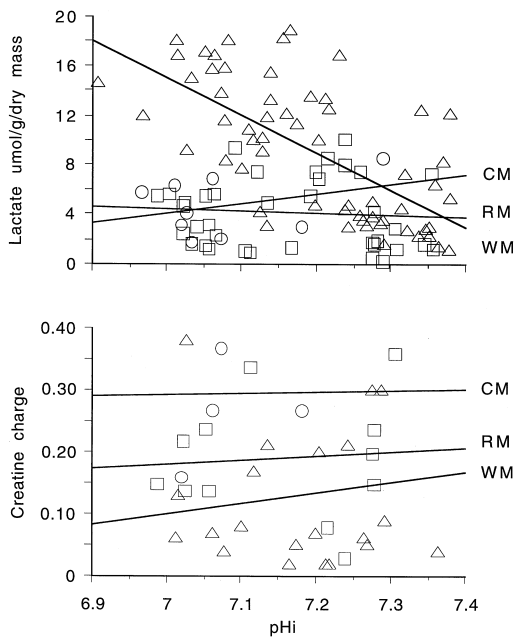


Fig. 3. Metabolite concentration for white (triangles), red (squares) and cardiac (circles) muscle. Top panel: intracellular lactate ($\mu\text{mol g dry wt}^{-1}$) as a function of intracellular pH (pH_i). The regression equations are $226-30.14 \times \text{pH}_i$, $R^2=0.45$ (WM), $15.2-1.55 \times \text{pH}_i$, $R^2=0.004$ (RM) and $-50.5+7.81 \times \text{pH}_i$, $R^2=0.11$ (CM). Bottom panel: as above, for creatine charge. Note the high variability in data.

The pH_i values of all fish groups examined are shown in Fig. 2, together with pN . The difference between sculpin and plaice at the same temperature emphasises that use of interspecific comparisons is unsafe. The $\delta\text{pH}_i/\delta T$ for white muscle between 5 and 11°C was $-0.035 \text{ U } ^\circ\text{C}^{-1}$ based on 5°C-acclimatised plaice, or $-0.022 \text{ U } ^\circ\text{C}^{-1}$ with 5°C-acclimatised sculpins, whereas the overall $\delta\text{pH}_i/\delta T$ over the temperature range 1–26°C was $-0.011 \pm 0.001 \text{ U } ^\circ\text{C}^{-1}$ ($P < 0.001$). Red muscle pH_i followed a similar trend to that of white muscle, and again values at or below 5°C were significantly greater than those above 5°C ($P < 0.05$). However, pairwise comparisons between species presumably reflect differences in habitat and lifestyle, as well as the underlying influence of environmental temperature, e.g. 4°C-acclimatised rainbow trout had pH_i values significantly higher than sculpin acclimatised to 5°C (7.321 ± 0.014 versus 7.187 ± 0.025 , respectively; $P < 0.05$), while the $\delta\text{pH}_i/\delta T$ between 5 and 11°C was $-0.031 \text{ U } ^\circ\text{C}^{-1}$ based on plaice at 5°C, or $-0.020 \text{ U } ^\circ\text{C}^{-1}$ for sculpin at the same temperature. The overall $\delta\text{pH}_i/\delta T$ over the temperature range examined was $-0.009 \pm 0.001 \text{ U } ^\circ\text{C}^{-1}$ ($P < 0.001$). The only effect temperature had on the pH_i of cardiac muscle was observed in 4°C-acclimatised rainbow trout, which was

Table 2

Intracellular pH and metabolic status of fishes from a range of thermal habitats. Mean \pm SEM (n)^a

| | <i>Notothenia</i> 1°C | Sculpin 5°C | Plaice 5°C | Trout 4°C | Trout 11°C | Trout 18°C | Tilapia 26°C |
|-----------------------|-----------------------|-------------------|-------------------|--------------------|--------------------|--------------------|--------------------|
| White muscle | | | | | | | |
| pH _i | 7.327 ± 0.015 (8) | 7.233 ± 0.020 (7) | 7.309 ± 0.017 (7) | 7.342 ± 0.015 (6) | 7.134 ± 0.026 (8) | 7.182 ± 0.017 (4) | 7.074 ± 0.023 (8) |
| [lac ⁻] | 2.741 ± 0.440 (8) | 4.859 ± 1.224 (7) | 3.920 ± 0.515 (7) | 12.193 ± 3.094 (6) | 14.220 ± 1.117 (8) | 13.829 ± 1.072 (8) | 12.048 ± 1.135 (8) |
| creatine charge | 0.133 ± 0.084 (3) | 0.105 ± 0.036 (4) | 0.355 ± 0.055 (2) | N.D. | 0.068 ± 0.023 (8) | 0.085 ± 0.37 (6) | 0.158 ± 0.05 (6) |
| Red muscle | | | | | | | |
| pH _i | 7.246 ± 0.020 (4) | 7.187 ± 0.025 (7) | 7.261 ± 0.023 (4) | 7.321 ± 0.014 (6) | 7.076 ± 0.012 (8) | 7.098 ± 0.027 (4) | 7.024 ± 0.008 (8) |
| [lac ⁻] | 5.395 ± 1.788 (4) | 6.226 ± 1.239 (7) | 4.963 ± 1.491 (4) | 2.092 ± 1.086 (6) | 2.942 ± 0.739 (8) | 2.300 (1) | 3.761 ± 0.511 (8) |
| creatine charge | 0.195 ± 0.045 (2) | 0.103 ± 0.050 (3) | 0.360 (1) | N.D. | 0.340 (1) | N.D. | 0.200 ± 0.028 (6) |
| Cardiac muscle | | | | | | | |
| pH _i | 7.120 ± 0.061 (2) | N.D. | N.D. | 7.299 ± 0.010 (2) | 7.024 ± 0.018 (5) | 7.090 ± 0.049 (4) | 7.022 ± 0.003 (2) |
| [lac ⁻] | 5.015 ± 1.985 (2) | N.D. | N.D. | 5.175 ± 3.475 (2) | 6.866 ± 2.986 (5) | 3.950 ± 4.885 (2) | 3.715 ± 0.485 (2) |
| creatine charge | 0.270 ± 0.001 (2) | N.D. | N.D. | N.D. | 0.390 ± 0.020 (2) | 0.370 (1) | 0.160 (1) |

^a pH_i , intracellular pH; [lac⁻], tissue lactate concentration ($\text{mmol.g dry mass}^{-1}$); creatine charge = $[\text{PCr} + \text{Cr}]$; N.D., not determined.

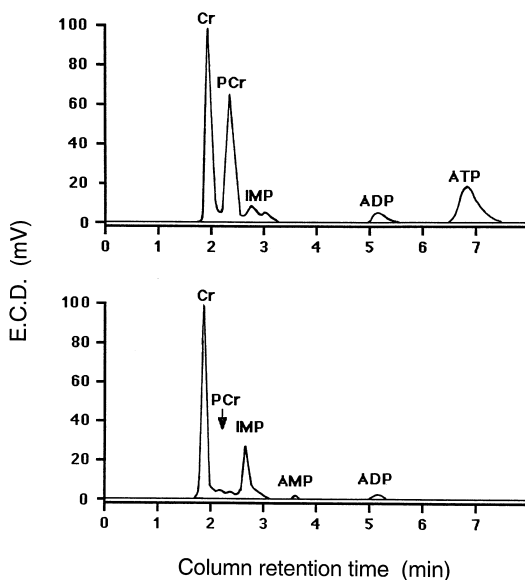


Fig. 4. HPLC chromatograms illustrating the variability in glycolytic muscle composition between a sedentary marine and active freshwater species. (A) plaice with high phosphocreatine and ATP levels, (B) trout with low PCr and ATP levels.

significantly higher than both the 26°C acclimatised tilapia and the 11°C acclimatised rainbow trout ($pH_i = 7.299, 7.022$ and 7.025 , respectively; $P < 0.05$). The overall $\delta pH_i / \delta T$ was $-0.004 \pm 0.003 \text{ U } ^\circ\text{C}^{-1}$ (not significant; n.s.).

When pH_i was expressed as $[H^+]$ (data not shown) the effects of temperature produced a similar $\delta[H^+] / \delta T$ for both red and white muscle of 0.158 and $0.166 \times 10^{-8} \text{ Eq l}^{-1}$, respectively. For cardiac muscle the slope was $0.077 \times 10^{-8} \text{ Eq l}^{-1}$, emphasising that intracellular acid–base regulation of cardiac muscle was relatively independent of temperature. There were no significant trends between freshwater and marine fishes, as pH values were not different, indicating that active regulation was common to species from a wide variety of habitats and lifestyles.

3.2. Muscle metabolites

Intracellular concentrations of lactate, $[\text{lac}^-]$, were generally higher in the white muscle (range $2.7\text{--}14.2 \mu\text{mol lac}^- \text{ g}^{-1}$ dry muscle mass) than other muscles within a given species. Only in white muscle was there any consistent relationship with intracellular pH : with a $\delta[\text{lac}^-] / \delta pH_i$ of -30.14 ± 4.45 , $P < 0.001$ (Fig. 3). Values of $[\text{lac}^-]$ were low in red muscle (range $2.1\text{--}6.2 \mu\text{mol lac}^- \text{ g}^{-1}$ dry muscle mass) and cardiac muscle (range $3.7\text{--}6.9 \mu\text{mol lac}^- \text{ g}^{-1}$ dry muscle mass) (Table 2) with slopes of -1.55 ± 4.09 and 7.81 ± 8.47 ,

respectively (n.s.; Fig. 3). Qualitatively similar relationships were evident when $\delta[\text{lac}^-] / \delta H^+$ values were calculated (data not shown). Interestingly, white muscle was also the only tissue where $[\text{lac}^-]$ varied with environmental temperature, having a $\delta[\text{lac}^-] / \delta T$ of 0.31 ± 0.06 ($P < 0.01$) whereas red muscle (-0.04 ± 0.05) and cardiac muscle (-0.09 ± 0.09) had slopes that were not significantly different from zero.

Technical difficulties may affect acid–base status, as described. Creatine charge $[\text{PCr} / (\text{PCr} + \text{Cr})]$ was low, especially in the white muscle compared with red and cardiac muscle (Fig. 3). Due to the great variability between samples the slopes for creatine charge against pH_i or temperature were not statistically significant, with the regression coefficient having values of $R^2 < 0.1$ in all cases. HPLC chromatographs (Fig. 4) of white muscle provided an explanation for the variability in values: either high levels of PCr and ATP were present or, where PCr and ATP were low, there was a high level of IMP. Low creatine charge was associated with ATP depletion and an increase in IMP concentrations consistent with the activation of adenylate kinase and AMP deaminase during the sampling procedure.

4. Discussion

4.1. Extracellular pH

In ectotherms, it is generally expected that in the short term blood pH varies inversely with temperature. In the present study pH_e (obtained from cannulated fish) deviates from that predicted for constant relative alkalinity and the alphastat hypothesis. There was a slightly steeper slope than for values from uncannulated fish, showing that the method of sampling can significantly affect the apparent thermal sensitivity of acid–base regulation. Randall and Cameron (1973), in an open (constant pCO_2) system, gave a predicted pH_e of 7.517 at 2°C . In contrast, the regression of present data gave predicted values of 7.785 (uncannulated fish) or 7.948 (cannulated fish) at 2°C , derived by extrapolation, whereas if pH_e changed in parallel with pN a value close to 8.1 would be predicted. However, the concept of relative alkalinity was developed to explain intra-specific responses to temperature change, and the inter-specific comparison would only be expected to follow a similar pattern if all species behaved in the same manner. Of more interest are the data for trout, showing a relative insensitivity to acclimatisation temperature at the lower range (Table 1).

In aquatic animals such as fish, a shift in pH due to changes in environmental temperature may result from three processes. First, the maintenance of pH_e at lower than predicted values in the cold may in part be due to

the complex buffering system within fish (Perry, 1986). The passive acid–base regulation by intracellular buffers appears to play only a minor role in regulating pH (van Dijk et al., 1997). Second, ventilation rates may be reduced, resulting in accumulation of carbon dioxide in the blood at lower temperatures and the elevated $p\text{CO}_2$ leading to respiratory acidosis. Most species acclimatised to temperatures below 11°C in the present study are bottom dwelling fish, with ventilatory systems designed to avoid the intake of sand, faeces and exhaust respiratory water. It has been reported that in the long-horned sculpin, *Myoxocephalus octodecemspinosus*, ventilation rate decreased at temperatures below 10°C (Roberts, 1973), which may lead to accumulation of CO_2 and the lower pH_e observed. Rainbow trout, however, is an active species and such a ventilatory response would be detrimental to the supply of O_2 to the tissues, and there was no significant difference recorded in $p_a\text{CO}_2$ for 4°C trout when compared with those acclimatised to 11 or 18°C (Taylor, 1995). Rather, a constant $p_a\text{CO}_2$ suggests either that rates of CO_2 excretion match production, or that active regulation of pH_e at the lower temperatures may be involved. Finally, active ion transport may be used for uptake or removal of acid–base equivalents. Cameron (1989) concluded that rainbow trout regulated pH_e by adjusting plasma $[\text{HCO}_3^-]$ through ionic exchanges (Cl^- for HCO_3^- , and Na^+ for H^+ or NH_4^+) in the gills and kidneys, while van Dijk et al. (1997) concluded that such active regulation of pH accounted for 2/3 of the change in pH with temperature found in eelpout.

4.2. Intracellular pH

For all tissues examined the intracellular pH was substantially lower than that of arterial blood pH. This is a general phenomenon, with pH_i typically around 0.3 pH units below pH_e for human (Sahlin et al., 1978), turtle and bullfrog (Malan et al., 1976), channel catfish (Cameron and Kormanik, 1982), tilapia (Johnston et al., 1983) and rainbow trout (Milligan and Wood, 1986). This may be accomplished by active maintenance of low intracellular bicarbonate concentrations (Cameron, 1989) with the more alkaline tissues associated with higher HCO_3^- levels, and maintained by $\text{Na}^+/\text{H}^+/\text{Cl}^-/\text{HCO}_3^-$ exchange (Heisler et al., 1976; Roos and Boron, 1981; Thomas, 1984).

In the present study the $\delta\text{pH}_i/\delta T$ of each tissue and $\delta\text{pH}_e/\delta T$ relationship between 1 and 26°C suggests an uncoupling in regulation of both intra- and extracellular acid–base states from the change in pN with temperature. In contrast, the change in pH_i for red and white muscle of round-bodied fish was significantly increased between 11 and 4°C, giving a $\delta\text{pH}_i/\delta T$ of $-0.02 \text{ U } ^\circ\text{C}^{-1}$ for both tissues, which is consistent with the alaphstat hypothesis. Such maintenance of constant

relative alkalinity may indicate sustained functional integrity over a wide range of temperatures (cf. abdominal muscle of crayfish; Whitely and Taylor, 1993). However, interspecific comparisons should only be made with caution, e.g. a more pronounced $\delta\text{pH}_i/\delta T$ was observed if 5°C-acclimatised plaice pH_i values were included: $-0.035 \text{ U } ^\circ\text{C}^{-1}$ (white muscle) and $-0.031 \text{ U } ^\circ\text{C}^{-1}$ (red muscle), which are greater than the change in pN over this temperature range ($\delta pN/\delta T = -0.017$), and may relate to the difference in body form and lifestyle of the plaice, as well as difficulties with obtaining unstressed samples.

The overall $\delta\text{pH}_i/\delta T$ for white muscle across a 25°C range of environmental temperatures was similar to that of red muscle, being approximately half that of $\delta pN/\delta T$. The red and white muscle of the American eel also had similar values between 5 and 20°C ($-0.008 \text{ U } ^\circ\text{C}^{-1}$; Walsh and Moon, 1982), and in the cardiac muscle of dogfish between 10 and 23°C ($-0.0098 \text{ U } ^\circ\text{C}^{-1}$; Heisler et al., 1976). In a recent review, Ultsch and Jackson (1996) demonstrated a $\delta\text{pH}_i/\delta T$ relationship of $-0.006 \text{ U } ^\circ\text{C}^{-1}$ in muscles of freshwater fishes. The $\delta\text{pH}_i/\delta T$ of cardiac muscle was significantly lower than that of skeletal muscle in the present study, and pH_i remained relatively constant with changing temperature. Unlike skeletal muscle, cardiac myocytes readily oxidise lactate and hence reduce the influence of this important metabolite on acid–base status. However, the relative contribution of fatty acid oxidation to the cellular energy budget is inversely related to environmental temperature in fishes (Sidell et al., 1995), with a reduced turnover potentially contributing to a depression of pH_i in the cold. The same range of values have also been reported in the cardiac muscle and hepatopancreas of the freshwater crayfish between 1 and 12°C (-0.0087 and $-0.006 \text{ U } ^\circ\text{C}^{-1}$, respectively; Whitely et al., 1995). These authors suggest that the maintenance of a set pH_i value may serve to down regulate intermediary metabolism during winter, to preserve glycogen stores when food supplies are low. Walsh and Moon (1982) commented that the eels in their study had reduced spontaneous activity levels at low temperatures, due to pH_i being sub-optimal for enzyme function, thereby reducing metabolism. Butler and Day (1993) concluded that such an argument would not hold for brown trout, as the resting metabolic rate and swimming ability were only slightly reduced in fish acclimated to cold temperatures, compared with warm acclimated fish (Butler et al., 1992). However, Taylor et al. (1996) showed that in seasonally acclimatised rainbow trout swimming performance was optimised in the middle of the temperature range (11°C), and that the relative acidosis in winter animals was indeed associated with impaired performance. In contrast, the limiting factor at 18°C may be oxygen availability in water (Brett, 1964), which resulted in a

significantly reduced arterial O₂ content (Taylor et al., 1996).

4.3. Muscle metabolites

Muscle metabolites (creatine charge and lactate concentration) were used to assess whether changes in intermediary metabolism may explain interspecific difference in p*H*_i. The initial hydrolysis of PCr (an alkaline reaction, as it consumes a proton) is stoichiometrically linked with the breakdown of ATP, and therefore acts as a buffer against rapid ATP depletion. However, in some samples of white muscle the chromatographs showed a near absence of PCr and ATP as well as high levels of IMP, indicating that anaerobic metabolism had occurred during sampling (Schulte et al., 1992). The buffering capabilities of IMP may negate the corresponding acidification due to anaerobic metabolism, which seems to be the case for the 4°C trout where homogenate p*H*_i values were comparable to weak acid distribution (dimethyl sulphoxide; D.M.O.)-derived p*H*_i values from the same tissue (data not shown). The hydrolysis of PCr during sampling was not limited to white muscle, however, as the creatine charge of red and cardiac muscle were low (ranges = 0.2–0.4), whereas values of approximately 0.5 are regarded as representative for white muscle in resting fish (Schulte et al., 1992).

Concentration of lactate in white muscle in the present study (range = 2–14 μmol lac⁻ g⁻¹ dry muscle mass), are similar to those reported in the literature for a variety of species: 3–15 μmol wet mass kg⁻¹ (Johnston, 1975; Turner et al., 1983; Dobson and Hochachka, 1987; Boutilier et al., 1988; Nelson, 1990; Schulte et al., 1992; Arthur et al., 1992). The high lactate levels are typical of fish acclimatised to warm temperatures, due to increased metabolic rate. However, the levels of [lac⁻] may not simply be due to a temperature-dependent mechanism, as rainbow trout acclimatised at 4°C have a similar high level of lactate as the warmer-acclimatised trout. Unlike p*H*_i, there seems to be a difference between habitats in [lac⁻], with the lowest values found in marine species. However, Tang and Boutilier (1991) found a small difference in the accumulated intracellular lactate during anaerobic stress between freshwater and seawater acclimatised fish, which certainly does not accord with the difference recorded in the present study. A more plausible explanation is that the marine species studied are primarily benthic, and are relatively inactive compared with the freshwater species. This may also explain the elevated [lac⁻] of 4°C-acclimatised rainbow trout. If aerobic metabolism is reduced at 4°C, due to the constant p*H*_i inhibiting oxidative activity enzyme, then anaerobic metabolism may be relatively enhanced. That the p*H*_i of 4°C-acclimatised

rainbow trout was not affected by the increased [lac⁻] may be due to the buffering effects of IMP formed in parallel with PCr hydrolysis. However, the buffering was incomplete as p*H*_i still deviated from the alphastat hypothesis.

4.4. Methodology

Previous reports comparing methods for estimating intracellular p*H* have shown that, for muscles with a high capacity for anaerobic function, estimated p*H*_i values agree between the homogenising and D.M.O. methods over a temperature range of 8–37°C (Pörtner, 1989; Pörtner et al., 1990; Schulte et al., 1992). Results from a recent study of white muscle of trout, within the temperature range 11–18°C, agree with these observations (Taylor, 1995). However, there are no published data concerning this relationship at lower temperatures, nor with respect to thermal acclimatisation or adaptation.

One potential problem with the present method was the time that elapsed before the muscle was frozen. If phosphocreatine (PCr) was hydrolysed in the white muscle to a variable extent within the time taken to excise the tissue, it may explain the relatively alkaline p*H*_i value with the homogenising technique compared with weak acid distribution (see above). Costhill et al. (1982) recommended that to avoid problems due to changing metabolite concentration, human muscle samples for determination of p*H*_i by the homogenising technique must be snap frozen within 30 s of biopsy. Spriet et al. (1986), also working on human muscle, reported that PCr was rapidly hydrolysed to 35% of control values after 30 s, and 23% after 2 min at 37°C. At low acclimatisation temperatures trout and other species show a rate compensation in intermediary metabolism (Hazel and Prosser, 1974), although the rate of change in metabolite concentration is still likely to be much slower than for human muscle.

The homogenising technique has certain advantages over the D.M.O. method as it can detect rapid p*H* changes (e.g. during exercise), and fish do not require the cannulation of a major blood vessel for the injection of isotopic markers. However, the differences between whole blood p*H* of cannulated and uncannulated fish clearly show that sampling via a cannula is advantageous in establishing a true *in vivo* reference, and the values obtained by cardiac/caudal artery puncture reflect acid-base disturbance during the sampling time that is probably metabolic in origin. The subsequent p*H*_i values obtained will, therefore, not reflect normal *in vivo* values, and may only be used with caution in comparative studies.

5. Conclusions

From these data we conclude that intracellular pH is regulated independently of temperature, and that the low $\delta p\text{H}_i/\delta T$ values observed in muscle tissue over a range of acclimatisation temperatures may serve to reduce metabolism when resources are low, e.g. during winter when the tissue will be relatively acidotic. HPLC data indicates that activation of AMP deaminase and creatine kinase affects H^+ balance during the sampling procedure of the homogenising technique. The absolute values of $p\text{H}_i$ reported with this technique must be interpreted with caution—the expected inverse relationship between $p\text{H}_i$ and environmental temperature was observed for white muscle, but is less evident in muscles with high mitochondrial densities. The large H^+ gradient between mitochondria and the cytosol, which will be variable among tissues and species, would introduce greater variability in data obtained with oxidative tissue. Interestingly, the $p\text{H}_i$ values for glycolytic muscle of Antarctic fish were very similar using the homogenising technique (*N. coriiceps*, 7.33 ± 0.02 , this study) and the non-invasive ^{31}P -NMR (*Harpagifer antarcticus*, 7.36 ± 0.07 ; Moerland and Egginton, 1998), which are close to the value expected for $p\text{N}$ at physiological salt concentrations (ca. 7.34). Indeed, analysis of published data for glycolytic muscle $p\text{H}_i$, from various taxa and methods of determination, gave a $\delta p\text{H}_i/\delta T$ of $-0.14 \text{ U } ^\circ\text{C}^{-1}$ over the range 1–37°C which is not significantly different from that of $p\text{N}$. However, this masks an essentially biphasic relationship, with $p\text{H}_i$ varying markedly at high temperatures but very little at low temperatures, such that $p\text{H}_i \approx p\text{N}$ at around 0°C (Moerland and Egginton, 1998). These varied approaches, however, point to the same conclusion: $p\text{H}_i$ changes with environmental temperature do not parallel those of $p\text{N}$, and cannot be explained by the concept of alaphastat regulation.

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