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Differential recovery from exercise and hypoxia exposure measured using ^{31}P- and ^{1}H-NMR in white muscle of the common carp *Cyprinus carpio*

Troy M. Hallman, Anibal C. Rojas-Vargas, David R. Jones and Jeffrey G. Richards*

Department of Zoology, The University of British Columbia, 6270 University Boulevard, Vancouver, BC, Canada V6T 1Z4

*Author for correspondence (e-mail: jrichard@zoology.ubc.ca)

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SUMMARY

Phosphocreatine (PCr) was reduced to equivalent levels in carp white muscle by high-intensity exhaustive exercise and exposure to hypoxia at 15°C and 25°C in order to assess the influence of intracellular pH (pH_i), temperature and lactate levels on PCr recovery *in vivo*. High-intensity exercise resulted in a significantly lower pH_i compared with hypoxia exposure and the rate of PCr depletion and tissue acidification during hypoxia exposure was significantly higher in carp held at 25°C compared with those at 15°C. During recovery, PCr and pH_i returned towards normoxia/resting levels at a faster rate following hypoxia exposure than after exercise. The lower pH_i in exercised carp caused a greater perturbation to cellular energy status (assessed as the free energy of ATP hydrolysis; $\Delta fG'$) and resulted in a higher [ATP]/[ADP_{free}] ratio, which may limit mitochondrial ATP production and contribute to the slower recovery from exercise compared with recovery from hypoxia exposure. Rates of recovery from exercise and hypoxia exposure were not affected by acclimation temperature (15 and 25°C), suggesting that the processes involved in acclimation compensate for the Q_{10} effects of temperature on metabolic processes. Finally, using a dual ^{31}P-NMR and ^{1}H-NMR analysis technique, we demonstrated that the greater tissue acidification observed after high-intensity exercise compared with hypoxia exposure occurred at similar white muscle lactate concentrations.

Key words: fish, muscle energetics, phosphorylation, potential, recovery, temperature.

INTRODUCTION

ATP turnover in muscle during high-intensity exhaustive exercise and exposure to hypoxia is primarily supported by substrate-level phosphorylation [creatine phosphate (PCr) hydrolysis and glycolysis], which serves to compensate for the metabolic demands that exceed the capacity of mitochondrial oxidative phosphorylation (Hochachka and Somero, 2002). During hypoxia exposure, hypoxia-tolerant animals, such as the common carp, typically suppress ATP turnover because of an inhibition of mitochondrial function; they rely on substrate-level phosphorylation to maintain energy balance (Bickler and Buck, 2007). By contrast, ATP turnover in white muscle during exhaustive exercise can increase by up to 40-fold (Moyes and West, 1995; Richards et al., 2002a), well beyond the capacity of oxidative phosphorylation to supply ATP and therefore PCr hydrolysis and glycolysis are coordinately increased to support a power output that exceeds oxidative capacity. Although the reasons for the activation of substrate-level phosphorylation during exhaustive exercise or exposure to hypoxia differ, the end metabolic profiles are similar, with low muscle [glycogen] and [PCr], and high [lactate] and metabolic [H^+] (Richards et al., 2007; Wang et al., 1994). In spite of these similar metabolic profiles, no study has directly compared recovery metabolism following exhaustive exercise and exposure to hypoxia.

During recovery from exhaustive exercise and hypoxia, pathways must be activated to resynthesize PCr and glycogen. The recovery of these metabolites will be linked because of their dependence on mitochondrial ATP production and metabolic H^+ use. In particular, the rate of PCr and intracellular pH (pH_i) recovery following exercise or hypoxia exposure will be linked through the creatine kinase catalyzed reaction:

$$PCr^{2-} + MgADP^{-} + H^{+} \leftrightarrow MgATP^{2-} + Cr\ , \qquad (1)$$

where mitochondrial ATP serves as the phosphate donor for free creatine accumulated during the metabolic insult. Phosphocreatine hydrolysis will have an alkalinizing effect on the intracellular fluid, whereas PCr synthesis during recovery will have an acidifying effect. As a result, during recovery there is an almost complete recovery of PCr before pH_i and lactate begin to return to normoxic resting levels (Richards et al., 2002b; Schulte et al., 1992; van den Thillart et al., 1989; Wang et al., 1994). Creatine kinase is a near-equilibrium reversible enzyme (Lawson and Veech, 1979), thus the factors that determine whether PCr is hydrolyzed or synthesized are the local [ATP], [ADP_{free}] and [H^+]. In addition, changes in ATP, ADP_{free} and H^+ play a dominant role in regulating mitochondrial oxidative phosphorylation and glycogensis, and therefore these metabolites functionally link and coordinate metabolism during recovery.

Rates of metabolism and ATP production are strongly influenced by temperature in ectothermic animals, thus temperature acclimation may affect rates of metabolic recovery. Temperature acclimation has been shown to result in dramatic changes in muscle properties that allow many ectothermic fish to maintain activity over a wide range of environmental temperatures (Guderley, 2004). This is in part due to an inverse correlation between muscle mitochondrial volume density and acclimation temperature, which has been observed in carp [*Carassius carassius* (Johnson and Maitland, 1980)] and in other species (Johnson et al., 1998; Lucassen et al., 2006; Moerland, 1995). Non-mitochondrial enzymes also show temperature compensation with increasing protein content and decreasing binding capacity [e.g. red muscle PFK from the goldfish *Carassius auratus* (Huber and Guderley, 1993)] during cold acclimation. Pickeral (*Esox niger*) acclimated to 5°C had 45% greater creatine kinase activity than 25°C acclimated fish when assayed at a common temperature (Kleckner and Sidell, 1985).

Indeed, cellular plasticity during temperature acclimation works to minimize the Q_{10} effects of temperature and maintain metabolic capacity within narrow confines (Hochachka and Somero, 2002). As a result, recovery from high-intensity exhaustive exercise (Kieffer et al., 1994) or exposure to hypoxia (Borger et al., 1998) in fish acclimated to warm and cold temperatures occurs at roughly the same rate. However, to date, no study has examined the effects of temperature on substrate depletion during hypoxia exposure and directly compared the effects of temperature acclimation on the recovery from exhaustive exercise and hypoxia exposure.

The objectives of the present study were to use ^{31}P nuclear magnetic resonance (NMR) technology to examine the relationship between PCr and pH dynamics in white muscle following exhaustive exercise and exposure to hypoxia in carp acclimated to 15 or 25°C. The exhaustive exercise and hypoxia exposure protocols were chosen because they both elicited similar decreases in muscle PCr and therefore direct comparisons of recovery metabolism could be made. Furthermore, we developed a methodology to use ^{1}H-NMR to measure lactate and combined it with the almost simultaneous use of ^{31}P-NMR spectroscopy to better understand the relationship between changes in pH_i, PCr, ATP and lactate during exposure to hypoxia or during recovery from exercise. The use of NMR spectroscopy in fish allows the frequent measurement of metabolites in an almost non-invasive fashion. ^{31}P-NMR has been extensively used in fish during hypoxia exposure and recovery (Borger et al., 1998; van den Thillart et al., 1989; van Ginneken et al., 1995; Van Waarde et al., 1990), but ^{1}H-NMR has been used in only a few studies (Bock et al., 2002; Wasser et al., 1992a; Yoshizaki et al., 1981) and has not been extensively used in fish to examine metabolic recovery from exercise or hypoxia.

MATERIALS AND METHODS

Experimental animals

Common carp (*Cyprinus carpio*, L.) were caught in Lake Okanagan (British Columbia, Canada) and transported to flow through freshwater holding facilities at the University of British Columbia. Upon arrival, carp were treated for potential bacterial infections with chloramphenicol and acriflavin. Carp were maintained in normoxic water at seasonal temperatures (6–15°C) and fed to satiation once per week with Mazuri koi pellets (PMI Feeds, St Louis, MO). The animals ranged in size from 590 to 1270 g body mass and from 31.5 to 42.5 cm in length. All experimental procedures were performed in accordance with the University of British Columbia animal care committee guidelines and the British Columbia Ministry of Environment.

Two separate series of experiments were conducted. The first series (Series 1) involved acclimating carp to 15 and 25°C and then monitoring PCr, ATP and pH_i at their respective acclimation temperature using ^{31}P-NMR during exposure to hypoxia and recovery from hypoxia and exercise. The second series (Series 2), involved developing methodology for quantifying lactate using ^{1}H-NMR and using this methodology, along with ^{31}P-NMR, in a dual head format, to almost simultaneously monitor PCr, ATP, pH_i and lactate dynamics in fish during exposure to hypoxia or recovery from exercise.

Series 1

Temperature acclimation

For temperature acclimation, healthy fish were kept indoors in pairs in 501 fiberglass tanks supplied with well-aerated, flow-through, dechlorinated Vancouver tap water at a rate of 1 l min^{-1}. All tanks were maintained on seasonal photoperiod at ~12°C. Temperature of the holding tanks were increased at a rate of 1°C per day until final acclimation temperatures (15°C or 25°C) were achieved. Water temperature was altered and maintained at above ambient temperature by passing water through a stainless steel coil warmed in a water bath before entry into the tank. Once the desired acclimation temperature was achieved, all but one fish were held at these temperatures for 13 days. Experiments were performed on a single fish after only 8 days acclimation to 15°C, but the results obtained were not statistically different from those observed in fish acclimated for the full 13 days, thus the data were included in the data analysis. Fish were fasted 2 days before experiments were started.

Protocols

To exercise carp to exhaustion, individuals were placed into a Brett-style swimming respirometer at their acclimation temperature. After 3 h of acclimation to the tunnel, carp were exercise for 60 min at ~0.5 body length s^{-1} to adjust to swimming against a current. The carp were then exercised to exhaustion using a similar protocol to that described by Dobson and Hochachka (Dobson and Hochachka, 1987). Briefly, the swim protocol began by increasing the water velocity to the speed at which the fish swam in a burst-and-glide pattern (precise speed not determined, but ≥3 bL s^{-1}). As each fish fatigued and was carried to the back of the swim tunnel, water velocity was decreased until the fish regained position in the tunnel when velocity was increased until a burst-and-glide pattern was achieved. Exhaustion was achieved when the fish could no longer regain position in the tunnel set at 1 body length s^{-1}. The average time to exhaustion was 15 minutes. When the exercise protocol was completed, a fish was transported to the NMR facility, secured inside a clear watertight plastic box and placed on its side over the ^{31}P-NMR coil. The watertight box was constructed of 3 mm plastic, which measured 50 cm×12 cm×10 cm (l, w, h) and was fitted with a 6 mm plastic lid that was sealed by a 5 mm neoprene gasket and six plastic screws. Each fish was centered in the box by foam sponges and was slightly restrained by a thin plastic plate held in place by a balloon. This configuration allowed some freedom of movement, but not enough to obscure the NMR spectra. During the NMR trials, the box containing a fish was supplied with well-aerated dechlorinated tap water at the fish's acclimation temperature at a rate of 0.5 l min^{-1} from a common recirculated reservoir. Water temperature was maintained in the reservoir by immersion of a stainless steel coil connected to a Lauda heating/chilling unit and temperature was monitored with a standard thermometer. Aeration was achieved by bubbling air and water through a series of three 11.4 l gas exchange cylinders, which was fed to the box by an Eheim 1048 water pump. Installing the fish in the box and moving it from the respirometer to the NMR facility took ~10 min. Once in the NMR magnet, PCr, intracellular phosphate (P_i) and ATP levels were monitored throughout the recovery period until the integral of the PCr peak leveled off (remaining constant for at least 30 min) and just before P_i could no longer be confidently differentiated from background noise (see NMR protocols below). Fish that struggled during the NMR measurements were eliminated from the data sets.

After a period of at least 1 week at their acclimation temperature, the same fish used for the exercise study were used for the hypoxia study. Individual fish were placed in the clear plastic box and allowed to habituate for at least 8 h under normoxic flow-through conditions. One hour before each experiment, the box containing a carp was gently placed in the NMR over the coil and left to recover for at least 1 h. At time zero, the fish were exposed to severe hypoxia (P_{O_2}=20 Torr; 1 Torr≈133 Pa) by bubbling N_2 through the system

generally used for aeration. Hypoxia exposure to 20 Torr was chosen based on preliminary experiments that showed that this level of hypoxia resulted in a significant drop in muscle PCr within 2 h of exposure, but did not elicit any struggling (data not shown). Samples of water were collected in a gas tight syringe *via* a three-way valve in the line leading to the box and analyzed for water P_{O_2} using Clarke-type electrode attached to the PHM71 acid-base analyzer (Radiometer). Exposure to hypoxia was maintained until PCr depletion approximated that seen after exercise, at which point the water was returned to normoxic levels ($P_{O_2} \geq 150$ Torr) and the fish allowed to recover. NMR traces were gathered during the initial normoxic period, during hypoxia, and throughout recovery until the integral of the PCr peak leveled off and just before P_i could no longer be confidently differentiated from background noise.

The orientation of the fish during recovery was initially a concern because it was necessary for the fish to be placed on their side (dorsoventral axis being horizontal) owing to their size and the availability of appropriately arranged probes. Special care was taken to ensure that there was ~5 mm of clearance between the fish and the box to avoid compression and possible muscle ischemia. In our hands, the time taken for laterally oriented carp to rebuild PCr following hypoxia exposure are comparable with that observed by van den Thillart et al. (van den Thillart et al., 1989) and van Ginneken et al. (van Ginneken et al., 1995) where the probe design allowed the fish to recover in a vertical position.

^{31}P NMR

Phosphate metabolites were measured *in vivo* by ^{31}P-NMR spectroscopy using a 1.89 T, large bore horizontal superconducting magnet (Oxford Instruments, Oxford, UK) connected to a Nicotel 1280 spectrophotometer. The coil was placed along the midline of the body above the anal fin. The signal was detected by a 2.2 cm diameter double looped coil of 1.0 mm thick silver wire protected by polyethylene tubing and tuned for ^{31}P (32.5 MHz). Spectra (1024 data points) consisted of 256 individual scans accumulated over 5.07 min at a nominal 90° (width of 42 μs) and a delay between pulses of 1 s in a spectral window of ±1500 Hz. These parameters were adjusted on fish before the start of the experiments to achieve an adequate signal-to-noise ratio and resolution of metabolite peaks while minimizing the time necessary for a single spectrum. Before data analysis, concurrent raw signals were summed to improve the signal-to-noise ratio, allowing a more accurate analysis to be made. The baseline-corrected paired signals were smoothed by a Gaussian multiplication factor of 20, zero-filled to 4096 points, Fourier transformed and phase shifted before deconvoluting the PCr, P_i and β-ATP peaks to obtain the metabolite areas. Experiments were terminated when the amplitude of the PCr peak appeared constant and when the P_i peak could no longer be resolved. For data analysis, complete recovery was assumed when PCr was at least 95% of the mean resting value of each group of fish.

Intracellular pH was estimated by the chemical shift (δ) of the P_i peak relative to the PCr peak, which served as the internal standard and was set to zero. The pH_i measurements were calibrated using the following equations. At 15°C:

$$pH = 0.372\delta^3 - 4.890\delta^2 + 22.160\delta - 27.798 ; \quad (2)$$

at 25°C:

$$pH = 0.353\delta^3 - 4.712^2 + 21.673\delta - 27.549 . \quad (3)$$

The above equations were derived from data obtained from a carp muscle homogenate using protocols set out by van den Thillart and colleagues (van den Thillart et al., 1989). Briefly, carp white muscle was homogenized in a solution containing 300 mmol l^{-1} sucrose, 20 mmol l^{-1} EDTA, 10 mmol l^{-1} NaF and 50 mmol l^{-1} PCr. The homogenate solution was made fresh daily. pH was altered by titrating 20 to 60 μl of 1 mol l^{-1} HCl or 1 mol l^{-1} NaOH to the homogenate and was measured using a Corning Chek-Mite pH 30 pH meter fitted with a Fisher Calomel Microprobe combination electrode (Fisher Scientific). The pH meter was calibrated at either 15°C or 25°C with pH 4 or 7 buffer (VWR Scientific) and checked with pH 6 and 8 buffers to ensure proper calibration before measuring the homogenate pH using NMR. The range of pH measured was restricted to 6–8, representing the general physiological range of skeletal muscle. At lower pH values (<6.4), the homogenate PCr was depleted rapidly, and therefore the homogenate was supplemented with 15 mg aliquots of PCr periodically to maintain a visible PCr peak in the NMR spectra.

Following a pH measurement using the pH meter, a small homogenate sample was placed into a water jacketted cylinder (3 mm plastic, 2.9 cm inner diameter, 7.4 cm outer diameter, 5.3 cm high) on the NMR coil and a spectrum was acquired. Sample temperature was maintained at either 15 or 25°C using water circulated from a constant temperature bath through the outer jacket of the cylinder. Each spectrum (1024 data points) consisted of 128 individual scans at a 90° pulse with a 1 s delay in a spectral window of ±500 Hz. These parameters yielded a total time of 3.32 min per spectrum. Following each spectrum, a second pH measurement was taken using the pH meter. The baseline corrected raw signals were smoothed by a Gaussian multiplication factor of 20, zero-filled to 8192 points, Fourier-transformed and phase shifted. PCr was set to zero and the chemical shift of P_i was recorded. Owing to the subjectivity of manual phase shifting, each spectrum was analyzed three times in random order, thus producing a mean that was plotted against the mean of the two pH measurements. In some cases, owing to the lack of a discernable P_i peak, pH_i could not be determined in normoxic, resting muscle or fully recovered muscle.

Series 2

^{1}H- and ^{31}P-NMR

In order to obtain both ^{31}P and ^{1}H spectra from the same animal in close temporal and spatial proximity, we constructed a probe head with two independent radio frequency circuits for ^{31}P- and ^{1}H-NMR. The probe head was equipped with two surface coils of the same geometry (two turns, 2 cm diameter), separated by ~1 cm. The change from one nucleus to the other required the connection of the appropriate *rf* circuit and the corresponding *rf* filter, which usually took less than 1 min. A similar set up to that described above was used to house carp during recordings. Using large fish allowed us to assume that both coils were receiving signals from similar tissues.

The magnets were shimmed using the ^{1}H coil on the water signal of the carp muscle and because of the close proximity of the two coils; good quality spectra were obtained from both nuclei in most cases. The ^{31}P-NMR spectra were obtained using a simple 1-PULS sequence using the procedures described above. To acquire ^{1}H-NMR spectra and isolate the lactate signal, we used a modified binomial pulse sequence with null excitation at the center of the water signal and a maximum excitation around the lactate doublet. During optimization of the ^{1}H-NMR spectra, several phantoms were used, consisting of an inner culture tube containing 2.5 ml of a 1 mmol l^{-1} solution of potassium lactate, creatine chloride and sodium acetate, surrounded by an outer culture tube containing 2.5 ml of pure corn oil. Owing to the dimensions of the two tubes, a 1 mm layer of corn oil always surrounded the phantom solution.

From the phantoms, uncorrected ^{1}H-NMR spectra were dominated by signals from water and lipid, which masked the signals from the metabolites of interest (e.g. lactate). To suppress or eliminate these interfering signals, we used a combination of the following spin-echo sequences:

90(1331)-t-180-t-acquire

90(1331)t-180(2662)-t-acquire

using appropriate t values to yield spectra that differed in phase from all the other signals, except the lactate doublet. To limit the rebuilding of the water signal during the application of these two sequences, the experiment was designed to apply one sequence after the other. Following this step, a simple water suppression sequence was applied, delaying the acquisition of the FID, and including in its processing a sine function apodization or trapezoidal multiplication before the Fourier transformation, to minimize the intensity of signals of the faster relaxing lipids, while maintaining the signals from the longer relaxing metabolites. Furthermore, this allowed the optimization of the setting of the *rf* receiver. The inconvenience of dephasing the different signals was corrected by the application of a magnitude calculation during the processing of the FID. Although this procedure increased the broadening of the signals, decreasing the quality of the spectrum, it proved to be the most useful for the application to the fish studies because of its simplicity and sensitivity. We also performed the same optimization procedure on hypoxia-exposed carp to determine whether there were any interfering signals in the carp muscle. We found that shape and position of all resonance peaks were similar between the phantom and the carp tissue, strongly supporting the notion that there were no interfering peaks in the carp tissue.

Once the technique for the near-simultaneous recording of ^{1}H- and ^{31}P-NMR spectra was optimized, two sets of experiments were conducted to examine the utility of ^{1}H-NMR in fish tissues: recovery from exercise and exposure to hypoxia. The animal set up and handling for high-intensity exercise or exposure to hypoxia was similar to those given above. In some cases, hypoxia exposures were conducted on the same fish that experienced exercise, but carp were allowed to fully recover for at least 18 h following exercise before hypoxia exposure was initiated. All experiments using the dual ^{31}P- and ^{1}H-NMR head were conducted on fish acclimated to a common temperature of 20°C.

Calculations and statistical analysis

Cellular PCr is expressed as PCr/(PCr + P_i). Free cytosolic [ADP] was calculated according to published protocols (Golding et al., 1995; Teague et al., 1996) using the following equation:

$$\left[\mathrm{ADP_{free}}\right] = \frac{\left[\mathrm{ATP}\right]\left[\mathrm{Cr}\right]}{\left[\mathrm{PCr}\right]K'_{\mathrm{CK}}}. \qquad (4)$$

The equilibrium constant for creatine kinase (K'_{CK}) was corrected for experimental temperature, pH and free Mg^{2+} (assumed to be 1 mmol l^{-1}) (Golding et al., 1995; Teague et al., 1996). Cellular [PCr] and [ATP] were estimated from NMR spectra by the relative resonance intensities of PCr and β-ATP, starting from a normoxic [ATP] of 5.1 μmol g^{-1} wet mass (van Ginneken et al., 1995). Free [Cr] was estimated by subtracting [PCr] from published total creatine values [30 μmol g^{-1} wet mass (van Ginneken et al., 1995)]. Metabolite concentrations were then converted to molar concentrations (per liter of intracellular water) using a tissue water content of 0.70 ml g^{-1} wet mass (Richards, unpublished) (Wang et al., 1994). Cellular water content has been shown not to vary in response to exhaustive exercise in rainbow trout (Milligan and Wood, 1986; Wang et al., 1994); therefore, in the present study it was assumed that muscle water content would not change during recovery from exercise or hypoxia exposure in carp.

The Gibbs free energy of ATP hydrolysis ($\Delta_f G'$; kJ mol^{-1}) was determined using the following equation:

$$\Delta_f G' = \Delta_f G'^{\circ}_{\mathrm{ATP}} + RT\ln\frac{\left[\mathrm{ADP_{free}}\right]\left[\mathrm{P_i}\right]}{\left[\mathrm{ATP}\right]}, \qquad (5)$$

where R is the universal gas constant (8.3145 J K^{-1} mol^{-1}), T is temperature in K and $\Delta_f G'^{\circ}_{ATP}$ is the standard transformed Gibbs energy of ATP hydrolysis ($\Delta_f G'^{\circ}_{ATP} = -RT\ln K'_{ATP}$) at the measured pH and temperature and estimated free [Mg^{2+}]. Cytosolic free [P_i] was estimated using the observed changes in relative resonance intensity and assuming a resting level of 1 μmol g^{-1} wet mass and converted to molar concentrations.

Changes in PCr, ADP_{free} and $\Delta_f G'$ during recovery were fit to a mono-exponential function and used to calculate recovery rate constants (τ). Recovery rate constants for pH_i and [ATP]/[ADP_{free}] could not be determined due to the unusual shape of the curves; however, for pH_i, we removed the initial declining pH_i during the early part of recovery and fitted the remaining data with a standard linear regression to describe the effects of time on pH_i recovery. In all cases, regressions were chosen that yielded the highest correlation coefficient. It must be noted, however, that exercise recovery started 10 min before we were able to collect our first NMR recording, owing to the time required to transfer the fish from the respirometer to the NMR chamber. In an attempt to account for unknown variation in T=0 values (exhausted values) for PCr, ADP_{free} and $\Delta_f G'$ on τ, we performed sensitivity analysis. Briefly, for PCr, ADP_{free} and $\Delta_f G'$, we varied T=0 values from the exercise groups within a range of predicted values (0 to the values observed at T=10 min for PCr; 100 to the values observed at T=10 min for ADP_{free}; –45 kJ mol^{-1} to the values observed at T=10 min for $\Delta_f G'$) and recalculated τ. The resulting variation in τ was small (<20%) and did not affect the significance of our results or our data interpretation. Differences in time and rate constants were analyzed by two-way analysis of variance (ANOVA) with treatment (exercise or hypoxia) and temperature as categorical variables. Series 2 experiments were analyzed with one-way ANOVA. All data sets were tested for normality and homogeneity of variance and if the data sets failed either assumption, data were log transformed and analysis of variance repeated. Logarithmic transformation always yielded data of normal distribution and equal variance. Comparisons of one variable between two groups were made and analyzed using a two-tailed *t*-test. All values are reported as means ±standard error of the mean. Significance was accepted at $P<0.05$.

RESULTS

Series 1

Temperature acclimation

Acclimation to 15°C or 25°C had no effect on white muscle [PCr], [ATP] or pH_i in normoxic/resting carp.

Exercise and hypoxia exposure

No mortality was observed in response to our exercise protocol or hypoxia exposure. White muscle [PCr] was similarly depleted, by 60–70%, following both exhaustive exercise and exposure to

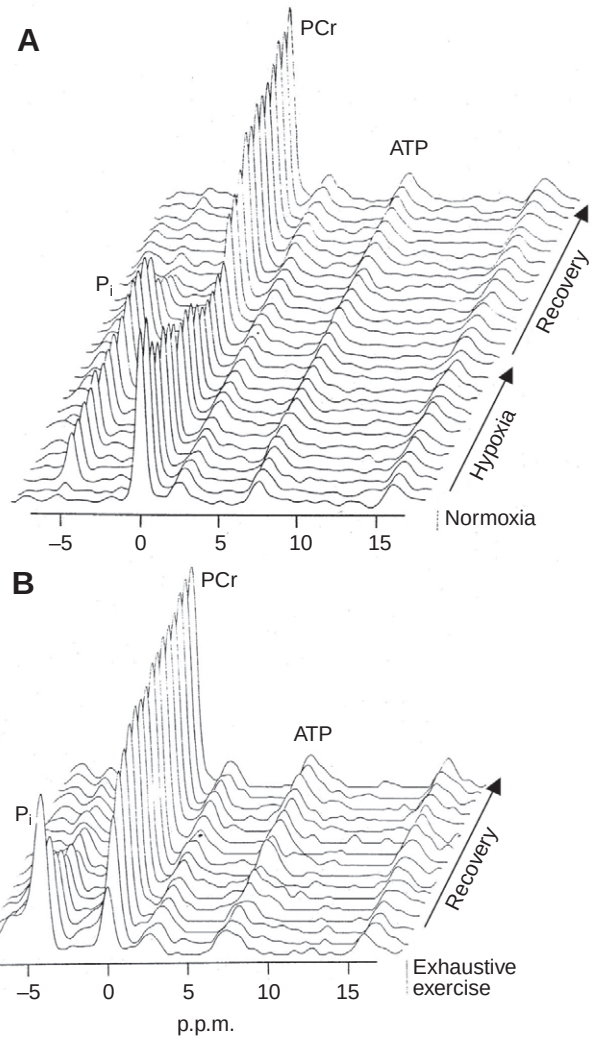

Fig. 1. Representative series of spectra obtained from white muscle of carp acclimated to 15°C and exposed to hypoxia followed by recovery (A) or during recovery from exhaustive exercise (B). The first spectra in panel A represent metabolite profiles in our control group (rest/normoxia) and the first spectra in panel B represents exhausted fish. PCr, phosphocreatine; P_i, intracellular phosphate.

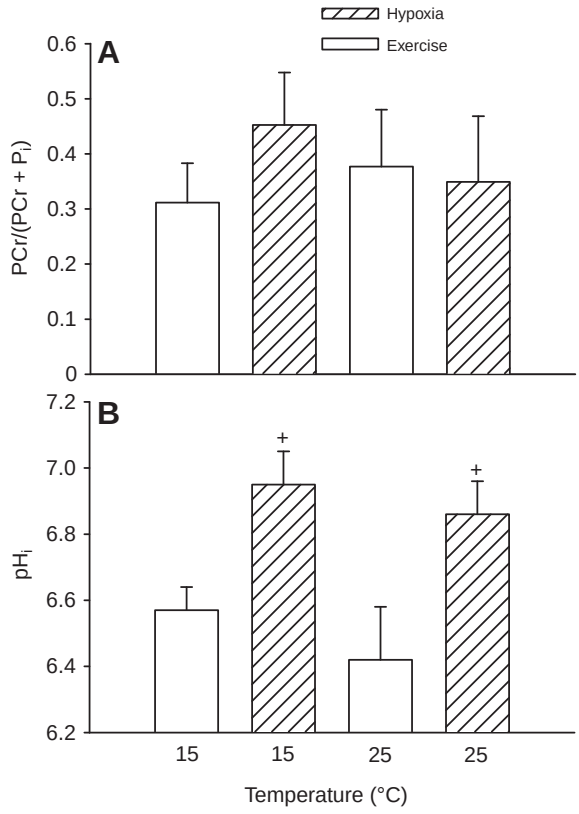

Fig. 2. White muscle PCr (A) and pH_i (B) following exercise (white bars) and exposure to hypoxia (striped bars) in carp acclimated to 15 or 25°C. Phosphocreatine levels are normalized to the sum of PCr and P_i, and expressed relative to normoxic/resting controls. NMR data were collected at the fishes' acclimation temperature. Each bar represents mean + s.e.m. Significant differences (P<0.05) between exercise and hypoxia at a given temperature are indicated by + (two-way ANOVA). From left to right in each panel, N=7, 7, 10 and 6.

hypoxia, and the extent of the depletion was not affected by acclimation temperature (Fig 1; Fig. 2A). Acclimation temperature did, however, affect the rate of PCr depletion during exposure to hypoxia with carp acclimated to 25°C depleting muscle PCr at a faster rate than carp acclimated to 15°C [rate constant (τ) at 15°C=0.64±0.36 *versus* 25°C=1.27±0.24; P=0.040, *t*-test] (Fig. 3A). Despite these changes in white muscle PCr, neither exercise nor exposure to hypoxia affected white muscle [ATP] in carp acclimated to 15°C or 25°C (data not shown).

In carp acclimated to 15°C and 25°C, white muscle pH_i was lower after exercise than after hypoxia exposure (Fig. 2B). In addition, the rate of white muscle pH_i decrease during exposure to hypoxia was greater in fish acclimated to 25°C compared with fish acclimated to 15°C [rate constants (τ) at 15°C=0.03±0.01 *versus* 25°C=0.05±0.01; P=0.014, *t*-test] (Fig. 3B).

Recovery from exercise and hypoxia

Temperature acclimation did not affect the time necessary for PCr to return to pre-exposure values following exercise or hypoxia exposure (Fig. 4A,B; Table 1). Muscle PCr increased logarithmically during recovery from exercise and hypoxia; however, the PCr recovery rate constant following hypoxia exposure was 3.5- to 4-fold higher than the PCR recovery rate constant following exercise (Table 1).

At the onset of recovery, muscle pH_i continued to decrease over the first 2.31±0.95 and 1.35±0.50 h in the exercised carp acclimated to 15°C and 25°C, respectively and for the first 1.03±0.27 and 0.76±0.30 h in hypoxia exposed carp at 15°C and 25°C, respectively (Fig. 5A,B). In all groups, the mean lowest pH_i was lower than pH_i at the beginning of the recovery period, and in both exercised and post-hypoxia exposed carp, temperature had no effect on the lowest pH_i observed, nor on the time needed to reach the lowest muscle pH_i. Once pH_i began to rise, acclimation temperature continued to have no influence on the rate of recovery after exercise or hypoxia exposure; however, at both acclimation temperatures, pH_i returned towards control values significantly faster after hypoxia exposure compared with the recovery from exercise (Fig. 5A,B; Table 1). When the exercise recovery experiments were terminated, muscle pH_i at 15°C and 25°C were still significantly lower than resting values of 7.36±0.13 (N=8; P<0.05; *t*-test).

Muscle ATP remained constant during recovery from intense exercise and hypoxia exposure in carp acclimated to both 15°C and 25°C (Fig. 6A,B). Muscle [ADP_{free}] decreased exponentially

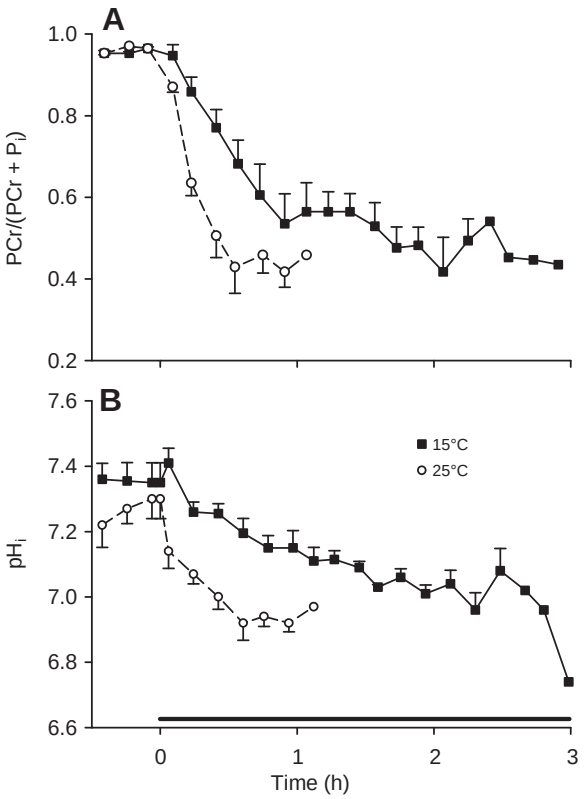

Fig. 3. White muscle PCr (A) and pH_i (B) in carp exposed to normoxia and hypoxia for up to 3 h at 15°C (squares) and 25°C (circles). Phosphocreatine levels are normalized to the sum of PCr and P_i, and expressed relative to normoxic controls. NMR data were collected at the fishes' acclimation temperature and for each time point an average consisting of 256 spectra gathered over 5 min is shown. The black horizontal bar immediately above the *x*-axis indicates the period of hypoxia exposure (P_{O_2}=20 Torr). Each point represents mean ± s.e.m. *N*=7–10 for each point.

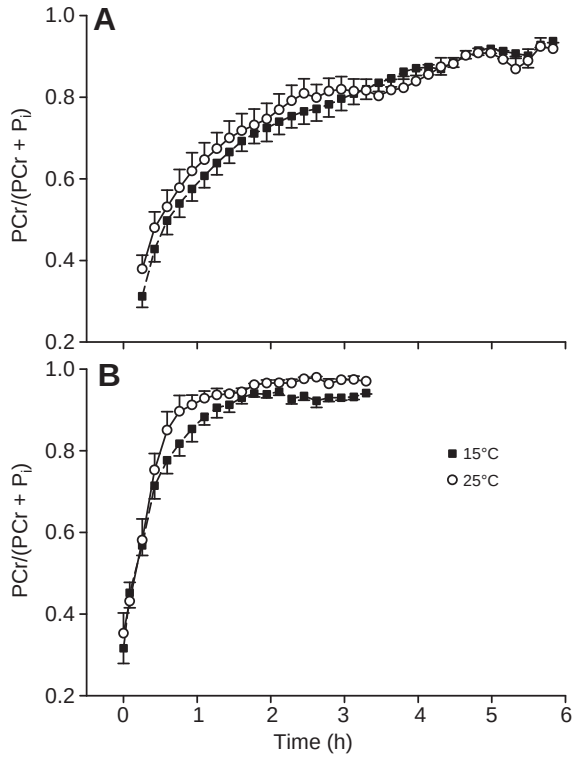

Fig. 4. Recovery of PCr following exercise (A) and hypoxia exposure (B) at 15°C (squares) and 25°C (circles). Phosphocreatine levels are normalized to the sum of PCr and P_i and expressed relative to normoxic/resting controls. NMR data were collected at the fishes' acclimation temperature and for each time point an average consisting of 256 spectra gathered over 5 min is shown. Time zero represents the point at which exercise stopped (A) or when fish were returned to normoxic water (B). In A, transfer of the fish from the exercise respirometer to the NMR took ~10 min, therefore the recovery trace does not start at zero. Each point represents mean ± s.e.m. *N*=7–10 for each data point.

during recovery from exercise and hypoxia exposure, and there was no difference in ADP_{free} recovery rate constants following exercise or hypoxia at either 15°C or 25°C (Fig. 7A,B; Table 1). Cytoplasmic [ATP]/[ADP_{free}] increased during the initial part of recovery from high-intensity exercise and hypoxia exposure; however, the shapes of the recovery curves were dramatically different (Fig. 8). Following hypoxia exposure, [ATP]/[ADP_{free}] peaked at ~700 during the first hour of recovery and then declined to values close to the values observed immediately following hypoxia exposure (Fig. 8B). By contrast, during recovery from high-intensity exercise, [ATP]/[ADP_{free}] increased over the first 2 h of recovery to ~1000, and remained at this elevated value for the remainder of the recovery period (Fig. 8A). There was no apparent impact of temperature on [ATP]/[ADP_{free}]. Free energy of ATP hydrolysis ($\Delta fG'$) recovered at a rate 1.6 to 2.5 times higher following hypoxia exposure compared with intense exercise (Fig. 9A,B; Table 1) and there was no effect of temperature on recovery rate constants (Table 1).

Table 1. Recovery rate constants (τ) for PCr, ADP_{free}, Gibbs free energy of ATP hydrolysis ($\Delta fG'$) and pH_i following high-intensity exercise and exposure to hypoxia in carp acclimated to 15°C and 25°C

	15°C		25°C		ANOVA	
Treatment	Exercise	Hypoxia	Exercise	Hypoxia	H *versus* E	Temperature
PCr (τ) (h^{-1})	0.54±0.03	2.26±0.08	0.68±0.05	2.46±0.12	*P*<0.001	*P*=0.205
ADP_{free} (τ) (h^{-1})	5.21±1.07	6.80±1.30	6.42±1.35	5.39±0.79	*P*=0.951	*P*=0.835
$\Delta fG'$ (τ) (h^{-1})	0.85±0.15	2.10±0.39	0.98±0.17	1.58±0.31	*P*=0.009	*P*=0.569
pH_i	0.12±0.01	0.30±0.03	0.16±0.02	0.36±0.08	*P*<0.001	*P*=0.177

Mono-exponential regressions were fitted to individual PCr, ADPfree and $\Delta fG'$ and rate constants calculated. For analysis of pH_i, the rate of recovery from the lowest measured pH_i to the end of the experiment was determined by standard linear regression. The choice of regression analysis was based on the model that yielded the highest correlation coefficient for each data set. H, hypoxia; E, exercise.

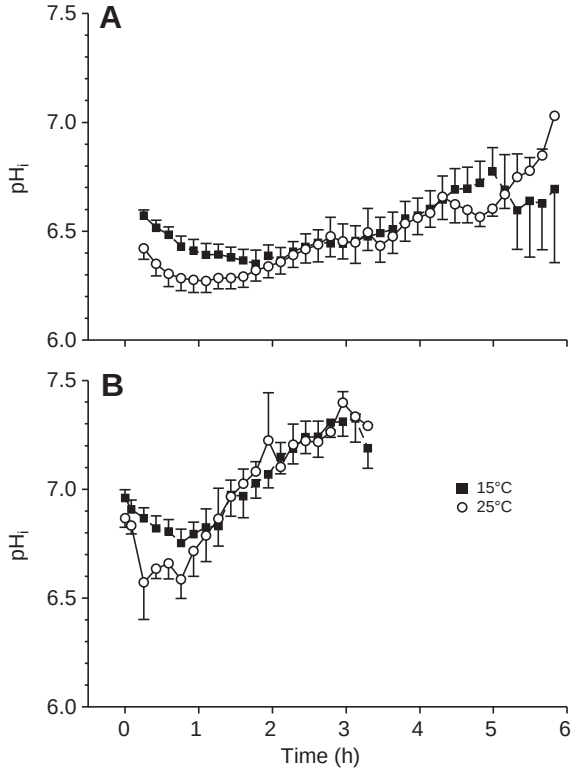

Fig. 5. Recovery of pH_i following exercise (A) and hypoxia exposure (B) at 15°C (squares) and 25°C (circles). Each point represents mean ± s.e.m. *N*=7–10 for each data point. See Fig. 4 legend for more detail.

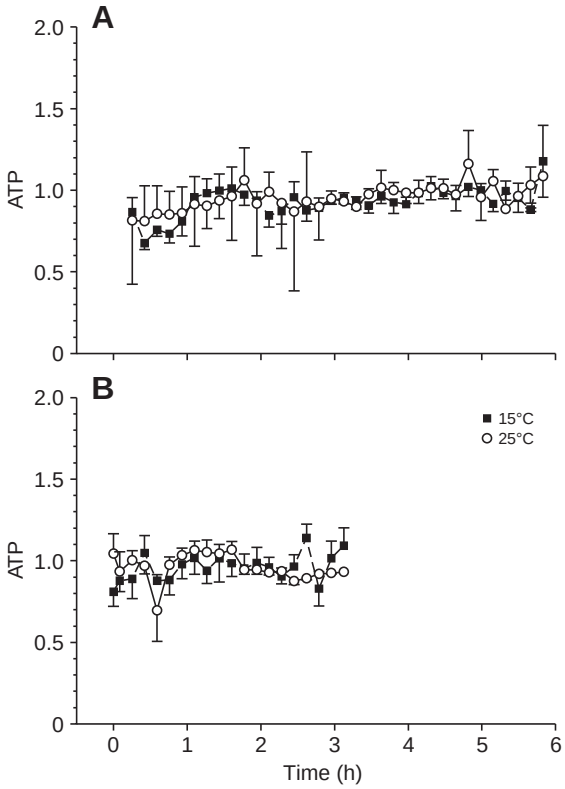

Fig. 6. Recovery of ATP following exercise (A) and hypoxia exposure (B) at 15°C (squares) and 25°C (circles). ATP levels are expressed relative to normoxic/resting controls. Each point represents mean ± s.e.m. *N*=7–10 for each data point. See Fig. 4 legend for more detail.

Series 2

Using the dual ^{1}H- and ^{31}P-NMR probe head, we were able to receive nearly simultaneous traces for the quantification of changes in lactate, PCr, ATP and pH_i in carp white muscle followed exercise or hypoxia exposure. These preliminary experiments using ^{1}H-NMR and 1331-delayed acquisition sequences yielded very good spectra with only two predominant signals (Fig. 10). The most intense peak, around 3.0 p.p.m., corresponds to the $–NCH_3$ of creatine and PCr, and its chemical shift was used later on the internal standard. The other signal on this spectra centered at 1.30 p.p.m. corresponds to the lactate doublet. In some cases, pyruvate was also detected at 2.10 p.p.m., although not in a consistent manner. The ^{31}P-NMR spectra allowed for the detection of P_i, PCr and ATP, and the spectral shifts in P_i, were used to calculate pH_i as described in Materials and methods.

The results obtained from carp exposed to hypoxia or recovery from exercise using the dual ^{31}P-NMR and ^{1}H-NMR head are in good agreement with the results from Series 1. White muscle PCr decreased to ~45% of normoxic values after an 8 h exposure to hypoxia, and this drop was matched by a similar drop in pH_i to that observed in Series 1, from ~7.3 to 7.0 (cf. Fig. 11A,B; Fig. 3A,B). Exposure to hypoxia did not affect white muscle [ATP] (Fig. 11C). Using ^{1}H-NMR, we detected a fourfold increase in white muscle lactate after an 8 h exposure to hypoxia (Fig. 11D).

White muscle from exhausted fish had ~40% of resting PCr level (Fig. 12A) similar to those observed after an 8 h exposure to hypoxia (Fig. 11A). White muscle PCr returned to resting levels within ~3 h of recovery. Intracellular pH was depressed to similar levels to that observed in response to exercise in Series 1 experiments (cf. Fig. 12B; Fig. 2B) and recovered to resting levels within ~3 h. There was no effect of exercise or recovery on white muscle [ATP] (Fig. 12C). At exhaustion, white muscle [lactate] was fourfold higher than in resting fish and returned to resting values within 3 h of recovery (Fig. 12D).

DISCUSSION

The present study yielded two novel findings. First, PCr and pH_i recovery from exposure to hypoxia occurred at a rate two to four times faster than recovery from exercise. Second, temperature acclimation significantly affected the rate of PCr depletion and tissue acidification during exposure to hypoxia, but had no effect on the rates of PCr or pH_i recovery.

Exercise and hypoxia

Exercise and exposure to hypoxia both resulted in similar decreases in white muscle PCr (Fig. 2A); however, the carp that underwent exercise developed a more severe metabolic acidosis than did the carp exposed to hypoxia (Fig. 2B). The cause of metabolic acidosis has been strongly debated for several decades (Pörtner, 1987), but recent views once again point to ATP hydrolysis as the primary source of metabolic acid production (Hochachka and Mommsen, 1983; Robergs et al., 2004) as shown by:

$$MgATP^{2-} + H_2O \rightarrow MgADP^{-} + HPO_4^{2-} + H^{+} . \quad (6)$$

Intense exercise and exposure to hypoxia both involve an activation of substrate-level phosphorylation to support an ATP turnover that exceeds the capacity of mitochondrial oxidative phosphorylation and H^+ use. Under conditions of limited

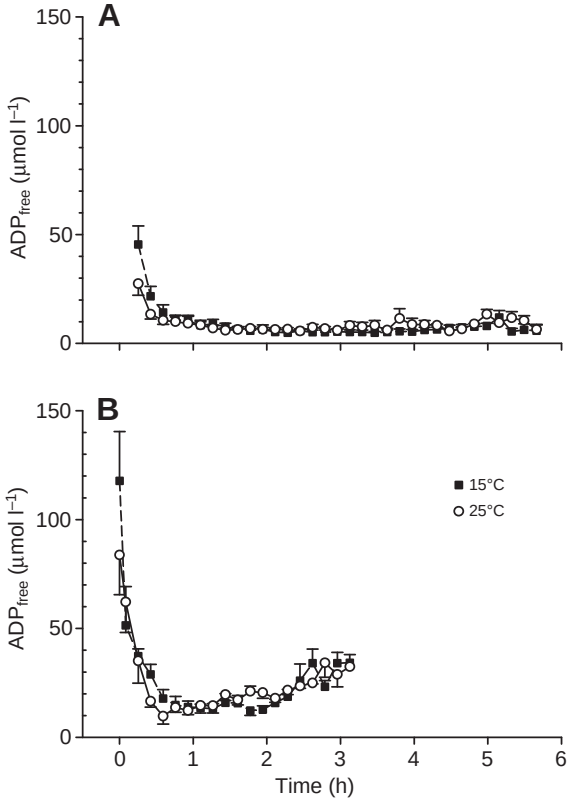

Fig. 7. Recovery of cytosolic free ADP following exercise (A) and hypoxia exposure (B) at 15°C (squares) and 25°C (circles). ADP_{free} levels are in units of µmol l^{-1} intracellular water (see Materials and methods for more detail). Each point represents mean ± s.e.m. *N*=7–10 for each data point. See Fig. 4 legend for more detail.

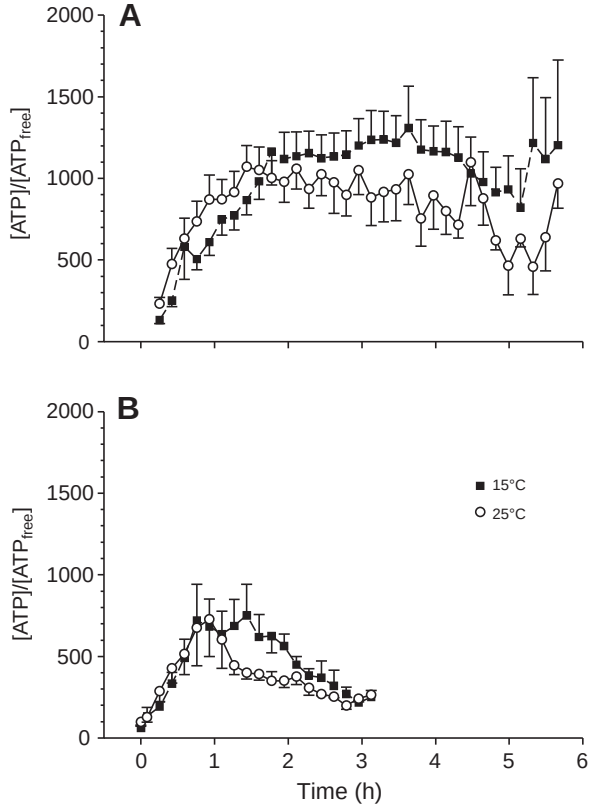

Fig. 8. Recovery of [ATP]/[ADP_{free}] following exercise (A) and hypoxia exposure (B) at 15°C (squares) and 25°C (circles). Each point represents mean ± s.e.m. *N*=7–10 for each data point. See Fig. 4 legend for more detail.

mitochondrial H^+ use, the degree of metabolic acid production will be dependent on the substrate used to rephosphorylate ADP forming ATP. ATP synthesis *via* PCr hydrolysis results in no appreciable H^+ production (balance of Eqns 1 and 6). By contrast, ATP synthesis *via* glycogenolysis/glycolysis yields net H^+ production because protons produced by ATP hydrolysis are not stoichiometically used by glycolysis.

In the present experiment, exposure to hypoxia and exercise yielded similar decreases in white muscle PCr (Fig. 2B) and accumulation of lactate (Fig. 11D; Fig. 12D), but a much greater metabolic acidification was observed following exercise compared with hypoxia exposure. Where, then, is the increased proton load following exercise coming from? The main differences between these two treatments that may account for the different muscle proton loads are rates of ATP use and the length of time required to reach the observed substrate depletion. During high intensity exercise, white muscle ATP turnover rates can exceed 3.7 µmol g^{-1} wet tissue s^{-1} (Richards et al., 2002a) and, as a result, this ATP turnover rate can only be supported for seconds to minutes. By contrast, survival in hypoxia requires a suppression of ATP turnover, which extends the life of an animal by hours, days or weeks (Boutilier, 2001). Membrane transport of metabolic protons has been proposed to be an important mechanism of cellular acid-base regulation (Robergs et al., 2004) and the differences in the rate of proton production (slow in response to hypoxia exposure relative to exercise) suggest that during hypoxia exposure, protons may be transported from the intracellular fluid to the extracellular space for buffering or shuttling to other tissues. By contrast, during a bout of high-intensity exercise, the rate of proton production probably overwhelms membrane transport and therefore protons are retained in the tissue. Wang et al. (Wang et al., 1996) have demonstrated that proton movement across the white muscle cell membrane after exercise is not linked to lactate movement and is dependent primarily on the pH gradient.

The greater metabolic acidosis observed in carp white muscle after exercise compared with hypoxia exposure would directly impact the rate of PCr recovery. Post-hypoxia-exposed fish required significantly less time, about one-quarter of the time, to rebuild PCr compared with exercised fish (see Fig. 4; Table 1). In fact, during recovery from hypoxia, carp consistently had higher PCr levels than those recovering from exercise at both temperatures. Phosphocreatine levels were recovered fully in the hypoxia-exposed fish before pH_i levels even began to rise in the post-exercise fish (Fig. 5). In all cases, pH_i did not begin to increase until white muscle PCr had attained at least 80% of full recovery, which was also observed in carp and goldfish during recovery from anoxia (van den Thillart et al., 1989). Furthermore, there was a strong correlation between the falling pH_i, continuing into the first part of the recovery period, and the rebuilding of PCr during that time in the exercised and post-hypoxic carp at 15°C (r^2=0.938 and r^2=0.989, respectively) and at 25°C (r^2=0.823 and r^2=0.631, respectively).

The more severe and prolonged acidosis following exercise, in the presence of unchanging [ATP] (Fig. 6), yielded dramatically different cellular energy profiles during recovery from exercise compared with hypoxia exposure, which may affect PCr recovery. Overall, cellular energy status, as determined from calculations of

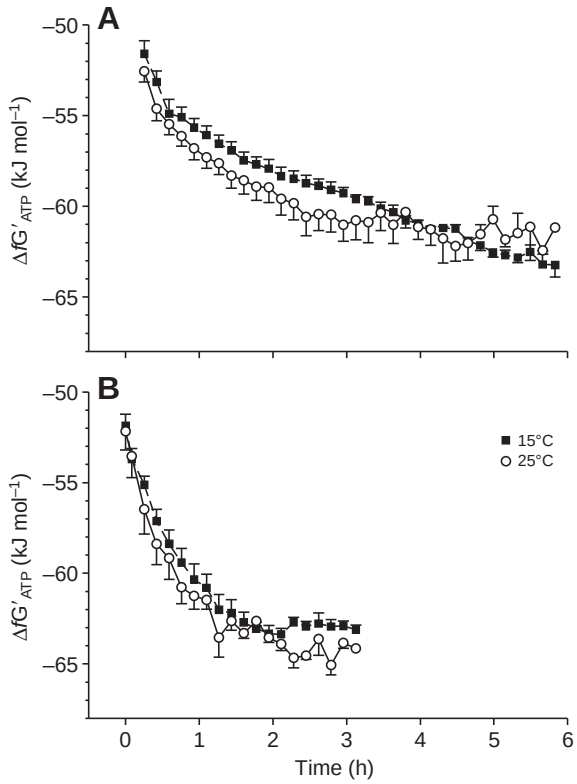

Fig. 9. Recovery of Gibbs free energy of ATP hydolysis ($\Delta fG'$; kJ mol^{-1}) following exercise (A) and hypoxia exposure (B) at 15°C (squares) and 25°C (circles). Each point represents mean ± s.e.m. *N*=7–10 for each data point. See Fig. 4 legend for more detail.

$\Delta fG'$, recovered at a rate two to three times faster after hypoxia exposure than after exercise (Fig. 9; Table 1). Recovery of PCr following exercise and hypoxia exposure is directly related to cellular energy status through its effects on the CPK substrate conditions (see Eqn 1). Although the recovery rate constants for [ADP_{free}] did not differ following exercise and hypoxia exposure (Table 1), higher [ADP_{free}] were noted throughout recovery from hypoxia exposure (21±1 μmol l^{-1} from 1 to 3.1 h) (Fig. 7B) compared with values observed during exercise recovery (8±1 μmol l^{-1} from 1 to 5.6 h; P<0.001, t-test) (Fig. 7A). As a result, muscle [ATP]/[ADP_{free}] was perturbed to a much greater degree following exercise than following hypoxia exposure (Fig. 8), which in theory could support a faster PCr recovery following exercise by shifting the CPK equilibrium towards PCr synthesis. This is clearly not the case, suggesting that the sustained decrease in white muscle pH_i during recovery from exercise (Fig. 5A) is the dominant factor that limits the rate of PCr recovery following exercise.

Mitochondrial respiration rate during recovery is controlled through the interactive effects of changes in [ADP_{free}], [ATP]/[ADP_{free}], [P_i] and pH_i (Moyes et al., 1992). During recovery from exercise the sustained elevation in muscle [ATP]/[ADP_{free}] (Fig. 8) should exert an inhibitory influence on mitochondrial respiration and potentially limit the overall rate of metabolic recovery. It should be noted, however, that [ATP]/[ADP_{free}] ratios greater than 200, as observed throughout most of the recovery from exercise and hypoxia, are expected to be inhibitory to mitochondrial respiration (Moyes et al., 1992). In trout white muscle mitochondria, P_i and pH are thought to be the major regulators of mitochondrial oxidative phosphorylation at high [ATP]/[ADP_{free}] with maximum stimulation of mitochondrial respiration occurring between 5 and 10 mmol l^{-1} P_i at acidic pH (~6.5) (Moyes et al., 1992). Changes in muscle [P_i] mirrored those of PCr during recovery (data not shown), starting at concentrations of ~20 mmol l^{-1} ICF and quickly returning to levels that should maximally stimulate mitochondrial oxidative phosphorylation. In fact, based solely on the change in muscle [P_i] and pH observed herein, the sustained elevation in [P_i] following exercise and the prolonged muscle acidification could have resulted in higher mitochondrial respiration during recovery from exercise compared with recovery from hypoxia exposure.

Our results showing a prolonged depression in cellular [ADP_{free}] following intense exercise (Fig. 7A) are in direct contrast with those recently reported by van Ginneken et al. (van Ginneken et al., 2008), who showed in carp muscle a transient depression in [ADP_{free}] at

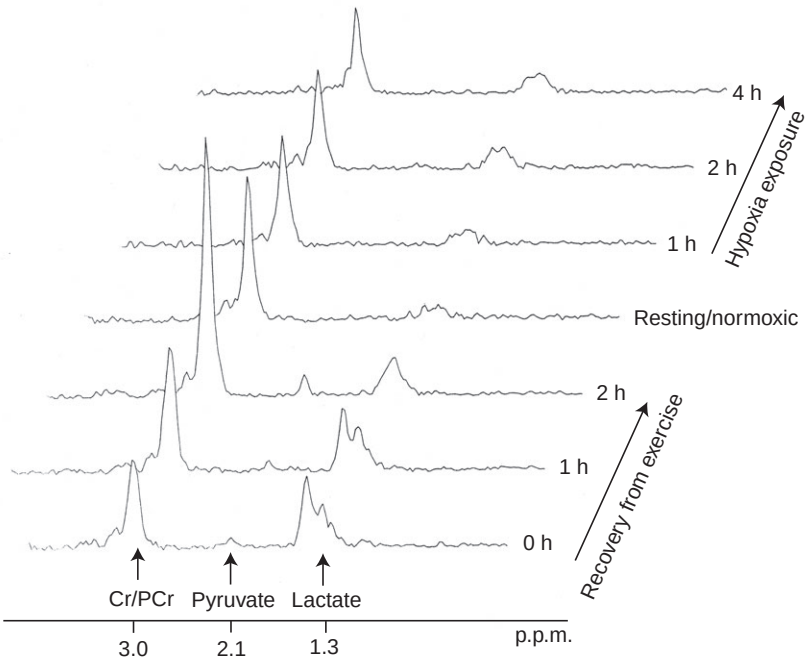

Fig. 10. Representative series of ^{1}H-NMR spectra taken on carp during a 2 h recovery from exercise followed by a 4 h exposure to hypoxia. The resting/normoxic trace was taken 24 h after the initial time 0 h reading following exercise. Peaks representing creatine (Cr)/phosphocreatine, pyruvate and lactate are identified. The size of the creatine/phosphocreatine peak is not quantitative.

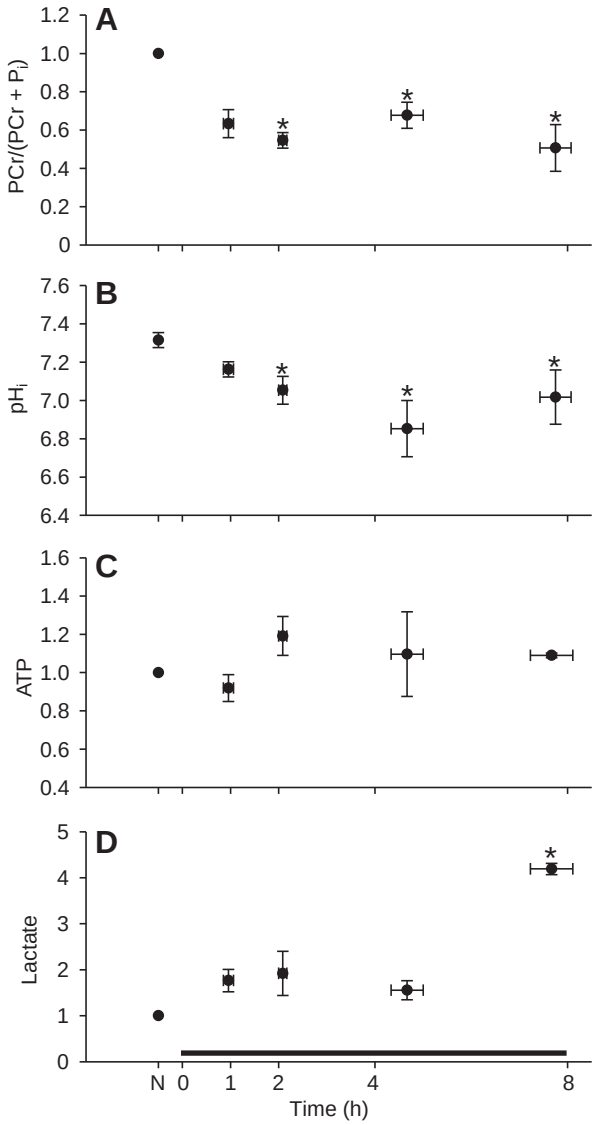

Fig. 11. White muscle PCr (A) pH_i (B), ATP (C) and lactate (D) levels from carp exposed to normoxia (N) and for up to ~8 h exposure to hypoxia. Phosphocreatine levels are normalized to the sum of PCr and P_i. PCr, ATP and lactate levels are expressed relative to normoxic controls. The black horizontal bar above the *x*-axis indicates the period of hypoxia exposure (P_{O_2}=20 Torr). Each point represents mean ± 1 s.d. Significant differences (P<0.05) between the resting/normoxic fish and hypoxia-exposed fish are indicated by an asterisk. From left to right, N=4, 10, 6, 3 and 4.

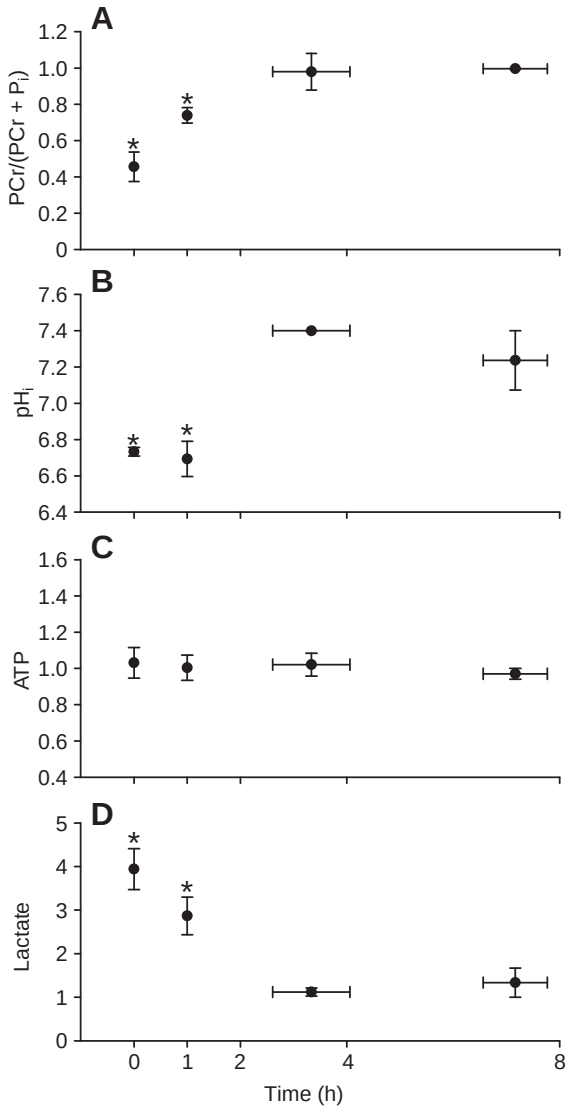

Fig. 12. White muscle PCr (A), pH_i (B), ATP (C) and lactate (D) levels from carp at exhaustion (time zero) and for up to ~8 h recovery. Phosphocreatine levels are normalized to the sum of PCr and P_i. PCr, ATP and lactate levels are expressed relative to normoxic controls. Each bar represents mean ± 1 s.d. Significant differences (P<0.05) between resting/normoxic fish and post-exercise recovery fish are indicated by an asterisk. N=3 for each point.

~1 h post exercise and recovery back to resting values by 2 h post-exercise. The rapid recovery of $[ADP_{free}]$ during recovery from exercise was probably due to the different exercise regime. van Ginneken et al. (van Ginneken et al., 2008) used a 'Ucrit' style swimming protocol involving incremental steps in swimming speed, taking more than 2.5 h to exhaust a fish. The result of this protocol was a less severe depletion of PCr (to ~60% of recovered values cf. <40% of resting vales in the present study) (Fig. 4A) and a low tissue acidification compared with the results observed in the present study (Fig. 5A) using a relatively short-duration intense exercise regime. Exercise intensity and the magnitude of the resulting metabolic perturbation have clear impacts on the rate of recovery.

Recovery from exercise has been extensively studied in many fish species (Wang et al., 1994) and the mechanisms that regulate metabolic recovery in white muscle of rainbow trout have mostly been established (Kieffer, 2000; Richards et al., 2002b; Schulte et al., 1992). Following exhaustive exercise, there is typically a rapid recovery of PCr and slower recovery of both pH_i and lactate. Throughout recovery, white muscle maintains an exaggeratedly high $[ATP]/[ADP_{free}]$ ratio (see Fig. 8A), which, together with elevated P_i, may limit mitochondrial oxidative phosphorylation, but is necessary for *in situ* glycogensis. In salmonids, a high $[ATP]/[ADP_{free}]$ ratio is thought to be necessary to provide an environment in which pyruvate kinase can function in reverse for *in situ* glycogensis from lactate (Schulte et al., 1992). Both trout and carp have been shown to have limited capacity for lactate shuttling to hepatic tissue for a mammalian-like Cori cycle (van Ginneken et al., 2004a); therefore, recovery of glycogen stores is dependent on *in situ* reversal of glycolysis for glycogensis. Therefore, during recovery from exercise there appears to be a

compromise between facilitating *in situ* glycogensis and providing sufficient mitochondrial ATP. By contrast, far less is known about the recovery from hypoxia exposure in fish. Studies using ^{31}P-NMR match our observations of a rapid recovery of PCr and pH_i, and a reduced [ATP]/[ADP_{free}] (Borger et al., 1998; van den Thillart et al., 1989).

Effects of temperature acclimation on muscle PCr and pH_i recovery

Acute temperature changes are known to have profound effects on the rates of biochemical and physiological processes in ectothermic animals. Profound temperature effects are minimized as an animal acclimates, thus reducing their impact on physiological and biochemical function (Guderley, 1990; Guderley, 2004). In the present study, however, we observed differential responses of temperature acclimation on metabolic processes. During exposure to hypoxia, fish acclimated to 25°C and exposed to hypoxia had depleted white muscle PCr (Fig. 3A) and accumulated metabolic H^+ faster (Fig. 3B) than carp acclimated to 15°C. By contrast, following exercise and exposure to hypoxia, acclimation temperature had no affect on rates of PCr and pH_i recovery (Table 1; Fig. 4A,B; Fig. 5A,B).

During exposure to hypoxia, the higher rates of PCr use and tissue H^+ accumulation in carp at 25°C are a likely consequence of higher temperature-dependent metabolic rates and a reduced capacity for metabolic suppression compared with carp acclimated to 15°C. The common carp are well known for their ability to survive prolonged periods of hypoxia exposure [e.g. overwintering under ice (Ultsch, 1989)]; however, some debate exists regarding whether the common carp employs metabolic suppression as a means for hypoxic survival. Reductions in muscle [PCr] and [ATP] and enhanced glycolytic flux has led some authors to suggest that common carp maintain a temperature-dependent metabolic rate during hypoxia through a strong activation of substrate-level phosphorylation (van Ginneken et al., 1995; Van Waarde et al., 1990). However, other studies (Zhou et al., 2000), in addition to the present study, demonstrate that hypoxia exposure does not affect muscle [ATP], suggesting metabolic suppression is occurring. A reduced capacity for metabolic suppression at warmer temperatures is consistent with work of Van den Thillart et al. (Van den Thillart et al., 1983), who demonstrated a negative relationship between survival time of goldfish exposed to anoxia and increasing acclimation temperature. Independently of whether carp are able to suppress their metabolism, carp acclimated to 25°C will have a higher metabolic rate then carp acclimated to 15°C, and will therefore require higher ATP turnover during hypoxia exposure, yielding greater substrate use and tissue acidification (Fig. 3).

Despite the profound effects of temperature acclimation on the rates of white muscle PCr depletion and acidification during exposure to hypoxia, the rates of PCr or pH_i recovery were independent of temperature (e.g. Q_{10} values for PCr recovery of 1.2 and 1.1 after exercise and hypoxia, respectively (Fig. 4; Table 1). This apparent lack of a temperature effect on recovery is consistent with the results of previous studies that examined recovery metabolism in fish following exhaustive exercise and exposure to hypoxia (Borger et al., 1998; Kieffer et al., 1994). In both of these studies, temperature acclimation did not affect the rates of PCr or pH_i recovery in white muscle of trout (*Oncorhynchus mykiss*) following exhaustive exercise (Kieffer et al., 1994) or in white muscle of carp following exposure to hypoxia (Borger et al., 1998). The lack of an effect of temperature acclimation on the rates of recovery suggests that temperature-dependent elevations in metabolic rate do not play a role in enhancing the rate of metabolic recovery. In line with this conjecture, Clutterham et al. (Clutterham et al., 2004) have demonstrated that fish do not actively select different temperatures during recovery from exhaustive exercise, suggesting there is no physiological benefit to modulating metabolic rate through temperature selection to enhance recovery.

Temperature acclimation is known to affect muscle morphology, mitochondrial volume density, membrane lipid composition and enzyme amount or enzyme isoform profiles, all contributing to the maintenance of tissue metabolic capacity across temperatures. Acclimation to cooler temperatures has been shown to increase the proportional area of muscle fibers occupied by mitochondria and increase enzyme concentrations to compensate for reduced catalytic capacity (see Guderley, 1990; Guderley, 2004). Specifically, 2 months of acclimation to 2°C resulted in a fivefold increase in white muscle mitochondrial volume density in Crucian carp compared with 28°C acclimated fish (Johnson, 1982), suggesting a higher aerobic capacity in cold acclimated *versus* warm acclimated animals. Furthermore, ATP is typically maintained at a higher concentration in 2°C acclimated carp compared with 28°C acclimated carp (Johnson and Maitland, 1980), which may further act to compensate for decreased metabolic rate and activity due to the cooler temperatures (Eggington and Sidell, 1989). These 'beneficial' effects of temperature acclimation poise the metabolic machinery of carp muscle for equivalent rates of metabolic recovery, which are not dependent on the elevated metabolic rate associated with warm acclimation. Elevated oxygen consumption in response to warm acclimation is the result of enhanced oxidative phosphorylation to keep pace with the Q_{10} effects of temperature on basal metabolic processes that consume ATP, and these processes are clearly not associated with enhanced metabolic recovery.

^{31}P- and ^{1}H-NMR to measure metabolic effects on tissues

Phosphorous NMR has been used extensively to examine the metabolic profiles in fish muscle during or following exercise or hypoxia exposure (Bock et al., 2002; van den Thillart et al., 1989; van Ginneken et al., 2008; van Ginneken et al., 1995); however, ^{1}H-NMR has only been used in a handful of studies to examine lactate dynamics in muscle (Seo et al., 1983; Wasser et al., 1992b; Yoshizaki et al., 1981) and fewer studies have used ^{1}H-NMR in fish (e.g. Bock et al., 2002). We successfully developed a methodology for monitoring changes in [lactate] *in vivo* using ^{1}H-NMR (Fig. 10) and coupled its detection in an almost instantaneous fashion with the acquisition of ^{31}P-NMR traces for the quantification of PCr, ATP and pH_i.

The main issue with the use of ^{1}H-NMR in living tissue is the dominating signal from water (~90% of the spectrum) and lipid. Without modification, small metabolites such as lactate and amino acids overlap the lipid signal and are therefore masked. Pulse sequences based on differences in spin–spin relaxation times were used to reduce these broad signals thus allowing the detection of signals from small molecules such as lactate even in intact tissue (Fig. 10). Using a dual ^{1}H- and ^{31}P-NMR head design, we successfully recorded changes in lactate, PCr, ATP and pH_i in carp muscle during exposure to hypoxia and recovery from exercise. The results obtained are in general agreement with the results from series 1, indicating the hypoxia exposure and exercise do not affect white muscle ATP, but in general result in a strong activation of substrate-level phosphorylation (PCr hydrolysis and glycolysis yielding lactate accumulation) (Fig. 11) to support ATP turnover. The magnitude of the white muscle lactate accumulation is small in comparison with studies on many salmonid fish (Richards et al.,

2002a), but are in agreement with studies on the sluggish common carp which report a ~4-fold increase in white muscle lactate during exercise (Driedzic and Hochachka, 1976; van Ginneken et al., 2004b). Measurements of muscle [lactate] on sampled tissues would have been useful to confirm our ^{1}H-NMR results. Be that as it may, during recovery from exercise, there is a close temporal association between proton recovery, PCr resynthesis and lactate recovery (Fig. 12).

In summary, exercise and exposure to hypoxia result in dramatically different cellular energy profiles, primarily owing to the greater tissue acidification production during exercise, which negatively impacts the rate of recovery from exercise. During recovery from hypoxia, $[ADP_{free}]$ was high and ATP/ADP_{free} was low compared with exercised carp, which would stimulate mitochondrial ATP production and enhance the rate of metabolic recovery from hypoxia exposure. Rates of recovery from exercise and hypoxia exposure were not affected by acclimation temperature (15 and 25°C), suggesting that the processes involved in acclimation compensate for the Q_{10} effects of temperature on metabolic processes.

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