PHOSPHOCREATINE RESYNTHESIS AFTER EXERCISE AND HYPOXIA IN THE CARP *(CYPRINUS CARPIO)*: A ^{31}P-NMRS STUDY

by

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ABSTRACT

Phosphocreatine (PCr) was reduced in carp muscle by exercise or hypoxia at 15°C and 25°C in order to assess the influence of pH and temperature in PCr synthesis *in vivo*. Intramuscular PCr levels and intracellular pH (pH_i) values were determined by ^{31}P-NMRS. Exercise resulted in a significantly more acidic pH_i than following hypoxia, differing by 0.38 and 0.44 pH units at 15°C and 25°C, respectively. The lower pH values in exercised carp slowed the recovery of PCr compared with post-hypoxic fish, requiring a four-fold increase in the amount of time necessary to rebuild PCr levels. Throughout the recovery period. PCr levels following hypoxia were consistently and significantly higher than those following exercise. Intracellular pH continued to fall for up to over two hours, dropping to less than 6.3 and 6.7 following exercise and hypoxia, respectively. This continuing decline in pH_i was correlated with the rise in PCr levels up to the time when PCr was nearly recovered. During hypoxia, 25°C acclimated carp depleted PCr stores more quickly than those at 15°C, temperature had no effect on the time for recovery of either PCr or pH_i.

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INTRODUCTION

Phosphate Metabolites and the Enzyme That Drives Them

Adenosine-triphosphate (ATP) is the universal energy currency for sustaining life, more specifically, for fueling metabolic or mechanical work. ATP is necessary for movement, allowing for the contraction and relaxation of skeletal muscle. It is a catalyst for numerous, necessary reactions in the cell and is a fuel for the active transport of molecules across the cell membrane. ATP also acts to maintain the requisite electrical gradient along a nerve axon through the action of various cation-coupled ATPases. In all these functions, hydrolyzing ATP in the process of doing work yields adenosine-diphosphate (ADP), inorganic phosphate (P_i) and a proton (H^+):

$$ATP \quad <---> \quad ADP + P_i + H^+ \qquad (1).$$

The concentration of ATP maintained within a tissue is limited, only about 5 $\mu M.g^{-1}$ tissue, which can be exhausted in a matter of seconds at high work/metabolic rates (Kushmerick 1995). The importance of ATP in cellular function requires the concentration to be at reasonable levels at all times, so ATP utilization must be balanced by a parallel, metabolic pathway yielding ATP (Hochachka 1985). This alternate pathway must coordinate quick and efficient ATP production when muscle work is at its maximum and be catalyzed by an enzyme that operates over the entire physiological range of conditions. If the partnership between ATP flux and the ATP generating reaction fails, due to the under- or over-production of ATP, muscle rigor sets in or ATPases are downregulated, respectively, and normal functioning of the

cell grinds to a halt. This important support system in vertebrates is provided by phosphocreatine (PCr) metabolism.

$$PCr \quad <---> \quad Cr + P_i \qquad (2).$$

An inorganic phosphate, a creatine (Cr) molecule, and a proton are formed for each PCr molecule hydrolyzed by creatine kinase (CK). The integration of the hydrolyses of ATP (1) and PCr (2) are linked via P_i, which is united with ADP, from the just broken ATP, to resynthesize another ATP. Nearly instantaneously, ATP is hydrolyzed yielding ADP, PCr is hydrolyzed by CK, and ADP is phosphorylated to ATP. This series of reactions occurs very rapidly due to the high affinity of CK for ADP (Gadian *et al.* 1981, Shoubridge *et al.* 1984).

The involvement of CK in maintaining the proper ATP/ADP ratio and PCr pool size in tissues makes it the key enzyme in muscle energetics. The work potential of skeletal muscle is correlated to the PCr and ADP concentration in the muscle (Chance *et al.* 1985, Kushmerick and Meyer 1985). Consequently, ATP utilization and concentration remains constant (Hubley *et al.* 1997) and is independent of a changing PCr concentration until PCr is nearly depleted (Kushmerick and Paul 1976, Crow and Kushmerick 1982, van den Thillart *et al.* 1989). In fact, in the absence of PCr, ATP is depleted immediately and requires a much longer time for complete resynthesis relative to when PCr is present in the tissue (Miller *et al.* 1993). This partnership between ATP usage and PCr hydrolysis is effective because PCr is depleted at a faster rate than ATP, thus acting like a "buffer" for maintaining constant ATP concentrations in the cell (McGilvery *et al.* 1974, Meyer *et al.* 1984). The coupling of these two reactions is catalyzed by CK:

$$PCr + ADP + H^+ \quad <---> \quad Cr + ATP \qquad (3).$$

Creatine kinase catalyzes nearly all of the phosphate transfer between PCr and ATP (adenylate kinase accounts for 7%) (Dzeja *et al.* 1996), therefore, newly generated ATP molecules are processed by the CK-system from the hydrolysis of PCr. Across a wide range of physiologically relevant pH values and temperatures, the equilibrium constant of the CK reaction is generally high in both directions of the reaction (Lawson and Veech 1979), and the determining factor as to whether PCr is hydrolyzed or synthesized is the local concentration of other members of the reaction, ATP, ADP, P_i, and H^+ (Teague and Dobson 1992). It has been calculated that 80% of CK activity is in the direction of ATP synthesis and 20% in the direction of PCr synthesis in a diverse array of vertebrate species (Newsholme *et al.* 1978).

There are four isozymes of CK: one specifically associated with muscle (MM-CK), one found in relative high concentrations in the brain (BB-CK), one primarily associated with mitochondria (Mi-CK), and the other is a hybrid of the muscle and brain isozymes (MB-CK). Although found in different areas of the body and even in different parts of the cell, the four function identically, i.e. phosphate transfer from PCr to ATP and the reverse. As their names suggest, the highest relative concentrations of CK isozymes are located in specific regions of the body, however, small amounts of other isozymes are found in each area. For instance, muscle has a very large proportion (91%) of the MM-CK isozyme, but the other three isozymes are also found in measurable quantities: BB-CK consists of 1% of the total CK, Mi-CK 5%, and MB-CK 3% (Yamashita and Yoshioka 1991). Previously, it has been shown that CK has higher levels of activity in typically glycolytic muscle compared with oxidative muscle in several species (Raggi *et al.* 1969, Newsholme *et al.* 1978).

Integration of PCr with Other Metabolic Pathways

Since PCr has a direct impact on the amount and maintenance of ATP in the cell, and thus the energy production for the cell, its link to other energy producing pathways is crucial. PCr in resting muscle is high compared with ATP, usually between 20 and 30 $\mu mol.g^{-1}$ muscle tissue (Table 1), and is typically higher in fast muscle fibers than in slow fibers in many species (Walesby and Johnston 1980, Johnston 1981, Edström *et al.* 1982, Parkhouse *et al.* 1988). During the first few seconds of intense maximal exercise or during sustained "aerobic" swimming in fish, intramuscular PCr is only minimally decreased (Johnston and Goldspink 1973, Parkhouse *et al.* 1988). Anaerobic ATP production must be rapidly activated if intense exercise is sustained for more than a few seconds (Krause and Wegener 1996). These bursts of activity are powered exclusively at the expense of intramuscular PCr. Glycolysis is the primary anaerobic energy-producing pathway in vertebrates (Bennett 1978), although there are numerous other anaerobic pathways used by invertebrates (Hochachka 1980, Hochachka and Somero 1984). If the duration of the work is extended past a few seconds, PCr is depleted and glycogenolysis will follow (Dobson *et al.* 1987) in order to maintain ATP at operational levels:

$$\text{glucose} + 2ADP + 2P_i \text{ <---> } 2\text{lactate} + 2H^+ + 2H_20 + 2ATP \quad (4).$$

It has been shown that following intense anaerobic stress (e.g. exercise resulting in an oxygen deficit or from an acute exposure to a hypoxic environment) leading to a severely depleted glycogen level, PCr replenishment is delayed, suggesting a

TABLE 1. Changes in phosphocreatine levels from rest to after various anaerobic activities in selected teleost white muscle.

Species	°C	Resting PCr	Activity	Duration	End of stress PCr	Reference
C. auratus	20	8.31 ± 2.34	anoxia	>6 hr.	3.15 ± 2.02	van den Thillart et al. 1976
	20	8.7	anoxia	12 hr.	2.7	van den Thillart et al. 1982
K. pelmis	25	30*	exercise	15 min.	8*	Arthur et al. 1992
O. mykiss	4-6	27.03 ± 1.29	exercise	10 sec.	21.36 ± 0.76	Dobson et al. 1987
	4-6	19.83 ± 0.92	exercise	10 min.	1.56 ± 0.59	Dobson et al. 1987
	4-6	19.83 ± 0.92	exercise	30 min.	1.51 ± 0.85	Dobson et al. 1987
	8	13.05 ± 0.71	exercise	"exhaustion"	0.54 ± 0.14	Mommsen and Hochachka 1988
	8-12	22.69 ± 2.69	exercise	"exhaustion"	4.26 ± 1.14	Schulte et al. 1992
	9	16.58 ± 1.09	hypoxia (33 Torr.)	24 hr.	10.80 ± 1.12	Boutilier, et al. 1988
	10	19.9 ± 1.6	exercise	30 min.	15.9 ± 0.9	Parkhouse et al. 1988
	10	19.9 ± 1.6	exercise	"exhaustion"	1.8 ± 0.6	Parkhouse et al. 1988
	15	23*	exercise	"exhaustion"	2*	Milligan and Wood 1986
	15	28*	exercise	"exhaustion"	17*	Wang et al. 1994
P. flesus	10	22.2 ± 3.10	hypoxia (15 Torr)	7 hr.	10.0 ± 5.0	Jørgensen and Mustafa 1980b
	10	22.2 ± 3.10	hypoxia (15 Torr)	29 hr.	16.5 ± 7.6	Jørgensen and Mustafa 1980b

* estimation taken from a figure
PCr measured in µmol.g-1 white muscle

relationship between glycogenolysis/glycolysis and the recovery of PCr stores (Milligan and Wood 1986, Dobson and Hochacka 1987, Mommsen and Hochachka 1988). Furthermore, PCr synthesis and oxidative phosphorylation are linked via Mi-CK, together being very important in transfering energy (in the form of PCr) from sites of production to sites of utilization. This coupling of ATP-producing reactions to the CK system is the basic tenet underlying the "PCr shuttle" hypothesis first hypothesized by Bessman and Geiger (1981).

Briefly, the PCr shuttle facilitates the movement of energy, in the form of high energy phosphate molecules, through the cell to match with the energy demand of the muscle cell (reviewed in Meyer *et al.* 1984, Bessman and Carpenter 1985, Wallimann *et al.* 1992). PCr is the first metabolite to be hydrolyzed, before glucose or glycogen, to maintain ATP at or near resting levels during muscle work. PCr is able to act as an ATP buffer because it is stored at much greater concentration than ATP and because CK has a catalytic capacity many times greater than ATP hydrolysis (Newsholme 1978). Creatine kinase has been found to be an important enzyme in the regulation of glycolysis (Davuluri *et al.* 1981). As PCr is depleted, glycolysis is initiated (Hoult *et al.* 1974) due to rising concentrations of ADP (Chance *et al.* 1985, Brooks and Storey 1988). PCr breakdown also acts to provide a substrate for glycogen phosphoylase, i.e. P_i, a prerequisite for maximal activation of glycolysis (Meyer *et al.* 1986). Furthermore, PCr hydrolysis consumes a H^+, limiting its release and preventing the inhibition of phosphofructokinase (PFK) at an important regulatory step in glycolysis (Krause and Wegener 1996). Once anaerobic metabolism begins, the ATP generated from glycolysis is not used directly by the ATPases driving cellular processes, but is immediately transphosphorylated by CK to PCr in the attempt to maintain PCr at operating levels (Scopes 1973). Hochachka and Mommsen (1983) and van Waarde *et al.* (1990) have reported that at high rates

of ATP usage resulting in a rise in ADP levels and a decrease in PCr concentrations, the CK reaction and anaerobic glycolysis are effectively coupled by a proton as the intermediate. During recovery from anaerobic activity, glycolysis halts as oxidative phosphorylation takes over. Although the pathways, glycolysis and oxidative phosphorylation, are different, the purpose is essentially the same, i.e. to produce ATP to drive the synthesis of PCr (Bessman and Geiger 1981).

The distribution of CK in the cell also provides circumstantial evidence for the PCr shuttle hypothesis. Glycolytic enzymes have been shown to be grouped together in a complex bound on the actin-myosin complex (Bronstein and Knull 1981, Brooks and Storey 1988). Furthermore, a very high proportion of MM-CK within a muscle cell is also located here, specifically at the I-band of the myofibril (Wallimann *et al.* 1989). Kupriyanov *et al.* (1980) demonstrated that when Cr is added to an *in vitro* preparation of glycolytic enzymes, lactate and PCr are produced. They also showed that if elevated levels of PCr were present glycolysis was inhibited, presumably by the build up of ADP. The same also holds true for Mi-CK and oxidative enzymes. In relatively higher concentrations in aerobic muscles, Mi-CK is found primarily at the inner membrane of mitochondria, also the site of many oxidative enzymes (Wallimann *et al.* 1992). The MM-CK isozyme is found at its highest levels, relative to the other isozymes, in glycolytic fibers with 91% versus 78% of total CK located in fast glycolytic fibers and slow oxidative fibers, respectively (Yamashita and Yoshioka 1991).

However, following the anaerobic stress, i.e. during the normoxic recovery period, the coupling of the CK reaction and glycolysis is lost as oxidative phosphorylation commences (van Waarde *et al.* 1990). The final piece to the puzzle that is the PCr

shuttle is the interaction between oxidative metabolism and PCr (Walliman *et al.* 1992). One of the major products, probably the most important, is ATP:

$$\text{Glucose} + 36\text{ADP} + 36\text{P}_i + 36\text{H}^+ + 6\text{O}_2 \;\text{<---->}\; 6\text{CO}_2 + 36\text{ATP} + 6\text{H}_2\text{O} \qquad (5)$$

Oxidative phosphorylation is, like glycolysis and PCr hydrolysis above, at least partially controlled by concentrations of ADP (Mahler 1985, Chance *et al.* 1986, Moerland and Kushmerick 1994). Being far more efficient than anaerobic pathways in terms of ATP production, using oxidative phosphorylation when oxygen is available is a more advantageous path to replenish cellular metabolites (Hochachka 1985).

Nuclear Magnetic Resonance Spectroscopy

In the past few decades, the single most important tool used in studying cellular metabolism, specifically the interaction between PCr and total energy utilization, has been Nuclear Magnetic Resonance Spectroscopy (NMRS). Although its initial applications were physical in nature (Bloch *et al.* 1946, Purcell *et al.* 1946), NMRS has now become an essential technology in the biological sciences and medicine, as the following plethora of reviews attest (Gadian 1982, Meyer *et al.* 1982, Balaban *et al.* 1987, Becker and Fisk 1987, Gillies 1992, Radda 1992, Malhorta and Shapiro 1993, Andrew 1994, Kushmerick 1995, van den Thillart and van Waarde 1996). Due to the virtually innumerable and broad subject base of the NMRS literature, the following commentary will be brief and concentrate on the areas immediately applicable to the current investigation, i.e. the basic theory of nuclear magnetic resonance and its application to muscle energetics and physiology.

Every atom has its own magnetic nucleus, called a magnetic moment (m). When confronted with an external magnetic field (B_0) powered by a superconducting magnet, the nucleus will spin around in the axis of the field. Many atoms are dipoles, thus within B_0 these dipoles must spin parallel with B_0 and occupy one of two energy levels (Figure 1a). The sum of all the individual magnetic moments rotating around B_0 is known as the magnetization vector (M) (Figure 1b). If this spinning atom is irradiated, e.g., by a radio-frequency pulse, the measure of the frequency required to flip the dipole from one energy state to the other is known as the resonance frequency. For instance, the phosphorus atom is a dipole and the required resonance frequency of the pulse needed to flip the ^{31}P dipoles between energy states in a 1.89 Tesla (T) magnetic field is 32.51 MHz. The application of this pulse forces M away from B_0 (Figure 1c), a 90° pulse drives M to a position perpendicular to B_0 (Figure 1d). M, now in the x-y plane, generates an electromagnetic frequency, which is detected by the receiver coil on the probe. The time necessary for M to "relax" and move back to its original orientation parallel with B_0 is known as the spin-lattice relaxation time (T1). The distance M travels back toward its original station, before the subsequent pulse, is proportional to the number (or concentration) of the target nuclei in the sample. Ideally, the delay between pulses should be 4-times T1, insuring that all of the nuclei in the sample be detected (Gadian 1982). Typically, multiple pulse-delay intervals, known as free-induction decays (FIDs), are sent through the probe to the spectrometer where they are amplified. Acquiring numerous FIDs allows for greater resolution and increased signal-to-noise ratio, which yields a "cleaner" spectrum. Once the multi-frequency signal is Fourier transformed, the collection of FIDs takes the appearance of an NMR spectrum (Figure 2).

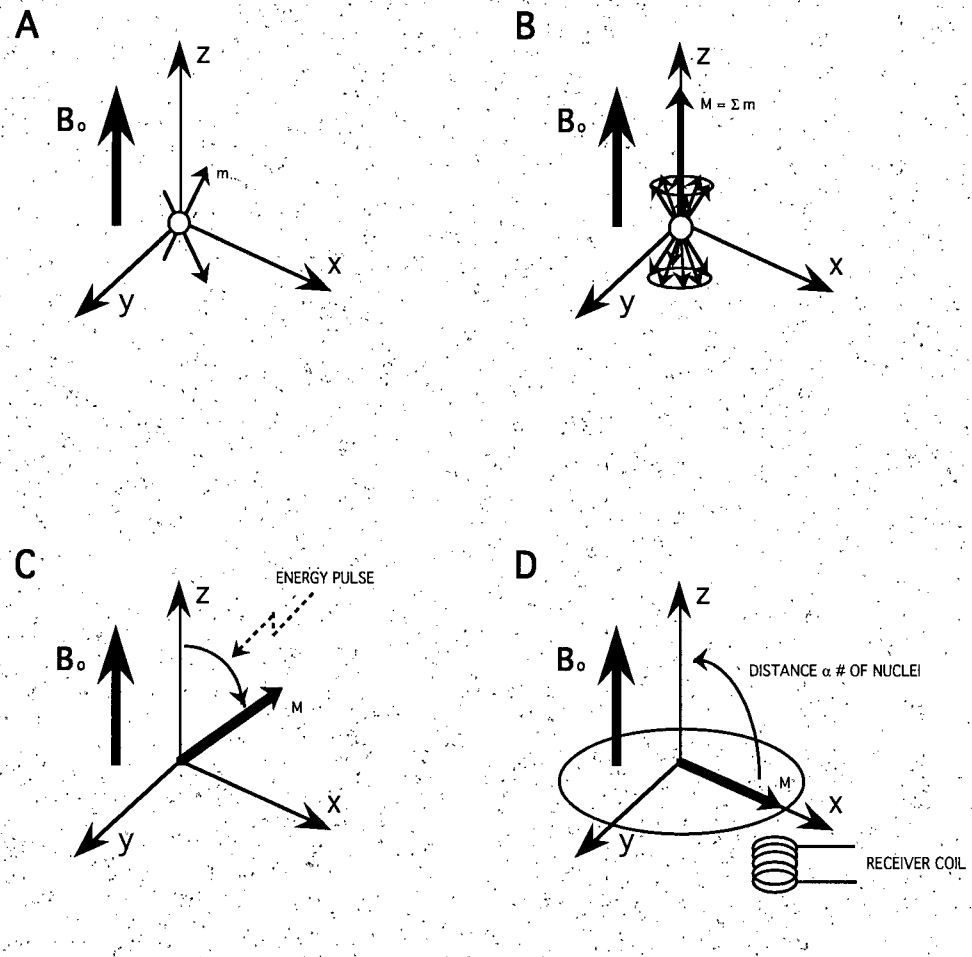

FIGURE 1. Behavior of magnetic moments (m) and resultant magnetization vector (M) within a magnetic field (B_0) before and after stimulation by a high frequency energy pulse. See text for explanation. Modified from Meyer *et al.* 1982.

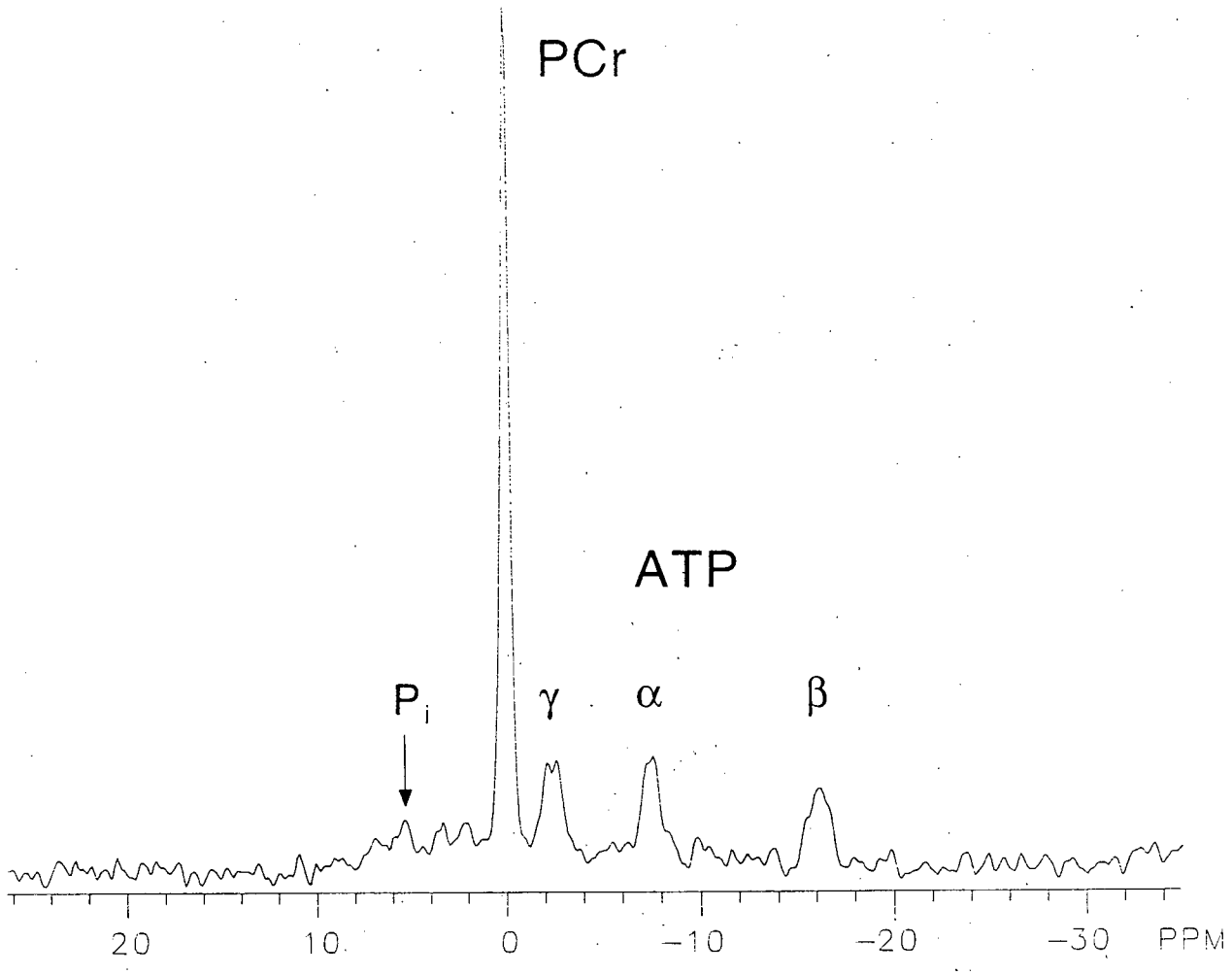

FIGURE 2. Typical spectrum taken from a resting carp acclimated to 15°C. The PCr peak is set at zero, while the chemical shift of all other peaks (P_i and each ATP) are reported as a distance relative to PCr.

Although much smaller than the superconductor B_0, each nucleus also has it own local magnetic field (Bα). Depending on the adjacent atoms of the target molecule, up to 2-3 bonds away, Bα can vary slightly. By shielding or screening the target atom, the other atoms alter the electromagnetic microenvironment, and the individual resonance frequency, of the target atom. It is this alteration that allows NMRS to differentiate between molecules with similar constituent atoms, i.e. distinguishing the uniqueness of the ^{31}P atom in PCr from the ^{31}P atom in P_i. The difference between these frequencies of two different molecules (one is usually referred to as the standard) is known as the chemical shift of the molecule (δ). The chemical shift is generally measured in the dimensionless unit parts per million (ppm), but occasionally Hz are also used. In ^{31}P-NMRS, PCr has been arbitrarily chosen as the internal (within the sample) standard in terms of calculating chemical shifts of other ^{31}P associated molecules. Specifically, one of the most useful applications of chemical shifts in ^{31}P-NMR is that between PCr and P_i. The resonance frequency of P_i is dependent on how many protons are surrounding the phosphate molecule (Moon and Richards 1973) and the temperature of the sample (Kost 1990), thus, when corrected for temperature, the chemical shift of P_i is an accurate method of determining the intracellular pH (pH_i) of the sample.

Not only will Bα recognize specific atoms in different molecules, but will also identify atoms within a molecule. In ^{31}P-NMRS, the prime example of this phenomenon is ATP. Each ATP molecule has three phosphate groups; α, β, and γ; and in the center of each group is a ^{31}P atom. Because of the different combinations of adjacent atoms, each of the three ATP phosphates have a different chemical shift, and thus are seen as three distinct peaks in a ^{31}P spectrum (Figure 2). Interestingly, when ATP is hydrolyzed, the γ phosphate (the third from the adenosine ring) is cleaved off, leaving the α and β phosphates on the remaining ADP. In ADP, the β

phosphate, now at its furthest location from the adenosine, has a very similar frequency to that of the γ-ATP. α-ADP, being so close to the adenosine, has a comparable chemical shift to α-ATP. Thus, of the three ATP phosphates, β-ATP is the only peak that contains only a ^{31}P signal from ATP, without any "contamination" from ADP. This is why whenever the quantities of ATP are studied in a sample, the β-ATP peak is generally examined.

Since PCr is in high concentrations and ATP is easily detectable concentrations in resting muscle, the role ^{31}P-NMR plays in the study of muscle physiology is undeniable. By being able to make *in vivo* measurements of the intracellular concentrations of the three phosphorus compounds most directly related to muscle energetics, ATP, PCr, and P_i, researchers can more easily study the effects of stress (exercise, hypoxia, or ischemia) on muscle physiology (reviewed by McCully *et al.* 1994). Two of the most alluring features of NMRS are that the radio-frequency pulses used in the measurement of the relaxing magnetization vectors of the target atoms can be used on *in vivo* samples and that NMRS is a non-destructive and non-invasive method of detection. The need for biopsy sampling and freeze clamping of excised tissue has been diminished by the advent of NMRS. NMRS eliminates the presence of excision and preparation artefacts due to the rapid breakdown of high energy phosphates when performing biochemical assays.

Furthermore, studies of muscle metabolism can now concentrate on changes in the state of individual subjects over the course of an experiment. In this instance, the study subject is essentially serving as its own control, thus the need for a separate "control" group is unnecessary, and the inherent variability seen in a population should be reduced. Also, at a time when the political activism directed at animal research is frequently in the headlines, the reduction in the sample size in any given

experiment, by eliminating the control group, is a necessary concession that should be considered by researchers (Mann *et al.* 1991). In direct relevance to human research, the non-invasive nature of NMRS has made it the current standard in human exercise physiology (Sapega *et al.* 1987, Cozzone and Bendahan 1994).

Due to the usefulness of NMRS in human biomedicine, most of the non-human research has been conducted on mammals. Some pioneer NMR biological experiments used excised frog muscles (Burt *et al.* 1976, Hoult *et al.* 1974), others have used turtles to study the effects of hypoxia on phosphate metabolites (Jackson *et al.* 1995), but not until recently has NMR technology been used to study the *in vivo* muscle physiology of whole fishes (reviewed by van den Thillart and van Waarde 1996).

Ecological Significance

In a natural setting, the inability to move rapidly can be disastrous, especially if a predator is nearby. Endurance capacity and maximal speed are important determinants of the "ecological characteristics" of an organism (Weber 1992), as the probability of survival will increase as swimming performance increases (Taylor and McPhail 1985, Webb 1986, Jayne and Bennett 1990). An increased ability to convert ATP to work is key in muscle and locomotory performance (Hubley *et al.* 1997). The ATP demand of or its production by an organism depends on the locomotory mode of the organism (Schmidt-Nielsen 1972). The escape response of a fish is generally a rapid, bursting, anaerobic activity, therefore using PCr as the primary means to drive muscle function. Predator avoidance is the most important factor in survival in adult fish (Pitcher and Hart 1982). Development of anaerobic capacities can play a major and cumulative role in predator-prey relationships

(Beckoff 1987). Since PCr is depleted in a short time when fish are maximally exercised (Dobson and Hochachka 1987, Dobson *et al.* 1987), it would be in their best interests to be able to replenish their PCr stores to allow for another flight if the need occurs. Individuals with a high anaerobic threshold, such as specialized organisms or trained athletes, will tend to recover from anaerobic stresses quite quickly (Sharkey 1984). If activity continues and PCr drops too low or recovery is too prolonged, maintenance of ATP levels will falter and the muscle will fail, jeopardizing an organism's survival.

Not only do PCr levels affect muscle function by directly affecting ATP levels, but low PCr and high P_i concentrations in muscles can directly contribute to a decrease in maximal force of a working muscle (Allen *et al.* 1995). In rats, depletion of the total creatine pool, PCr and Cr, resulted in a 50% decrease in force generation when compared with control subjects (Levine *et al.* 1996). Furthermore, reduced pH_i, typically seen in intensely worked muscle, also causes decreases in the maximal force a muscle can attain (Fitts 1994). Reduction in maximal force of a swimming muscle will lead to a reduced ability to escape predators.

Weibel *et al.* (1991) have recently introduced the term symmorphosis, which contends that parts of a living system must be co-adapted for a biologically economic role. This explanation fits nicely to the idea that basic muscle physiology and biochemistry directly affect a fish's locomotory ability and thus its survivability. For instance, burst swimming is a beneficial attribute when the need for predator avoidance by a prey species or prey capture by a predatory species arises. The down-side is that this intense activity can severely deplete PCr stores within the muscle, endangering ATP levels, producing P_i, and thus hindering muscle function. Also, intense exercise is generally an anaerobic event, utilizing glycolysis for

energy repletion, but also yielding protons and an acidic pH_i. A reduced pH_i alters enzyme activities and offsets the rate at which muscles can be "re-energized". The circle completes because PCr levels must be increased to functional levels as quickly as possible in order to fuel burst swimming to avoid a subsequent attack by predators. A balance must be achieved between performance, energy economy, and the ecological significance of both to permit an organism to successfully function and survive in its habitat. Therefore, metabolic energy pathways and their various regulatory steps and specific molecular events can be directly linked to important dimensions of an organism's ecology (Weber 1992).

Statement of Purpose

Fish white muscle represents the bulk of the total myotome of a fish (Johnston and Maitland 1980), and thus the majority of whole body metabolism. White muscle is made up of fast glycolytic fibers, is used to power strenuous swimming where burst speeds are necessary (Nag 1972), and is mostly an anaerobic tissue (Bone 1966). Compared with red muscle, white muscle has only 40% of the number of mitochondria, has smaller mitochondria, and has less actomyosin, yet it has three-times the ATPase activity of red muscle (Nag 1972). Anaerobic capacity of a tissue is generally thought of in terms of the potential production of anaerobic end-products. In glycolysis, these are lactate and protons from muscle glycogen (equation 4). As long as ATP levels remain constant, there is no net release of protons from ATP hydrolysis (equation 1). and the breakdown of PCr actually consumes protons (equation 2). Therefore, the accumulation of protons during intense muscle work is due primarily to glycolysis.

Stoichiometrically, the reverse CK reaction (equation 3), the building of PCr, is pH dependent (Lawson and Veech 1979) *in vitro*. When there are more protons, thus a lower pH, production of PCr will be less favored than at a relatively less acidic pH. Fish exercised to the point of exhaustion typically produce more lactate and more protons than during hypoxia (Table 2). In exercised carp, white muscle lactate concentrations are 260% to 340% of those at rest (Driedzic and Hochachka 1976, West *et al.* 1994), whereas in white muscle following hypoxia there is only a 25% increase in lactate levels relative to resting concentrations (Driedzic and Hochachka 1975). Therefore, because of a lower pH_i from glycolytic end-products, an exercise-induced depletion in PCr should be rebuilt more slowly *in vivo* than a quantitatively similar PCr depletion following hypoxia. The current series of experiments was designed to test this hypothesis.

Temperature effectively changes the reaction rates of enzymes involved in energy metabolism (Hazel and Prosser 1974). Additionally, it has been shown that temperature can affect the high energy phosphate content, depletion and rebuilding rates in excised muscle (Binzoni *et al.* 1990). Many investigations have observed pH variations in different fish tissues when subjected to a varied temperature regime (reviewed by Cameron 1984). Cooler temperatures relate to better burst swimming and also better sustained swimming performance in fish (Rome *et al.* 1985). Mitochondrial density increases at cooler temperatures (Johnston and Maitland 1980) and may bring about a lower demand for ATP than at higher temperatures. With lower temperatures, as the distance between mitochondria decreases there is a corresponding decrease in ATP and PCr concentration (Walesby and Johnston 1980, Hubley *et al.* 1997). In cyprinids, acclimation to cold temperatures also increases myosin-ATPase activity (Johnston *et al.* 1975, Heap *et al.* 1986) thus improving contraction kinetics (Johnston *et al.* 1985). An increased

TABLE 2. Changes in lactate levels from rest to after various anaerobic activities in selected teleost white muscle.

Species	°C	Resting [lactate]	Activity	Duration	Recovery [lactate]	Reference
C. auratus	4	0.7	anoxia	60 hr.	3.5	Shoubridge 1980
	20	5.82 ± 1.42	anoxia	>6 hr.	12.14 ± 4.06	van den Thillart et al. 1976
	20	1.50 ± 0.83	anoxia	12 hr.	3.17 ± 1.28	van den Thillart et al. 1982
C. carassius	15	6*	hypoxia (17 Torr)	90 min.	15*	Johnston 1975a
C. carpio	10-13	5.1 ± 1.2	exercise	>2 hr..	13.2 ± 4.9	West et al. 1994
	12	3.71 ± 0.17	exercise	"exhaustion"	12.58 ± 1.18	Driedzic and Hochachka 1976
	25	9.4 ± 1.2	anoxia	death	26.2 ± 2.3	Smith and Heath 1980
	12	9.6	hypoxia (15 Torr)	4 hr.	12.02	Driedzic and Hochachka 1975
K. pelamis	25	7.3 ± 3.8	exercise	15 min.	75.8 ± 4.8	Arthur et al. 1992
O. mykiss	4-6	1.79 ± 0.43	exercise	10 sec.	2.20 ± 0.37	Dobson et al. 1987
	4-6	5.76 ± 0.49	exercise	10 min.	35.88 ± 1.30	Dobson et al. 1987
	8	4.07 ± 0.31	exercise	"exhaustion"	33.72 ± 7.36	Mommsen and Hochachka 1988
	8-12	3.92 ± 0.94	exercise	"exhaustion"	41.72 ± 2.63	Schulte et al. 1992
	9	3.33 ± 0.60	hypoxia (33 Torr)	24 hr.	8.33 ± 2.09	Boutilier et al. 1988
	14	5.07 ± 1.02	exercise	5 min.	27.02 ± 1.94	Milligan and Girard 1993
	15	9.61 ± 1.18	exercise	"exhaustion"	33.51 ± 3.49	Milligan and Wood 1986
	15	1*	exercise	"exhaustion"	26*	Wang et al. 1994
P. flesus	10	7.75 ± 1.3	hypoxia (15 Torr)	7 hr.	8.61 ± 0.69	Jørgensen and Mustafa 1980a
	10	7.75 ± 1.3	hypoxia (15 Torr)	29 hr.	15.1 ± 4.3	Jørgensen and Mustafa 1980a
P. americanus	13	4.93 ± 0.62	exercise	"exhaustion"	15.02 ± 0.81	Girard and Milligan 1992

* estimate taken from a figure
[lactate] expressed in µmol.g-1 white muscle.
"Recovery [lactate]" signifies the lactate concentration immediately following the "activity".

ATPase activity would also subsequently require increased CK activity, i.e. primarily PCr breakdown, to maintain the elevated level of ATP usage. In support of this point, Kleckner and Sidell (1985) have shown that 5°C-acclimated pickerel have a 45% increase in CK activity over 25°C-acclimated fish. When combining the results from the pair of previously mentioned studies it is reasonable to infer that PCr will be rebuilt more rapidly at warmer temperature. With decreasing temperatures, the mitochondria density in muscle increases, allowing for a lesser decrease in PCr and a delayed onset of fatigue (Hubley *et al.* 1997). There does not seem to be definitive proof, in the form of published data, that temperature plays a major role in PCr replenishment *in vivo*, thus the current set of experiments were designed to test this hypothesis. The rate and level of PCr rebuilding seems to be a good model to determine how quickly a fish has "recovered", as it is directly related to its ability to flee a predatory threat or to make another strike. Not only is adequate PCr necessary to supply ATP, but the recovery of power output, and thus swimming speed, is linked to PCr availability (Hitchcock 1989, Gaitanos *et al.* 1993) and is initially parallel with the recovery of PCr levels (Bogdanis *et al.* 1995).

The common carp (Cyprinus carpio) seem to be ideally suited to serve as a model to test these hypotheses. Since carp are "cold-blooded", it is generally understood that their muscle physiology will vary with changing temperatures. Carp are moderately hypoxia-tolerant, being able to survive at least 45 minutes of anoxia, compared with rainbow trout which only survive about 10 minutes (Smith and Heath 1980). This time was too short to consider trout as a potential study subject since attaining proper resolution of changes of phosphate metabolites using NMRS requires more than 10 minutes with the available equipment. Carp use glycolysis as a means of dealing with anaerobic metabolism during hypoxia (reviewed by van den Thillart and

van Waarde 1985) and rely heavily on anaerobic metabolism to power intense swimming, more so than other fishes (Jones 1982). Carp are active over a wide range of temperatures (Johnston *et al.* 1975). Lastly, the typical temperament of carp make them easy to work with. They tend to be calm when handled before (while resting) and during the experimental procedures.

The rebuilding of PCr has been shown to be a rapid process. In human and other mammalian muscle, a severely depleted PCr content can be restored within a few minutes, while in teleosts it could take as little as 30 minutes (Table 3). To develop an accurate model of this process it is necessary to take numerous samples throughout the recovery period, especially in the first portion of the recovery period. The only other viable alternative would have been taking tissue biopsies, but this was unacceptable for two reasons; 1) there have been recent analyses questioning the accuracy of this technique (van den Thillart *et al.* 1990), and 2) numerous samplings would have resulted in either extreme distress to an individual fish or in the sacrifice of a large number of fish (Mann *et al.* 1991). The inherent advantages of ^{31}P-NMR satisfied both of these concerns. As mentioned above, NMRS is non-destructive, allowing a single fish to be sampled, while intact, throughout the recovery period. The signal the spectrometer receives and displays from the fish is proportional to the *in vivo* concentration of the metabolite. To obtain a comparable amount of data, sampling by biopsy would have multiplied the number of fish used considerably. Furthermore, the number of exercise and hypoxia bouts would have also increased by the same multiple resulting in unnecessary time spent on experiments. For ethical concerns and simple efficiency, this would not have been acceptable.

TABLE 3. Time necessary for PCr levels to return to resting values following differing grades of hypoxia and exercise in various teleost white muscle and mammalian skeletal muscle.

Species	°C	Anaerobic treatment	Duration	PCr (%)	Time to Recover	Reference
C. auratus	20	anoxia	3.5 hr.	45	2.5 hr.	van den Thillart et al. 1989
	20	hypoxia (22 Torr)	80 min.	20	>8 hr.	van Ginneken et al. 1995
C. carpio	20	anoxia	30 min.	11	2.5 hr.	van den Thillart et al. 1989
	20	hypoxia (31 Torr)	80 min.	22	2.67 hr.	van Ginneken et al. 1995
	15	**hypoxia (20 Torr)**	**106 min.**	**45**	**1.23 hr.**	**---**
	25	**hypoxia (20 Torr)**	**56 min.**	**35**	**0.92 hr.**	**---**
	15	**exercise**	**"exhaustion"**	**31**	**4.68 hr.**	**---**
	25	**exercise**	**"exhaustion"**	**38**	**3.69 hr.**	**---**
K. pelamis	25	exercise	15 min.	27*	1.67 hr.*	Arthur et al. 1992
O. mykiss	15	exercise	"exhaustion"	7*	< 4 hr.	Milligan and Wood 1986
	8	exercise	"exhaustion"	4	> 25 hr.	Mommsen and Hochachka 1988
	15	exercise	"exhaustion"	60*	30 min.	Wang et al. 1994
	8-12	exercise	"exhaustion"	19	< 2 hr.	Schulte et al. 1992
S. mossambicus	20	hypoxia (22 Torr)	80 min.	29	> 8 hr.	van Ginneken et al. 1995
H. sapiens	37	exercise	5 min.	44*	100 sec.*	McCully et al. 1992
	37	exercise	3 min.	38*	< 13 min.	Bendahan et al. 1990
	37	ischemic stimulation	10 min.	54	3 min.*	Blei et al. 1993 (AJP)
	37	ischemic stimulation	8 min.	48*	200 sec.	Blei et al. 1993 (PNAS)
Wystar-Kyoto rat	40	stimulation	2 min.	16*	<6 min.*	Kemp et al. 1992

* estimation taken from a figure

Bold type represents data from the present investigation.

"PCr (%)" signifies the PCr level at the beginning of the recovery period relative to resting values.

Consequently, a recent review (van den Thillart and van Waarde 1996), indicated that the current study is the first utilizing NMRS to study the muscle metabolism of exercised fish and is part of a short list of investigations directed at the rebuilding of PCr following exercise in teleosts (Milligan and Wood 1986, Mommsen and Hochachka 1988, Arthur *et al.* 1992, Schulte et al. 1992, Wang *et al.* 1994).

METHODS

Animals

Carp were caught in Lake Okanagan, BC and transported to the University of British Columbia in June of 1995 and 1996. After their arrival at U.B.C. they were quarantined and treated for *Pseudomonas* spp. and *Aeromonas* spp. bacterial infections with an antibacterial solution of chloramphenical and acriflavin. They were held outdoors in a 2000 l fiberglass tank with normoxic, non-recirculating, dechlorinated water at outdoor seasonal temperatures (6° to 15°C). Fish were fed to satiation once per week with Mazuri® koi pellets (PMI Feeds Inc., St. Louis, MO).

Healthy fish were moved indoors and kept in pairs in 50 l fiberglass tanks on a seasonal light cycle. Normoxic, non-recirculated, dechlorinated Vancouver tap water was flowed through the tanks at a constant rate of 1 $l \cdot min^{-1}$. Over a one to two week period, the fish were acclimated to either 15°C or 25°C from the indoor seasonal water temperature (about 10°-15°C) by raising the temperature of the water 1°C per day until the acclimation temperature was achieved. Fresh water flowed through a stainless steel coil warmed in a water bath prior to being emptied into each tank. The fish were then held at 15°C or 25°C for one week before and during the experiments. The animals ranged from 590 to 1270 g body mass and 31.5 to 42.5 cm total length. These fish were also fed to satiation once per week with koi pellets, but fasted two days prior to an experiment.

All experiments and holding of live animals were performed in accordance with the University of British Columbia Animal Care Committee Guidelines and the British Columbia Ministry of Environment.

Experimental Protocols

The fish were placed in a Brett-style respirometer at U.B.C. at a water temperature of 15°C or 25°C for at least 3 hours before being exercised. The exercise protocol started with 60 minutes of slow (less than 0.5 Body Length·s^{-1}) sustained swimming to accustom the fish to the current. The fish were then exercised by maintaining the velocity at the maximum speed the fish could swim in a burst-and-glide pattern. The fish were considered exhausted when they could not swim against 1 BL·s^{-1} current when stimulated by an electrified grid located at the rear of the swim tube. When the exercise protocol was completed, the fish were transported to the NMR facility, secured inside a clear plastic box, and placed on their side over the NMR ^{31}P coil. Moving the fish from the respirometer to the NMR took about 10 minutes. PCr and ATP, and P_i were monitored through the recovery period. The experiment continued until the integral of the PCr peak leveled off (remaining constant for at least 30 minutes) and just before P_i could no longer be confidently differentiated from background noise.

After a rest period of at least one week, the fish were placed on their side in the clear plastic box for at least 8 hours to eliminate handling stress (lowered PCr, elevated P_i, and/or lowered pH_i) and then sited in the NMR over the NMR coil for another hour. The fish were then subjected to a period of extreme hypoxia (less than 3% PO2) which lasted until PCr depletion approximated that seen following exercise in the same fish. Levels of PCr, ATP, and P_i in the carp were followed at rest, while hypoxic, and throughout the recovery period.

All experiments were performed in a water tight box, constructed of 3 mm plastic, which measured 50 x 12 x 10 cm (l·w·h), with an inflow tube through which non-recirculating, dechlorinated fresh water at 15°C or 25°C with a constant water flow of 0.5 $l \cdot min^{-1}$ flushed the gills. The effluent water was directed down the drain. A 5 mm neoprene gasket separated the box and the lid (6 mm plastic), which was secured to the box by 6 non-magnetic screws. Each fish, while in the box, was centered by foam sponges and slighting restrained by a thin plastic plate held in place by a balloon, which allowed some freedom of movement (a few mm), but not enough to obscure the NMR spectra. During rest and recovery periods the water was maintained normoxic by bubbling air through a series of three 11.4 l gas exchange cylinders. The water was delivered from the third gas exchanger to the box by an Eheim 1048 water pump. Hypoxic water was administered by bubbling N_2 through the same system.

Not all experiments were successfully completed. In a case where a fish was not restrained properly, subsequently moving too much (in a sense, exercising) or causing the area of muscle to become ischemic, the experiment was stopped and excluded from the pool. After a one week "rest period" these fish were reused in a repeated experiment. There were two cases, both at 25°C, where the fish died as a result of the hypoxia treatment and thus were excluded from the hypoxia data pool, while these individuals' data from the previous exercise experiment was still applied to the data pool. There were also two fish acclimated to 25°C that escaped from their tank prior to the hypoxia experiments. Consequently, sample sizes were n=7 for both experiments at 15°C, but n=10 and n=6 for the exercise and hypoxia groups at 25°C, respectively.

^{31}P NMR Spectra

Phosphate metabolites were measured *in vivo* by ^{31}P NMR spectra using a 1.89 T, large bore horizontal superconducting magnet (Oxford Instruments, Oxford, UK) connected to a Nicolet 1280 spectrometer. 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). Homogeneity of the magnetic field was optimized by shimming on the ^{1}H signal of the intracellular water of the carp. The same fish was used for shimming and hypoxia, whereas a similarly sized fish was used to shim in place of the exercised fish.

Spectra (1024 data points) consisted of 256 individual scans accumulated over 5.07 minutes at a nominal 90° pulse (width of 42 μs) and a delay between pulses of 1 second in a spectral window of ±1500 Hz. These parameters (number of scans, pulse width, delay, and window width) were adjusted on fish prior to the start of the experiments (and remained unchanged for all experiments) to achieve an adequate signal-to-noise ratio and resolution of metabolite peaks while minimizing the time necessary for a single spectrum. A low signal-to-noise ratio would lead to inaccurate data, while a lengthy time spectrum duration might not resolve changes in metabolite levels. A saturation curve (Figure 3) showed that with a 1 second delay it was possible to detect 55.4% and 64.5% of the unsaturated values of the PCr and ATP signals, respectively. Prior to analysis, concurrent raw signals were added together to improve on the signal-to-noise ratio, which allows more accurate analyses to be

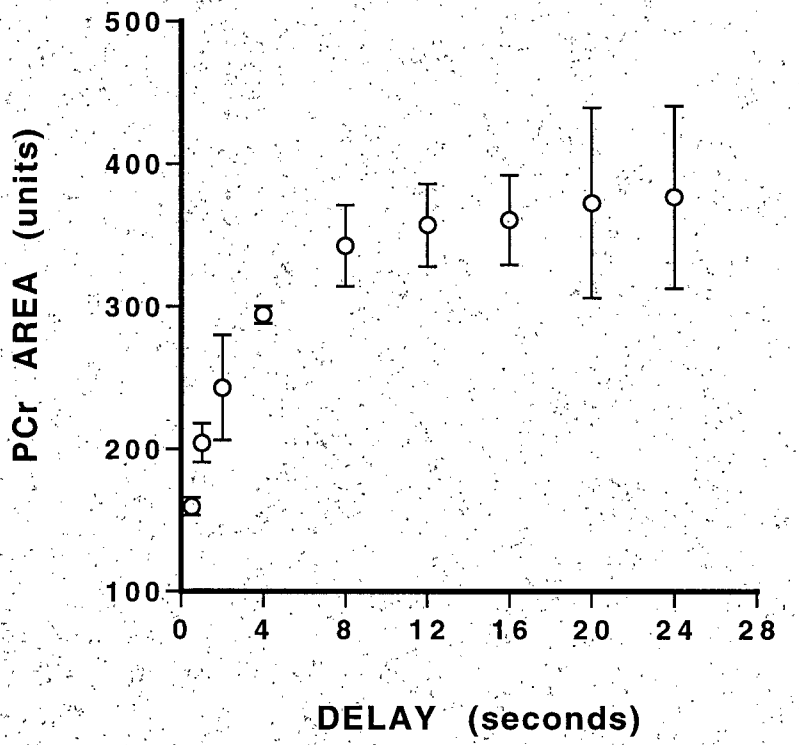

FIGURE 3. Saturation curve for PCr. The total number of nuclei detected by the NMR spectrometer, measuring 370 units, is independent of the length of the delay after 20 seconds. At a delay of 1 second, 55.4% of the PCr nuclei are detected. Values are means of three different analyses ± one standard deviation.

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 seemed to remain constant and when the P_i peak could no longer be resolved. For data analysis, recovery was assumed to be completed when PCr was at least 95% of the mean resting value of each group of subjects.

pH_i Measurements

Intracellular pH of the sample was estimated by the chemical shift (δ) of the inorganic phosphate peak relative to the PCr peak, which was the internal standard and was set to zero. The pH_i measurements were calibrated using one of the following equations:

$$@15°C \quad pH = 0.372\delta^3 - 4.890\delta^2 + 22.160\delta - 27.798 \quad (6)$$

$$@25°C \quad pH = 0.353\delta^3 - 4.712\delta^2 + 21.673\delta - 27.549 \quad (7).$$

The above equations were derived from data obtained from a carp muscle homogenate (Figure 4). The recipe used was modified from van den Thillart *et al.* (1989) and included: 300 mM sucrose, 20 mM EDTA, 10 mM NaF, 50 mM PCr, and ground carp muscle (33% of the final mass of the solution). All ingredients were homogenized and placed into 20 ml vials. The homogenate solution was made fresh each day. pH was altered by titrating 20-60 µl of 1M HCl or 1M NaOH to the homogenate and was measured using a Corning Chek-Mite© pH30 pH meter outfitted with a Fisher Calomel Microprobe Combination electrode. The pH meter was calibrated at either 15°C or 25°C with pH 4.00 potassium hydrogen pthalate and pH 7.00 potassium

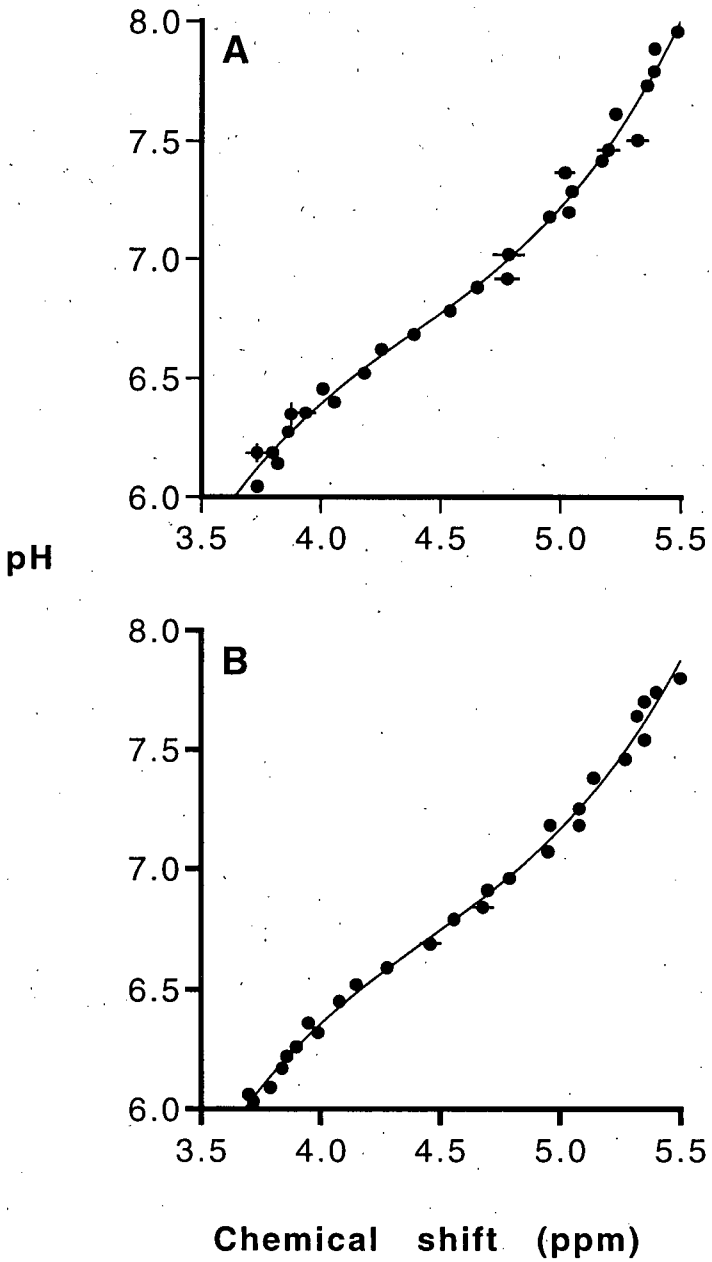

FIGURE 4. Muscle homogenate PCr-P_i chemical shift/pH measurements at 15°C (A, r^2=0.990) and 25°C (B, r^2=0.995).

phosphate buffers (VWR Scientific, West Chester, PA) and checked with pH 6.00 potassium hydrogen pthalate and pH 8.00 sodium phosphate buffers to insure proper calibration before measuring the homogenate pH. The range of pH measured was restricted to 6.0 to 8.0, representing the general physiological range of skeletal muscle. At lower pH values (<6.4), the homogenate PCr was depleted quite 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, the 20 ml homogenate sample was placed into a water jacket (3 mm plastic, 2.9 cm i.d., 7.4 cm o.d., 5.3 cm high) on the NMR coil and a spectrum was acquired. Each spectrum (1024 data points) consisted of 128 individual scans at a 90° pulse with a 1 second delay in a spectral window of ±500 Hz. These parameters yielded a total time of 3.32 minutes per spectrum. Following each spectrum a second pH measurement was taken. 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 zeroed and the chemical shift of P_i was recorded. Due to the subjectivity of manual phase shifting, each spectrum was analyzed 3-times, thus producing a mean that was plotted against the mean of the 2 pH measurements. All samples were maintained at either 15°C or 25°C by using a water bath (pH measurements) and the plastic water jacket (NMR measurements).

By placing the midline of the fish over the ^{31}P coil, the P_i peak from both the red and white muscle was detected and therefore, pH_i values for each muscle type. Late in the recovery periods, usually after the area of the PCr had stabilized, the amplitude of the P_i peak was too small to accurately distinguish it from background noise with a

reasonable level of confidence. Thus the profile of pH_i could not be followed past this point.

Statistical Analysis

PCr levels were standardized by reporting them as a proportion of $PCr \cdot (PCr+P_i)^{-1}$. All data points on graphs are plotted at 10 minute intervals, at the time represented by the end of the first and the beginning of the second in the pair of consecutive spectra that were added together. Following the exercise bout, the fish were put into the spectrometer as quickly as possible. This resulted in unmatched "start" times for the first NMR spectra (4-9 minutes) of the post-exercise recovery period. Whereas during the hypoxia experiments, the recovery period started consistently when the nitrogen bubbling through the gas exchanger was discontinued and was replaced by air. This point was designated time-0 of recovery and all successive data points, representing paired spectra, continued at 10 minute intervals following time 0. Therefore, data for post-exercise PCr and pH_i were interpolated between points by simple regression (CricketGraph III, Computer Associates International, Inc.), resulting in PCr levels and a pH_i representative of the same time intervals among exercised fish and the hypoxic recovery experiments that followed. These data were then analyzed for significant differences of PCr, pH_i, or time to recover between exercise and hypoxia, 15°C and 25°C, and over the recovery time using a two-factor ANOVA (SuperANOVA version 1.11, Abacus Concepts, Inc.). Comparisons of one variable between two groups were analyzed with a two tailed t-Test (Microsoft Excel 5.0, Microsoft Corporation), unless otherwise noted. All values are reported as means ± 1 standard deviation. All tests used a statistical significance of $p < 0.05$.

RESULTS

Experiments

Positioning the fish in the NMR and its orientation during recovery was initially a concern during preliminary experiments. In the present study it was necessary for the fish to be placed on its side (dorsal-ventral axis being horizontal); the sizes of the fish used, the concern of water leakage in the NMR, and availability of appropriately arranged probes were all considerations. Laterally oriented carp rebuilt PCr following hypoxia in times comparable with carp used by van den Thillart *et al.* (1989b) and van Ginneken *et al.* (1995), both using a probe requiring the fish to recover vertically (van den Thillart *et al.* 1989a). Physical restraint was necessary to immobilize the animals during the course of the experiment. Special care was taken to ensure that the fish was not compressed, causing ischemia of the muscle. In all experiments there was approximately 5 mm of clearance between the fish and the box and plate on either side of it. This allowed for enough immobilization to obtain repeatable and clear spectra, but also prevented ischemia of the muscle from excessive pressure. Furthermore, the times to rebuild PCr following exercise in this study do not seem to be different from times necessary for other species (Milligan and Wood 1986, Mommsen and Hochachka 1998, Arthur *et al.* 1992, Schulte *et al.* 1992) where orientation during recovery was not a factor. Thus, it is unlikely that being positioned laterally affects the time required to rebuild PCr following hypoxia or exercise.

NMRS allows repeated sampling over time in an individual subject. In this investigation our interests centered on the changes in PCr concentrations and the displacement of the P_i signal relative to that of PCr, a determinant in calculating pH_i.

To accurately follow the changes in PCr levels and the deviation of pH_i from its norm, several spectra had to be analyzed in succession for each experiment. Figures 5 and 6 illustrate the raw data from a typical completed exercise and hypoxia experiment, respectively. In these examples, and all other experiments, ATP levels remained unchanged through all stages of each experiment.

Comparison of biochemically analyzed muscle samples (measured in $\mu mol \cdot g^{-1}$) with deconvolution of PCr peaks (measured in dimensionless units) resulted in a weak relationship. Problems have been reported with sampling of muscle (Tang and Boutilier 1991), biochemical analysis (van den Thillart *et al.* 1990), or storage techniques (Madapallimattam *et al.* 1994) of muscle samples. It is possible that similar problems were experienced in this study. Therefore, the PCr data were reported as the ratio of PCr to the sum of PCr and P_i, rather than as a $\mu mol \cdot g^{-1}$ value. This method of reporting data has been used or is similar to recent NMR studies involving similar procedures and subjects (Chiba *et al.* 1990ab, Jackson *et al.* 1991, van den Thillart *et al.* 1989, van Waarde *et al.* 1990, van Ginneken *et al.* 1995).

Resting PCr and pH_i

Resting PCr levels between acclimation temperatures, 0.95±0.02 at 15°C and 0.96±0.02 at 25°C, were not significantly different from one another (p=0.225, two-tailed t-test). The cut-off point of recovery was assumed to be when PCr was at least 95% of the resting value of the carp in each temperature group. Thus, in terms of PCr levels, a fish was considered "recovered" when its PCr level reached 0.90 at 15°C and 0.91 at 25°C. This threshold was arbitrarily chosen because the PCr level of 3 of the 30 fish did not return to the mean resting value of the group,

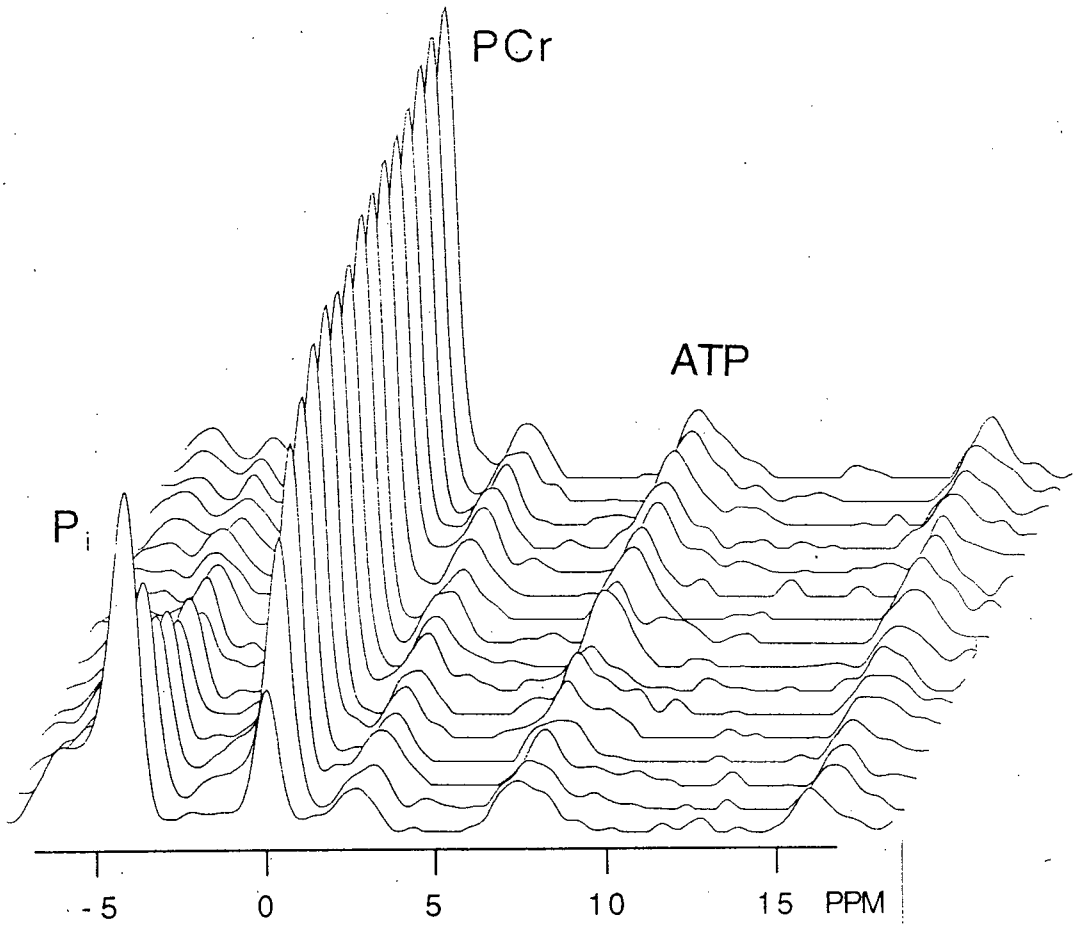

FIGURE 5. Typical series of spectra taken from carp acclimated to 15°C illustrating the progression of PCr rebuilding throughout the recovery period following exercise. As the recovery period begins there is a concurrent drop in P_i as PCr increases over time. Each plotted spectrum represent the summation of a consecutive pair of spectra and 10 minutes separate each plotted spectrum. Note that late in the recovery period the P_i peak shifts to the left, representing an increase in white muscle pH_i.

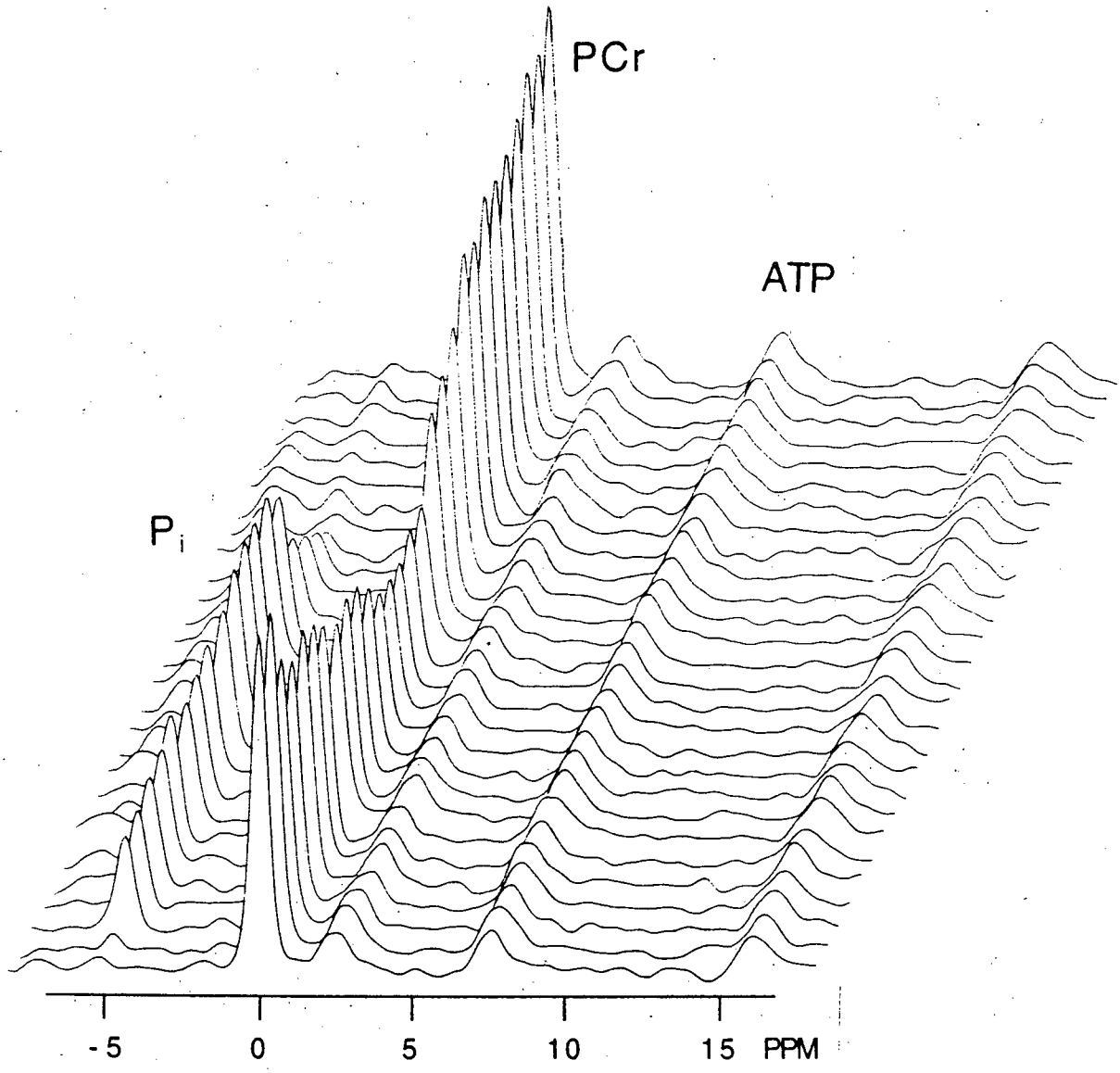

FIGURE 6. Typical series of spectra taken from carp acclimated to 15°C illustrating the progression of PCr depletion and rebuilding during and following hypoxia. The first spectra represents the metabolite concentrations at rest. The hypoxia period includes spectra 2 through 15, during which PCr drops and P_i rises. As the recovery period begins, at spectrum 16, there is a concurrent drop in P_i as PCr increases over time. Each plotted spectrum represent the summation of a consecutive pair of spectra and 10 minutes separate each plotted spectrum. Note that late in the recovery period the P_i peak shifts to the left, representing an increase in white muscle pH_i. ATP remained unchanged during hypoxia and during the recovery period.

but in all three cases PCr was rebuilt to at least 95% of the resting value. Furthermore, the "recovered" PCr values were not significantly different between either treatment ($p=0.083$) or temperature ($p=0.103$, two-factor ANOVA).

Intracellular pH values were calculated with the chemical shift of the P_i peak and equations 6 and 7. The resting pH_i values of white muscle of carp acclimated to 15°C and 25°C were 7.33±0.14 and 7.25±0.15, respectively, and were not significantly different from one another ($p=0.077$, two-tailed t-test). The experiments were typically allowed to continue for a considerable length of time after PCr levels had returned to normal, up to a total time of more than 6.5 hours, to allow the "recovery" of pH_i to be followed. This is a much slower process than PCr recovery, so the P_i peak of the spectra decreased to a point where it could no longer be accurately resolved. Therefore, analysis of pH_i ended with the spectrum just previous to this point.

Effects of Temperature on PCr Depletion and Rebuilding

Carp acclimated to 25°C required 44% less time (0.96±0.31 h.) than 15°C acclimated fish (171±0.70 h.) for their PCr stores to be significantly depleted by hypoxia ($p=0.017$, two-tailed t-test). Despite needing nearly twice the time to deplete PCr levels during the hypoxic periods, the decrease in PCr over time in carp during the hypoxic period at 15°C was not different from that in 25°C acclimated fish (Figure 7; $p=0.384$, two-factor ANOVA). PCr measurements at the initiation of the recovery period for exercise and hypoxia experiments using 15°C and 25°C acclimated fish were 0.32±0.07, 0.45±0.09, 0.38±0.11, and 0.35±0.12, respectively (Table 3, Figure 8). There were no significant differences between 15°C and 25°C ($p=0.670$) or between exercise and hypoxia at either temperature

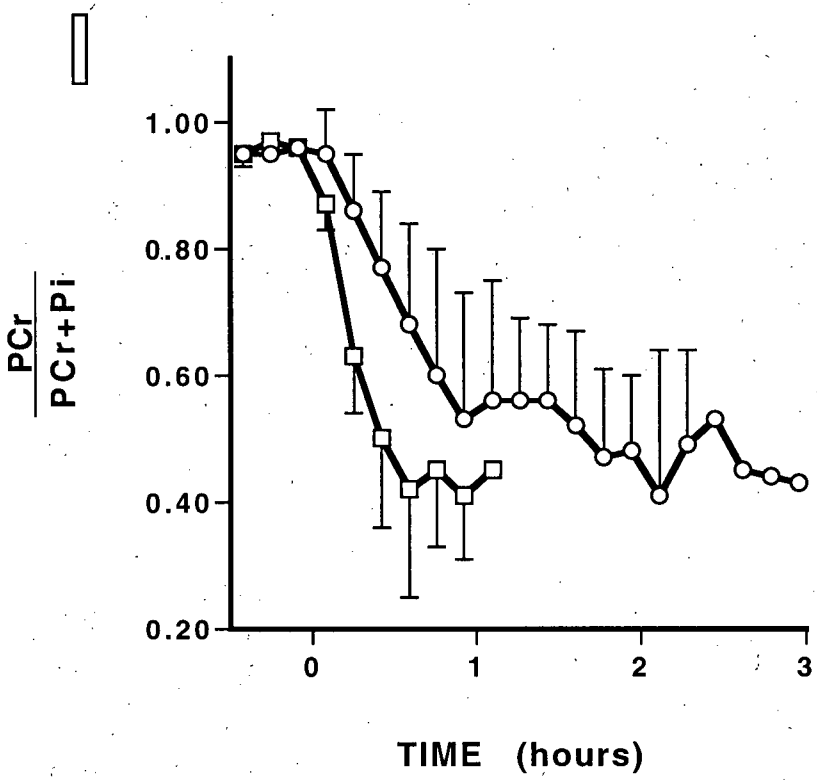

FIGURE 7. PCr levels in 15°C- (circles) and 25°C- (squares) acclimated carp during hypoxic exposure (20 Torr).

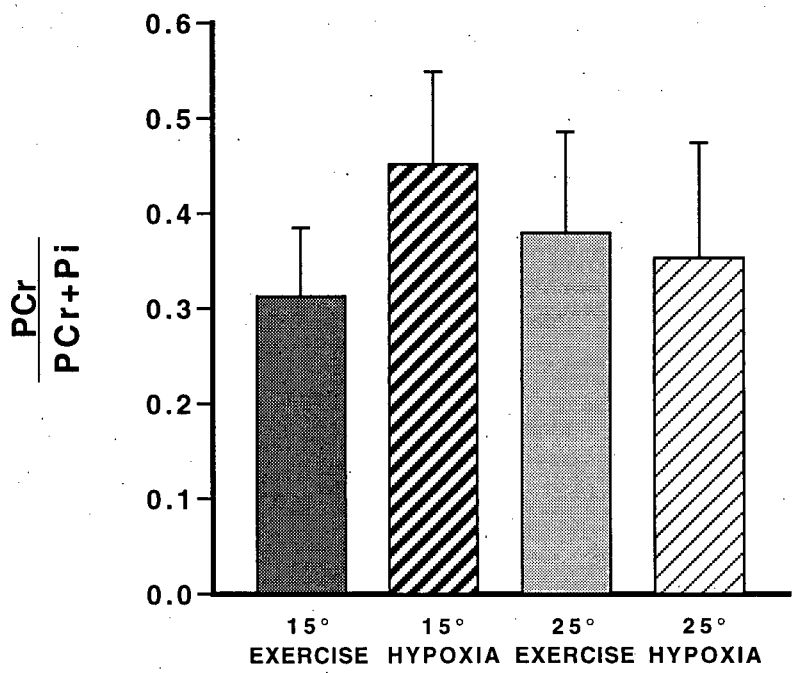

FIGURE 8. PCr levels at the initiation of the recovery period following either exercise or hypoxia in 15° and 25° acclimated fish.

(p=0.143, two-factor ANOVA). Temperature had no significant effect on the time necessary for PCr levels to return to 50% or 75% of recovered values following exercise or hypoxia, nor, in fact, for full recovery at either 15°C or 25°C (Figure 9; p>0.246, two-factor ANOVA).

Throughout the entire recovery period, the pattern of PCr rebuilding increased logarithmically. Temperature did not have a significant impact on the progression of PCr levels during recovery from either exercised (Figure 10a; p=0.285) or hypoxic fish (Figure 10b; p=0.613, two-factor ANOVA). Regression analysis of the data resulted in the following equations for exercised carp at 15°C and 25°C:

$$PCr = 0.437 \log TIME + 0.592 \qquad r^2 = 0.991 \qquad (8)$$

$$PCr = 0.338 \log TIME + 0.628 \qquad r^2 = 0.983 \qquad (9)$$

and post-hypoxic carp at 15°C and 25°C, respectively:

$$PCr = 0.302 \log TIME + 0.818 \qquad r^2 = 0.912 \qquad (10)$$

$$PCr = 0.332 \log TIME + 0.859 \qquad r^2 = 0.892 \qquad (11).$$

Effects of Temperature on pH_i

There was a 10 minute delay into the hypoxic period at both temperatures until pH_i values began to decrease (Figure 11). Once this progression began, the pH_i of carp white muscle at 25°C dropped at a more rapid rate, being consistently and significantly lower than pH_i in 15°C acclimated fish throughout the hypoxic period (p<0.001, two-factor ANOVA). At the onset of the recovery period the pH_i values of carp muscle at 15°C following exercise and hypoxia were 6.57±0.07 and 6.95±0.10

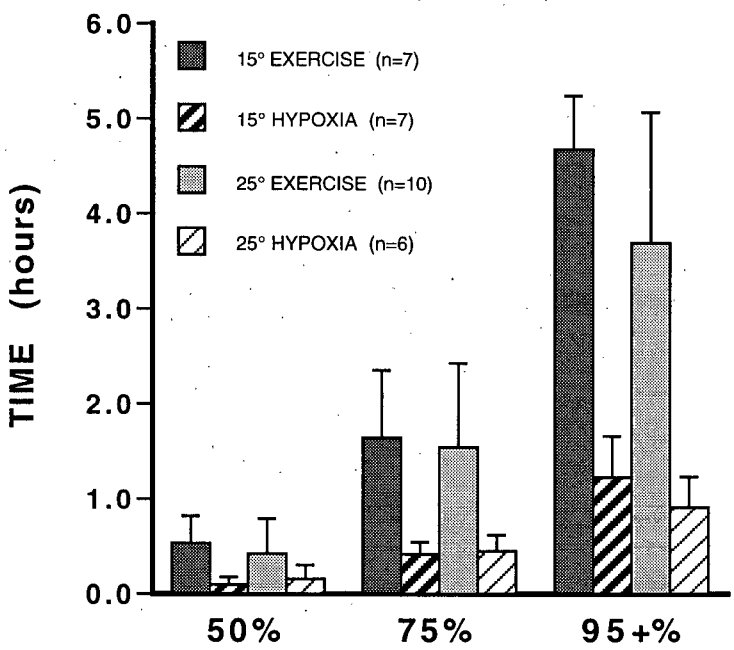

FIGURE 9. Comparison of the time necessary to recover PCr levels to 50%, 75% and 95+% of rest in 15° C and 25° C acclimated carp following exercise or hypoxia.

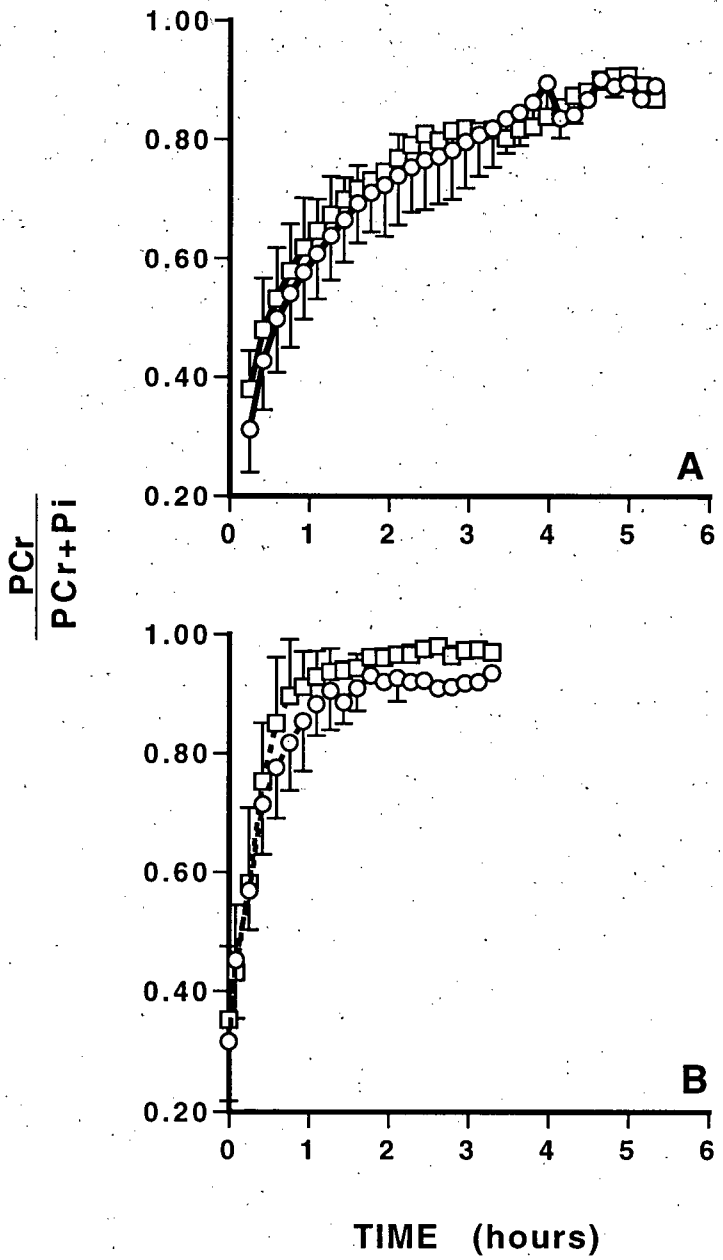

FIGURE 10. Time-course of PCr rebuilding in exercised (A) and post-hypoxic (B) fish acclimated to 15°C (circles) and 25°C (squares). Note that this figure contains the same data as Figure 15 but here temperates are paired within exercised and hypoxic groups to illustrate a lack of influence of temperature on the recovery of PCr levels.

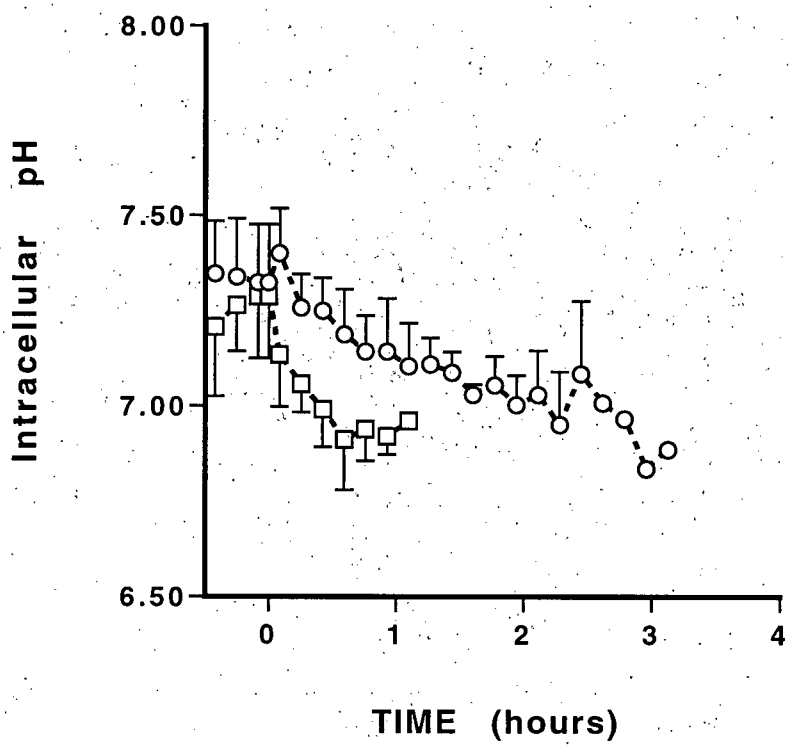

FIGURE 11. Time course of intracellular pH in 15°C (circles) and 25°C (squares) acclimated carp at rest and during hypoxia.

and in carp acclimated to 25°C the pH_i values were 6.42±0.16 and 6.86±0.10 in exercised and hypoxic fish respectively. All values were significantly different from resting pH_i values ($p<0.001$, two-factor ANOVA). Temperature had no effect on the severity of the decrease in pH_i in either exercised ($p=0.053$) or hypoxic fish (0.126, two-factor ANOVA), as the lowest pH_i values between temperature groups were not significantly different.

The pH_i continued to drop in each group, for 2.31±0.95 hours to 6.32±0.17 and for 1.03±0.27 hours to 6.71±0.15 in exercised and post-hypoxic carp at 15°C and for 1.35±0.50 hours to 6.23±0.17 and for 0.76±0.30 hours to 6.55±0.20 in exercised and post-hypoxic carp at 25°C (Figure 12). In all groups, the mean lowest pH_i value recorded was significantly lower than pH_i at the beginning of the recovery period ($p<0.021$, two-factor ANOVA). In both exercised and post-hypoxic carp, temperature had no significant effect on lowest pH_i ($p>0.120$, two-factor ANOVA), nor on the time needed to reach that pH_i value ($p>0.103$, two-factor ANOVA). Once pH_i began to rise, 15°C and 25°C exercised groups increase together as did 15°C and 25°C post-hypoxia groups, i.e. temperature did not have a significant influence on the recovery of pH_i following either treatment (15°C; $p=0.401$; 25°C; $p=0.127$, two-factor ANOVA). At both 15°C ($p=0.071$) and 25°C ($p=0.054$, two-factor ANOVA), pH_i returned to normal in post-hypoxic fish, while the P_i could still be resolved, 7.21±0.30 and 7.15±0.16, respectively. When the exercise experiments were finally terminated, the final pH_i values of exercised fish at 15°C and 25°C, 6.68±0.18 and 6.77±0.19 respectively, were still significantly different from resting values ($p<0.001$, two-factor ANOVA).

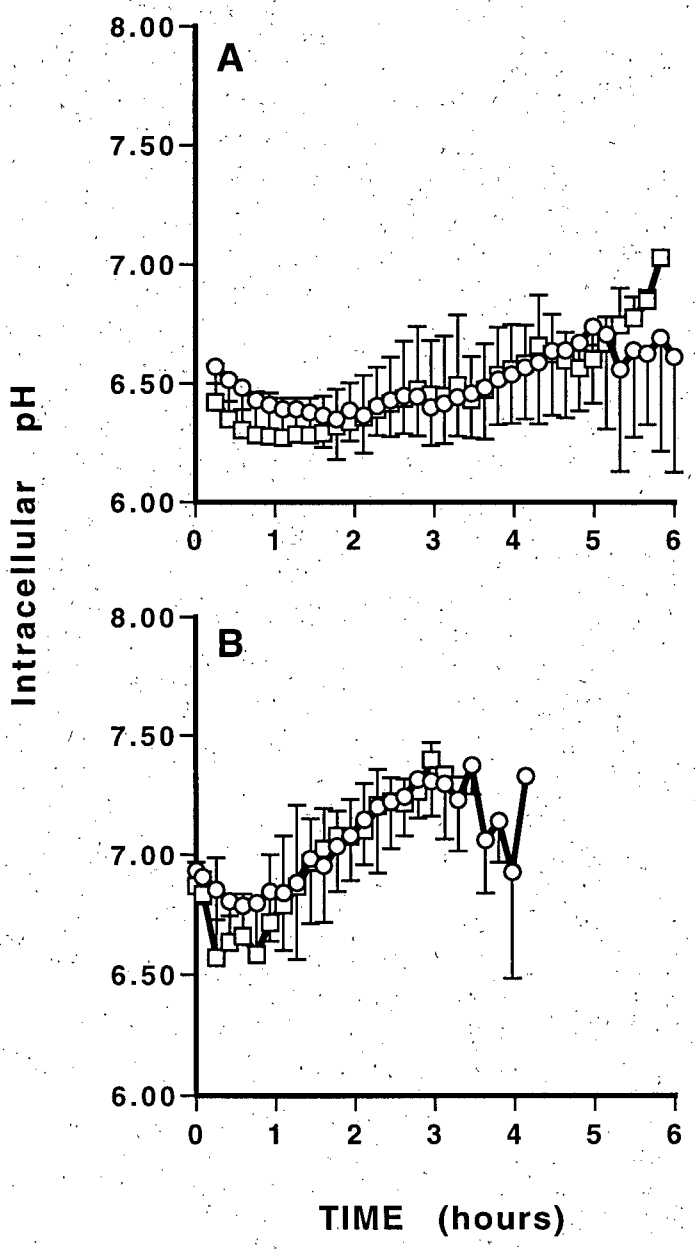

FIGURE 12. Time course of intracellular pH changes in white muscle of 15°C (circles) and 25°C (squares) acclimated carp following intense exercise (A) and hypoxia (B). Note that this figure includes the same data as presented in Figure 13, but temperatures are paired within exercise or hypoxia groups to illustrate the lack of influence of temperature on the recovery of white muscle pHi.

Effects of Treatment on pH_i

When subjected to exhaustive exercise, the pH_i values of carp acclimated to 15°C and 25°C were significantly different from that following extreme hypoxia (Figure 13). White muscle pH_i values immediately following the hypoxia period were higher than the initial pH_i values at the beginning of the post-exercise recovery rates at both 15°C and 25°C ($p<0.001$, two-factor ANOVA). The time to the end of the initial stage of the recovery periods, during which pH_i continued to drop, was significantly different between exercised and post-hypoxic carp at 15°C ($p=0.005$) and 25°C ($p=0.020$, two-factor ANOVA). Furthermore, the severity of the decrease in pH_i between exercised and post-hypoxic fish at either 15°C ($p<0.001$) or 25°C acclimated fish ($p=0.004$, two-factor ANOVA) were significantly different from one another. The rate and the level of pH_i recovery following hypoxia was consistently and significantly higher than that following exercise at both temperatures ($p<0.001$, two-factor ANOVA).

Interaction Between pH_i and PCr Rebuilding

The effect of the exercise or hypoxia treatments, manifested as a significantly lower pH_i in exercised fish relative to hypoxic fish, has direct ramifications on the rebuilding of PCr. Post-hypoxic fish required significantly less time, about one-quarter of the time, to rebuild PCr to 50%, 75%, and full recovery compared with exercised fish (Figure 9; $p<0.001$, two-factor ANOVA). Carp recovering from hypoxia consistently had a higher PCr level than fish recovering from exercise at both temperatures (Figure 14; $p<0.001$, two factor ANOVA). In fact, PCr had leveled off for well over one hour after the hypoxia experiments in both temperature groups, and the hypoxia experiments were actually terminated before exercised fish rebuilt their PCr to comparable levels. Regardless, the final PCr values at the end of

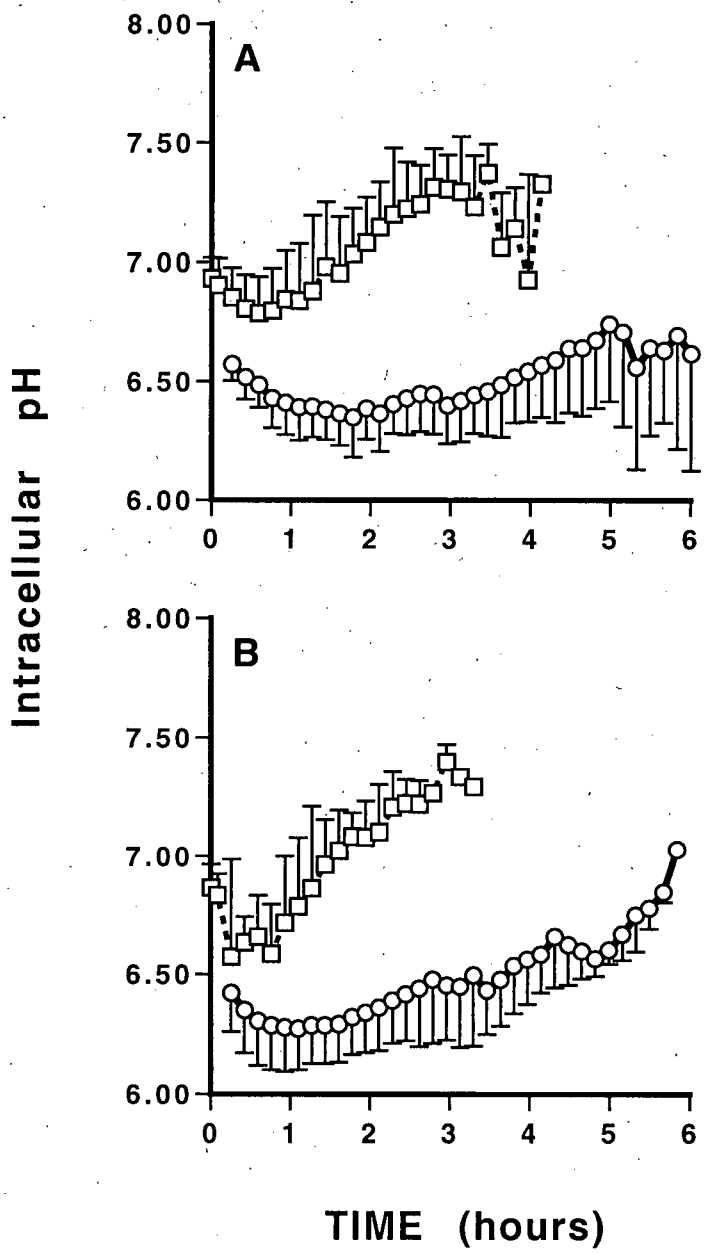

FIGURE 13. Time course of intracellular pH changes in white muscle of 15° C (A) and 25°C (B) acclimated carp following bouts of exercise (circles) or hypoxia (squares). Note that even after the recovery period began (at 0 hours) the pHi continued to fall. When shown with the PCr recovery profile of the same groups of fish it is evident that white muscle pHi does not begin to recover/increase until PCr levels have reached at least 81% of recovered values (see Figure 15).

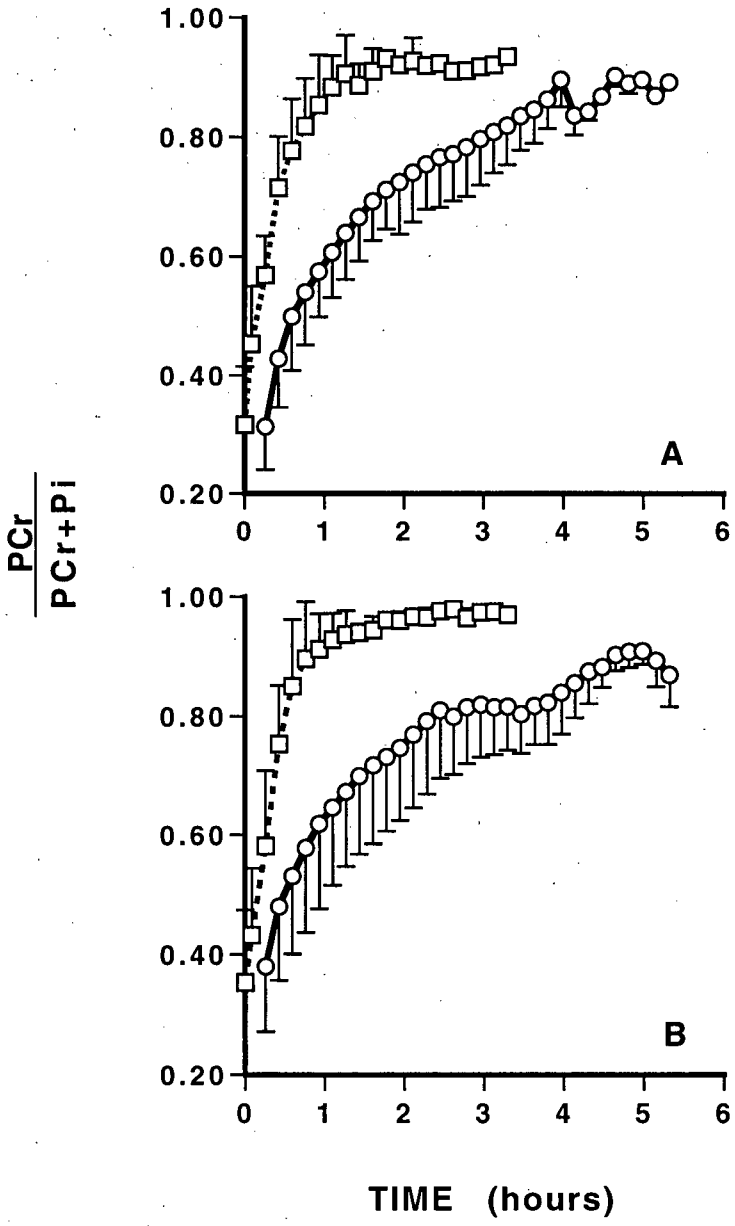

FIGURE 14. Time-course of PCr rebuilding in 15°C (A) and 25°C (B) acclimated carp following exercise (circles) and hypoxia (squares). There is a 10 minute time lag between the first reported points in the data from hypoxic and exercised fish, but the difference in PCr levels are real. Although there is no T0 data in exercised fish, since about 10 minutes were required to prepare the fish for the NMR procedure, the fish was still recovering during that time, thus the post-hypoxic and post-exercise values are comparable at any given time.

the experiments between exercised and post-hypoxic fish were not significantly different at either 15°C ($p=0.083$) or 25°C ($p=0.631$, two-factor ANOVA).

Figure 15 combines data from Figures 10, 12, 13, and 14 in order to more clearly illustrate the relationship between PCr level and the onset of pH_i recovery. Intracellular pH in 15°C acclimated carp (Figure 15a) did not consistently increase until PCr had attained 80.9% and 85.4% of full recovery in exercised and post-hypoxic carp, respectively. Similar values were also noted in 25°C acclimated fish (Figure 15b), requiring PCr to be 81.8% or 91.2% of full recovery in exercised and post-hypoxic fish before pH_i started to rise. There is a strong correlation between the descending pH_i continuing into the first portion of the recovery period and the rebuilding of PCr during that time (Figure 16) in 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).

Red Muscle pH_i

The pH_i of red muscle can be calculated from a second P_i peak (Figure 17). This peak was typically smaller than the white muscle P_i peak and was often difficult to resolve accurately. The range where the red muscle peak can usually be found overlaps with the range where sugar phosphates, like products of glycolysis, are also found. Thus, the changes in red muscle pH_i could be confidently followed in only a few experiments and even then variability was high. Due to the questionable nature of the analysis and a lack of confidence in these data no results concerning red muscle pH_i will be reported.

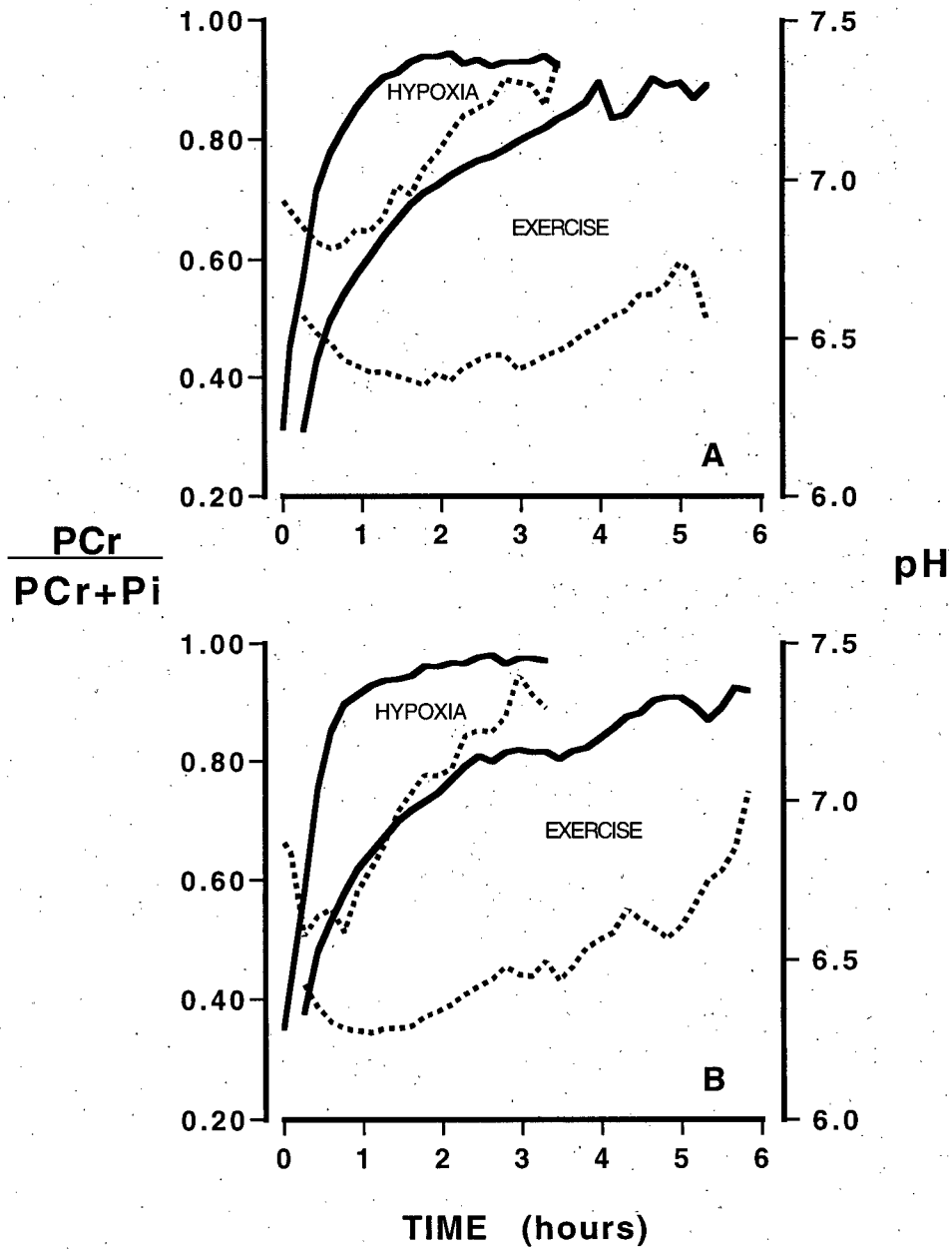

FIGURE 15. Time course of PCr (solid line) and white muscle pHi (dotted line) recovery in 15°C (A) and 25°C (B) acclimated carp following exercise and hypoxia. There is not a sustainable and continuous recovery/increase in pHi in any group until PCr stores have been rebuilt to at least 81% of recovered levels. This increase is not time dependent as this point ranges from 1 to 3.5 hours depending on the condition.

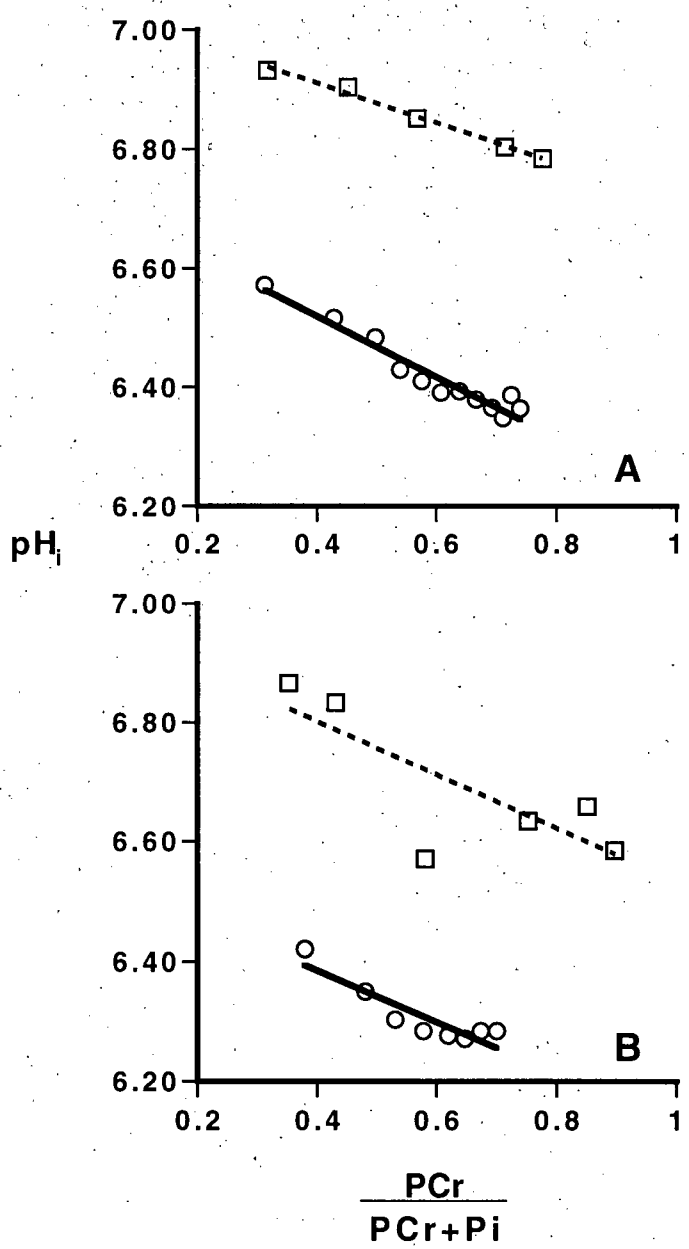

FIGURE 16. Correlation between the continuing decrease of intracellular pH and increase of PCr levels into the recovery period of exercised (squares) and post-hypoxic fish (circles) at 15°C (A) and 25°C (B). These data include readings of pH_i and PCr until the time when pH_i began to increase.

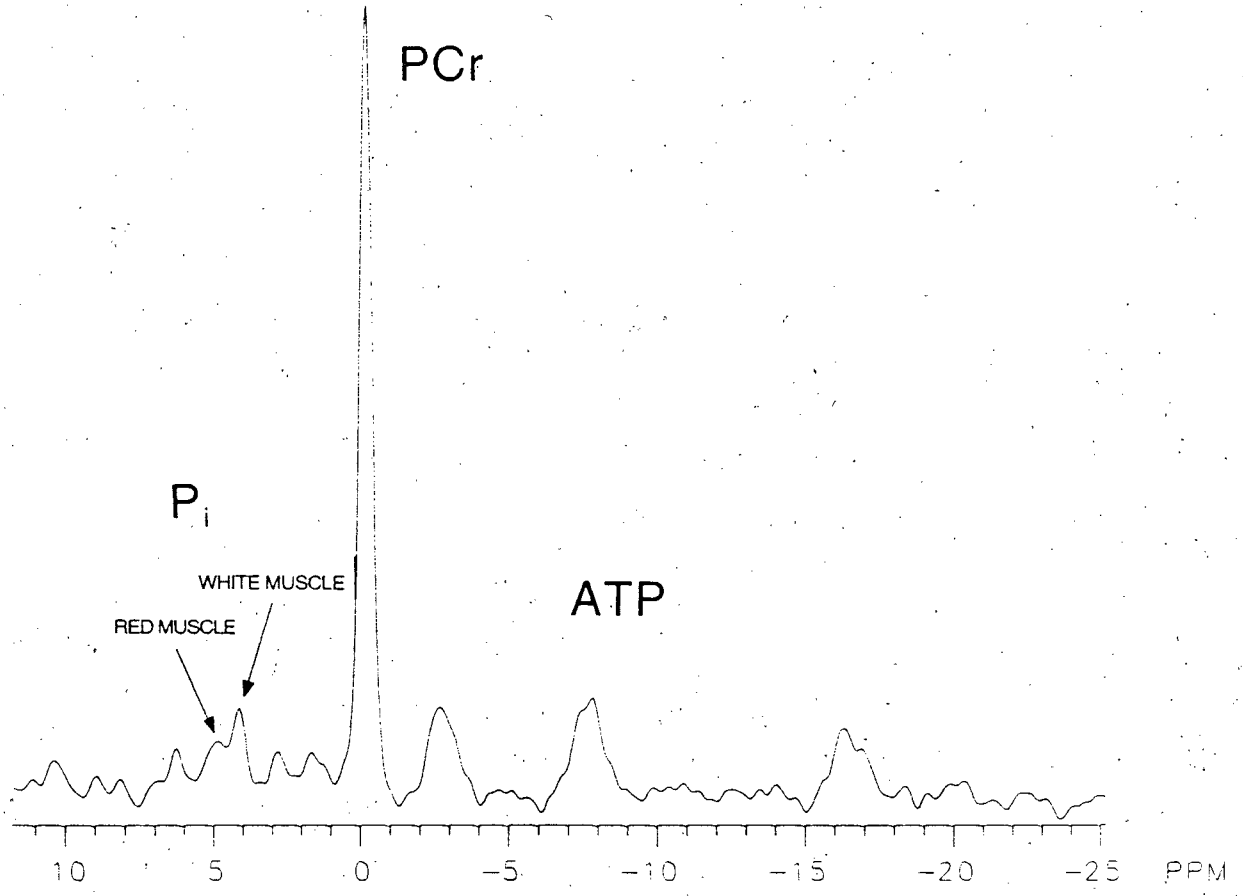

FIGURE 17. Typical spectrum taken from a carp acclimated to 15°C toward the end of its recovery period. Note the split P_i peak representing the two pools of P_i in white muscle and red muscle. The chemical shift of these peaks correspond to pH_i values of 6.49 and 7.14 in white and red muscle, respectively.

DISCUSSION

Relationship Between PCr and pH_i

When animals are presented with a relatively low oxygen level they unfailingly shift their energy producing pathways to those that produce protons, i.e. glycolysis. Carp white muscle develops a severe acidosis following intense exercise and hypoxia (Figure 13), more so during exercise, in spite of the consumption of a proton from the hydrolysis of a PCr molecule which, at least initially, causes an alkalization of the muscle (Meyer *et al.* 1986, 1991). This alkalization may be seen in these data (Figure 11). During the first 10 minutes of hypoxia, the pH_i remained stable in 25°C acclimated carp, whereas the pH_i increased by 0.08 units in 15°C acclimated fish. The end of this stage represents the initiation of glycolysis in a supporting role to PCr hydrolysis for ATP generation. Glycolysis does not start until after PCr levels have initially been perturbed (Dobson *et al.* 1987). A drop in PCr and subsequent rise in P_i are required to activate glycolysis, as the P_i is necessary as a substrate for glycogen phosphorylase and as an activator for phosphofructokinase (Radda 1996). This delay of glycolytic initiation is evident in Figure 11, illustrating a stabilization of pH_i for the first 10 minutes into the hypoxic period.

Carp acclimated to 15°C and 25°C showed a substantial white muscle acidosis following exercise and extreme hypoxia (Figures 12 and 13) compared with white muscle resting levels (Figure 11). In fact, exercised fish underwent a significantly larger drop in pH_i than did hypoxic fish at both 15°C and 25°C by the end of each anaerobic stress. The acidosis evident at the beginning of these recovery periods is primarily due to the protons accumulated as a result of glycolysis. The fish were exercised rigorously so that they would be forced to burst-and-glide, a method of

swimming powered anaerobically by white muscle (Bainbridge 1960, Black *et al.* 1962). Regardless of swimming technique, carp generally rely heavily on anaerobic metabolism to power swimming, more so than other fishes (Jones 1982). Furthermore, carp use glycolysis as a means of dealing with anaerobic metabolism during hypoxia (reviewed by van den Thillart and van Waarde 1985). During anaerobic glycolysis two end-products accumulate, lactate and protons. From Table 2, it is clear that there is less lactate produced, and thus, less glycolysis occurring, during hypoxia or anoxia compared with exercise. Since there is a 1:1 stoichiometric relationship between lactate and H^+ (Mommsen and Hochachka 1983), the concentration of lactate produced as a result of an anaerobic stress can serve as an estimate of the extent of the glycolytic activity that has occurred and a measure of the pH of the tissue. Therefore, one can infer that muscle pH_i following exercise will be more acidic than following hypoxia, a statement supported by the data in the current study (Figures 12 and 13).

Upon reoxygenation, the coupling of CK and glycolysis disappears as mitochondrial respiration commences (van Waarde 1990). In spite of the cessation of glycolysis as the fish begins to recover, pH_i continued to fall for up to 2.5 hours in exercised fish at 15°C and declined another 0.31 pH units in 25°C carp recovering from hypoxia. A plausible explanation for this decrease is the rebuilding of PCr (equation 3 in reverse). It has been suggested that the break-down of PCr causes an increase in pH_i (Amoreno *et al.* 1990), but the reverse seems to also be true, i.e. the rebuilding of PCr causing a decrease in pH_i. The formation of one molecule of PCr from creatine and P_i yields a proton, therefore it is reasonable that as PCr is being rebuilt, a further acidification of the muscle will occur (Meyer *et al.* 1986, van den Thillart *et al.* 1989, van Waarde *et al.* 1990, Curtin *et al.* 1997). This apparent dependence of PCr rebuilding on pH_i is best illustrated in Figures 15 and 16. The

time into the recovery period when pH_i begins to rise sharply corresponds closely to the time necessary for PCr to be rebuilt to near resting levels. It is not until PCr levels are replenished to 81-91% of recovered levels that pH_i begins to consistently increase. During this stage of recovery, the drop in pH_i is strongly correlated with the rise in PCr. The same trend, a delay in the recovery of pH_i until PCr has recovered to about 80%, has been seen previously in carp and tilapia subjected to graded levels of hypoxia (van den Thillart 1989, van Waarde *et al.* 1990). At this point, the rate of PCr rebuilding has nearly leveled to a plateau, thus the production of protons from PCr resynthesis is diminished, leading to an increase in pH_i as protons are siphoned from the pool by other reactions.

ATP is generated primarily by two pathways, glycolysis and oxidative phosphorylation. At rest, under normal environmental and physiologic conditions, ATP is synthesized effectively by oxidative phosphorylation. In times of anaerobic stress, i.e. during intense exercise or hypoxia, glycolysis takes over as the primary ATP generator. When an organism recovers from this stress ATP generation shifts back to oxidative phosphorylation as the organism rests and/or oxygen tensions of the tissue become normoxic. Therefore, the method of ATP generation, and thus support of the CK reaction, depends on the state of the organism, whether it is resting and aerobic or stressed and anaerobic (Bessman and Geiger 1981). Fish white muscle is used typically to power intense swimming, an event fueled by the coupling of MM-CK (highest in fast twitch muscle) and glycolysis, and thus, to support PCr-driven ATP maintenance during this time. Although, at rest or during recovery following anaerobiosis, oxidative phosphorylation coupled with Mi-CK (highest in mitochondria) maintains ATP levels within the cell.

At the beginning of the recovery period the mean differences in pH_i between exercise and hypoxia were 0.38 and 0.44 pH units at 15°C and 25°C, respectively. As each recovery period progressed, the difference in pH_i between exercised and post-hypoxic carp at each temperature ranged from 0.15 to 0.09 pH units (Figure 13). These differences in pH_i were the primary factors in the decreased rates of recovery and had direct influence on the times necessary to rebuild PCr between treatments, i.e. that post-exercise fish require more time, 300% more time, to rebuild PCr than post-hypoxia fish (Figure 9). Wallimann and co-workers (1984) have determined that the pH optimum for the CK reaction is about 6.7, with the activity dropping off quite dramatically as the pH increases or decreases. Figure 13 offers convincing evidence that because white muscle pH values hover around 6.7 following hypoxia, compared with pH_i values less than 6.4 during recovery from exercise, there is a more rapid rate of PCr rebuilding following hypoxia than exercise.

If it is assumed that resting white muscle has intramuscular PCr concentrations of approximately 30 $\mu mol \cdot g^{-1}$ white muscle (Hochachka 1985), and exercise depletes [PCr] by 70% at 15°C (current study), then 21 $\mu mol.g^{-1}$ of H^+ would be expected to be produced as PCr is rebuilt. Resting white muscle pH_i of 7.33 in 15°C-acclimated carp translates to a "background" $[H^+]$ of approximately 65 nM. Immediately following exercise the pH_i is 6.57, thus the $[H^+]$ is 257 nM. If all the H^+ from PCr synthesis directly contributes to the drop in pH during the first 1-3 hours of the recovery period, until PCr has been fully replenished, the pH_i would decline far below physiologic levels. Falling only to a minimum of 6.32, the net H^+ production from PCr replenishment is 63 nM, not 21 mM. The buffering capacity of the muscle is mostly responsible for preventing the drastic decrease in pH_i that would result if all the H^+ from PCr replenishment were unbuffered. Castellini and Somero (1981) have reported that the buffering capacities of "resting" rainbow trout and bass to be

60 and 62 respectively. Assuming that carp have a similar buffering capacity (60 ß), the muscle can buffer 60 mM worth of protons over the range of one pH unit, well above what is necessary to neutralize the $[H^+]$ produced by PCr resynthesis. However, as the pH_i drops lower and lower, the buffering of the muscle becomes less and less effective. Since the pH_i has, by this point, already debilitated the state of the muscle and reducing the buffering capacity of the muscle, the 60 mM H^+ that would be potentially buffered is a gross overestimate of the actual buffering capactity of the muscle, thus not all, but some of the protons produced by PCr resynthesis are not buffered and contribute to the further decrease of pH_i during PCr recovery.

Influence of Temperature on pH_i and PCr

Temperature affects pH_i during hypoxia, triggering a more rapid decrease in PCr (Figure 7) and pH_i (Figure 11). Hubley and co-workers (1997) report that in goldfish there is a six-fold increase in the V_{max} of CK at 25°C compared with those acclimated and tested at 5°C. Thus, one would expect to find that warmer fish deplete and rebuild PCr faster than cooler fish. This seems to be the case only in the depletion of PCr. During hypoxia PCr levels and pH_i decrease with time, dropping more rapidly in carp at 25°C than those at 15°C. This reduction in pH_i and PCr during hypoxia between 15°C and 25°C acclimated carp is the extent of the influence of temperature in this investigation. As the pH_i continues to fall, as a result of PCr rebuilding, following both exercise and hypoxia, neither the recovery of PCr levels, the time required to rebuild PCr, nor the changes in pH_i were affected by temperature. The influence of temperature on these reactions effectively becomes masked. The Q_{10} values are 0.77 and 1.10 between 15°C and 25°C in exercised and post-hypoxic groups, respectively, further elucidating that during the recovery periods of PCr and pH_i temperature is ineffectual. Hypoxic fish can recover at a

more rapid rate because their pH_i is higher than that of exercised fish (Figure 13), never falling far below the pH optimum for the CK reaction. The drastic decreases in pH_i evident in both temperature and treatment groups plays the primary role in dictating the reaction rates in exercised or post-hypoxic muscle.

Another likely, but secondary, contributing factor is to change the morphology of the carp musculature. White muscle of cold-acclimated cyprinids has a larger population of mitochondria compared with those fish accustomed to warm water (Johnston 1982). An elevated mitochondrial count means there are more metabolic enzymes present in the muscle. Furthermore, ATP is maintained at a higher level in cold acclimated fish (Johnston 1982), acting to drive the CK reaction in the direction of PCr resynthesis. This heightened concentration may act to compensate for a lessened activity due to the cooler temperature (Johnston and Maitland 1980). Furthermore, the more tightly packed the mitochondria are in the muscle cells, the less distance metabolites have to diffuse through the cytosol from one compartment to the next. The reduction in diffusion distance may compensate for lower diffusions coefficients in cold versus warm acclimated fish (Eggington and Sidell 1989). As 15°C and 25°C carp rebuild PCr at the same rate, the sum of these factors must play at least a limited role in compensating for a low body temperature.

PCr Rebuilding Across Class Lines

The present data exhibit a reasonable similarity to other teleost species at comparable temperatures with respect to PCr replenishment times (Table 3). The 15°C and 25°C acclimated carp subjected to hypoxia needed less time to rebuild PCr stores than other hypoxia-tolerant species, goldfish and tilapia. In exercised rainbow trout, a species considered more aerobic than carp, even less time is

required for white muscle PCr levels to reach resting values. A substanial disparity is evident in the rates of PCr replenishment when teleosts are compared with mammals. Fish, as a class, need far more time to rebuild PCr following depletion than do mammals. Generally, mammals rebuild PCr levels following anaerobic activity in the order of minutes (Table 3), while in most cases teleosts require well over an hour, if not many hours to rebuild PCr stores. Potential reasons for this tremendous dissimilarity of recovery rates are explained below.

Fish muscle, in terms of fiber types, is very homogenous, e.g., the white muscle region of fish is about 98% fast-twitch muscle, possessing very little mixing of fiber types (Randall and Daxboek 1982). This is not the case in mammals, where there is a great deal of heterogeneity of fiber types within a given muscle (Saltin 1988, Simoneau and Bouchard 1989). In mixed mammalian muscle, there can be a two- to five-fold increase in the amount of perfusion between fast- and slow-twitch fibers within a heterogenous muscle (Armstrong *et al.* 1989). Mejsnar and co-workers (1992) showed that by increasing the perfusion rate of a muscle, PCr concentration increases by 39%. With a greater capillary-muscle interface more metabolites (lactate, protons) could be moved away from the muscle. This diffusion of protons would allow for a quicker return of pH_i to resting values, and as pH_i rises closer to "normal", PCr will be rebuilt more quickly. This seems to be what is seen in in the mammalian system. Lactate can be transported from fast glycolytic to slow oxidative muscle fibers via the blood stream (Hochachka 1985). Although in fish, the lack of sufficient capillarization means that lactate and H^+ cannot be moved rapidly from white to red muscle in fish. This low amount of perfusion is a likely reason why metabolites produced in fish white muscle remains within white muscle, specifically, lactate and protons produced by anaerobic glycolysis are metabolized *in situ* (Milligan and Wood 1986, Moyes *et al.* 1992, Schulte *et al.* 1992, Milligan and

Girard 1992) and are not transported out of the cell. Moreover, lactate is actually sequestered by white muscle from the plasma in teleosts (Milligan and Girard 1992), presumably to provide a carbohydrate source for rebuilding muscle glycogen, the main source of muscular anaerobic energy in teleosts (Milligan and Wood 1986). At this final destination, lactate is oxidized, yielding ATP rather than spending it, as in anaerobic gluconeogenesis. The greater amount of available ATP during the recovery period, the quicker PCr should be replenished.

What seems to be the key factor in determining how quickly PCr can be restored in muscle is the amount of blood flow the muscle receives. Carp (Johnston 1982), and other teleosts (Mosse 1978) have a very low amount of capillarization in white muscle compared with red muscle. Crucian carp fast glycolytic muscle at 37°C has 0.87 capillaries supplying each fast-glycolytic fiber (calculated from Johnston 82). Since white muscle is poorly vascularized it does not rely on blood flow to deliver reaction substrates on demand, e.g. white muscle supports ATP turnover by glycolysis and endogenous glycogen (Krause and Wegener 1996). However, in humans, where the blood flow depends not only on the vascularization of the muscle, but also on the anatomy (fast- versus slow-twitch fibers) of the muscle (Fujita *et al.* 1977), the number of capillary contacts per fast glycolytic fiber ranges from 2.59 to 5.11 depending on gender and training (Plyley 1990). There are contradicting opinions as to whether perfusion of white muscle in fish is increased or decreased with exercise. Exhaustive exercise produces vast increases in perfusion of trout white muscle (Wardle 1978, Neumann *et al.* 1983), while during sustained swimming there is a ten-fold decrease in the perfusion of trout white muscle (Randall and Daxboeck 1982), whereas cod show no increase at all (Axelsson and Nilsson 1986). An increased perfusion would increase the amount of blood, and

thus oxygen, entering the tissue allowing oxidative metabolism to progress, thus permitting PCr levels to be replenished more efficiently.

Teleosts have 4.5- to 15-fold (flounder and trout, respectively) higher CK activity than humans (Newsholme *et al.* 1978, Simoneau and Bouchard 1989). Based on the shuttle hypothesis, having CK is not enough, there needs to be a source of ATP, without sacrificing the substrate for ATPases, for the synthesis of PCr by CK (Wallimann *et al.* 1992). Since this ATP directed toward PCr resynthesis comes from either glycolysis or oxidation, the amount and activity of metabolic (glycolytic and oxidative) enzymes in mammalian and fish muscle should contribute to the rebuilding of PCr. In general, across class lines (in the phylogenetic sense of the word), glycolytic enzymes are higher in white/fast-twitch muscle fibers than in red/slow-twitch fibers and regarding oxidative enzymes, the reverse is true (Hamoir *et al.* 1972, Johnston 1977, Johnston and Moon 1980, 1981, Bylund-Fellenius 1984, Tesch *et al.* 1987, Moyes *et al.* 1989). Comparisons of specific activities of representative glycolytic (HK, PFK, and LDH) and oxidative enzymes (CS and CO) show that concentrations of "aerobic" enzymes in mammals are 5- to 20-times that of fish (Johnston and Moon 1981, Bylund-Fellenius *et al.* 1984, Tesch *et al.* 1987, Moyes *et al.* 1989), whereas the "anaerobic" enzymes are no more than twice that in mammals compared with fish. (Avelar 1978).

It appears that the close linkage of perfusion and oxidative phosphorylation seems to be the answer to why so much time is necessary for PCr to be rebuilt in white muscle. Perfusion delivers oxygen to the tissue and oxidative phosphorylation requires oxygen as a substrate. Less perfusion means less oxygen delivery and, subsequently, less oxidative phosphorylation. At identical perfusion rates, PCr-depleted excised mammalian slow twitch muscle had a 6-times faster rate of PCr

rebuilding than a primarily fast twitch muscle, requiring only 10 minutes for a full recovery (Meyer *et al.* 1991). In this same investigation, pH_i also returns to resting values much quicker in slow-twitch muscle fibers relative to fast-twitch fibers, in fact, by the time the trials ended, fast-twitch muscle pH_i still had not returned to baseline values. Since in this case (Meyer *et al.* 1991) the rate and volume of perfusion was the same, perfusion alone cannot account for rate differences of PCr rebuilding between muscle groups. Based on these, and other data (Blei *et al.* 1993), oxidative phosphorylation is an important influence on how quickly a muscle can rebuild its depleted PCr store.

Red Muscle pH_i

Through the 1980s, with the increased popularity of NMRS as a tool for studying muscle metabolism, two theories were suggested attempting to explain why there seems to be multiple P_i peaks in exercised muscle. Initially, it was propsed that the double peak was dependent on the activity of the muscle, the more acidic right-most peak representing the active fiber and the less acidic peak representing the inactive, "resting" muscle (Taylor *et al.* 1983). Park *et al.* (1987) presented an alternative hypothesis, i.e., that the splitting of the P_i peaks is due to the NMR spectrometer detecting a signal from two different types of muscle, specifically, slow and fast-twitch, which was soon supported by other investigations (Mizuno *et al.* 1990). An extensive literature search has uncovered only one study where multiple P_i peaks were seen in fish (van Waarde *et al.* 1990), and in this case three P_i peaks were actually seen. Since the NMR coil was placed over the gills, these peaks were suggested to represent the P_i in plasma, muscle, and gill epithelium coinciding with pH_i values of 7.78, 7.27, and 6.98, respectively. In the present investigation the NMR coil was placed over the midline of the carp. The intention of this was to try to

decipher the P_i peaks of the three different muscle groups in fish, i.e., white, pink, and red muscle. Although complete success was not achieved, there were definitely two distinct peaks seen in recovery (Figure 17). Based on literature values of red and white muscle of other freshwater teleosts (Dobson *et al.* 1987, Parkhouse *et al.* 1988, van den Thillart *et al.* 1989, van Ginneken *et al.* 1995, Schulte *et al.* 1992), it seems very likely that the second P_i peak seen in this data does in fact represent the concentration of P_i in red muscle of these carp as the chemical shifts of the peaks coincide with the reported pH_i of the red muscle. This investigation appears to be the first to report the detection of fish red muscle pH_i using NMRS.

Conclusions

Carp, and fish in general, require a prolonged recovery to fully rebuild PCr following anaerobic activity induced by exhaustive exercise or hypoxia. The timecourse of PCr replenishment in the present data show a strong correlation between treatments and the recovery of PCr levels over time (equations 8-11, $r^2>0.892$), thus providing a model for PCr recovery in carp following exercise and hypoxia. Although recovery from hypoxia is far more rapid than that following exercise, about one hour is still required until PCr reached levels similar to those at rest. In terms of rebuilding PCr, fish white muscle just cannot compete with mammalian muscle. Although divisions between muscle fiber types are clearly present, mammalian muscle is more highly perfused than fish white muscle, promoting the efficient removal of metabolic by-products, most importantly protons and lactate from glycolysis. These protons are responsible for the huge decreases in carp white muscle pH_i induced by exercise or hypoxia. Intracellular pH continued to drop as PCr recovery progressed, mostly as a result of continued glycolysis, providing the fuel for PCr resynthesis. The decline in pH_i was so severe, nearly 1 pH

unit, that the influence of temperature on the reaction rates was effectively and completely masked. It was only in the first part of the hypoxia period, when pH_i was still close to normal, that the effect of temperature was noticed.

This slow rate of PCr recovery and low post-stress pH_i have serious ramifications on the survival strategy of the fish. Following a rigorous bout of exercise, whether it is an example of predator avoidance or capturing prey, a fish must "recover" so as to be successful in the next chase. Low PCr, high P_i and an acidic pH_i have been all been previously shown to reduce maximal output of muscle, effectively hindering burst swimming. These data suggest that a fish may only have one attempt at escape or capture before a long rest is needed to replenish PCr and allowing pH_i to return to normal. If intense activity persists PCr and pH_i may reach a level, as in this study, that requires a lengthy recovery period, which is not compatible with survival when there is a predatory fish nipping at your tail.

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