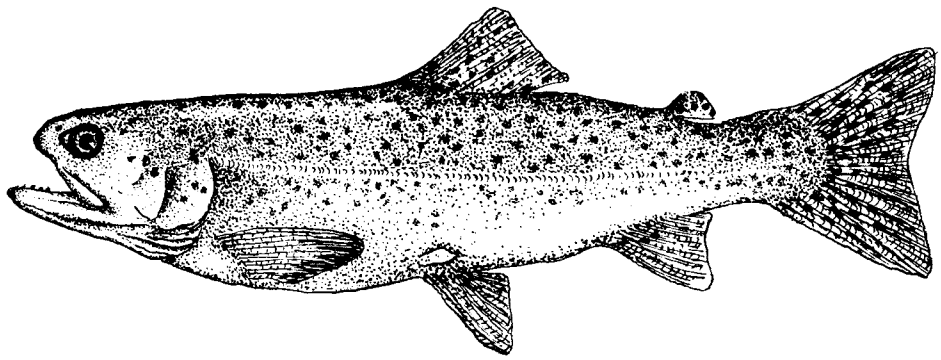
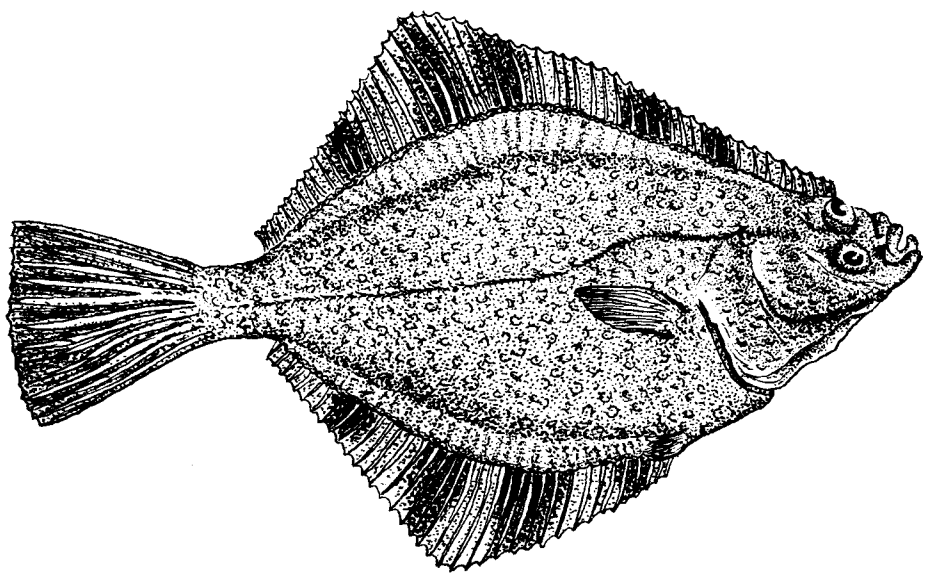
EXERCISE, ACID-BASE BALANCE & METABOLISM IN FISH



Salmo gairdneri



Platichthys stellatus

INTRACELLULAR pH REGULATION, ACID-BASE BALANCE, AND METABOLISM AFTER EXHAUSTIVE EXERCISE IN RAINBOW TROUT (*Salmo gairdneri*) AND STARRY FLOUNDER (*Platichthys stellatus*).

By

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ABSTRACT

This thesis examined the effects of exhaustive exercise on acid-base and metabolite status in the intracellular and extracellular compartments of two very different fish species: the active, pelagic rainbow trout, and the sluggish, benthic starry flounder.

In both species, exhaustive exercise resulted in an acidosis in the extracellular compartment of mixed respiratory and metabolic origin. Despite the reduction in pHe, red blood cell pHi was well regulated, though more precisely in trout than in flounder. Catecholamines were mobilized into the blood after exercise in trout but not in flounder. Circulating catecholamines may play an important role in regulating red blood cell (RBC) pHi in trout after exercise.

In trout, lactate appeared in the blood in excess of H^+; the reverse pattern was observed in flounder. H^+ appearance was similar in both species. Differential release of lactate from the muscle mass was apparently responsible for this discrepancy.

After exhaustive exercise, both trout and flounder experienced a severe intracellular acidosis in the white muscle, as measured by ^{14}C-DMO (5,5-dimethyl -2,4- oxazolidinenione) distribution. H^+ and lactate were not produced in equimolar quantities, with H^+ produced in excess of lactate. Muscle lactate and H^+ production was about 3-fold lower in flounder than in trout.

The muscle intracellular acid-base disturbance was corrected more rapidly in flounder (4-8h) than in trout (8-12h). In flounder, this occurred prior to , but in trout after , correction of the

extracellular acidosis. In flounder, a more rapid correction of muscle metabolite status was associated with the more rapid correction of the intracellular acidosis.

After exercise there was a reduction in the whole body extracellular fluid volume and expansion of the intracellular fluid volume, largely reflecting changes within the muscle. This fluid shift resulted in a general hemoconcentration.

Exercise led to a transient increase in net H^+ excretion in both trout and flounder. Negligible amounts of lactate were transferred to the water. In flounder, about 20% of the total H^+ load produced passed through the extracellular space and was transiently stored in the water, which appeared to hasten correction of the intracellular acid-base disturbance. In contrast, in trout, a much smaller portion of the acid load (about 6%), though about the same absolute amount as in flounder, was transferred to the water. This appeared to expedite correction of the extracellular acidosis.

The results of this thesis argue against a prominent role for the Cori cycle in the final disposition of the lactate burden produced during exercise. Instead, it is suggested that the bulk of the lactate was metabolized in situ , either by oxidation or glyconeogenesis. In flounder, this was almost the sole fate of lactate, as very little appeared in the blood space. In trout, a significant portion of the lactate was exported to the blood, which was taken up and metabolized by aerobic tissues.

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LIST OF ABBREVIATIONS

DMO: 5,5-dimethyl-2,4-oxazolidinedione
e: extracellular
i: intracellular

H^+: fixed, non-volatile or metabolic acid

ΔH^+m: metabolic acid load

ΔLa^-: lactate load
ATP: adenosine triphosphate
ADP: adenosine diphosphate
AMP: adenosine monophosphate
CrP: creatine phosphate
NTP: nucleoside triphosphate
IMP: inosine monophosphate
NADH: nicotinamide adenine dinucleotide (reduced)
EDTA: ethylenediaminetetraacetic acid
a: arterial
v: venous
P_{O_2}: oxygen tension

P_{CO_2}: carbon dioxide tension

C_{O_2}: oxygen content

C_{CO_2}: carbon dioxide content

αO_2: oxygen solubility constant

αCO_2: carbon dioxide solubility constant

Hb: hemoglobin
Ht: hematocrit
MCHC: mean cell hemoglobin concentration
Nad: noradrenaline
Ad: adrenaline
ECFV: extracellular fluid volume
ICFV: intracellular fluid volume
ISFV: interstitial fluid volume
BV: blood volume
PV: plasma volume
r_{H^+}: transmembrane distribution ratio for H^+

i.u.: international unit
h: hour
min: minute
g: gram

l: liter
ul: micro-liter
meq: milli-equivalent
L/sec: body lengths per second
: buffer capacity
uCi: micro-Curie
mCi: milli-Curie

^{14}C: carbon-14, a radioactive isotope of carbon

^{3}H: tritium, a radioactivie isotope of hydrogen

^{125}I: iodine-125, a radioactive isotope of iodine

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CHAPTER I

GENERAL INTRODUCTION

Man has a long history of interest in fish locomotion. Since the time of Aristotle (fourth century B.C.), differences in the locomotory habits of fish have been recognized and in the early years A.D., attempts were made to correlate fish body form with locomotion and habitat (Lindsey, 1978). In an ancient Hindu medical work (dating from about 100 A.D.) "river fish are said to be bulky in the middle because they move with their head and tail; pool fish, having little space to move about, are deep-bodied; torrent fish are flattened because they crawl with their chests on the bottom" (Hora, 1935).

Swimming performance in fish is divided into three major categories: sustained, prolonged and burst (Beamish, 1978). Sustained swimming is that which can be maintained for greater than 200 min without fatigue. Prolonged swimming is of shorter duration (less than 200 min) and eventually ends in muscular fatigue. Burst swimming can be maintained for only very short periods (less than 20 sec) and usually represents the highest speeds at which fish can swim. In practice, it is difficult to distinguish between prolonged and sustained swimming. The two are often considered together, as extended or long-term swimming in contrast to short-term, burst-type swimming (eg. Beamish, 1978; Butler, 1985).

This thesis is concerned with the physiology of recovery from short-term, burst-type activity leading to exhaustion.

Fish Muscle Fiber Types

In fish, 'red' and 'white' fibers form discrete muscle groups. The 'red' fibers comprise a superficial sheet of muscle along the lateral line, which runs the length of the fish. As the name implies, 'red' fibers are well vascularized, rich in myoglobin and mitochondria, have a high lipid content, and rely heavily on aerobic metabolism (Hamoir, Focant and Disteche, 1972; Bone, 1978; Mosse, 1979). These fibers have alkaline-labile, acid-stable, myofibrillar ATPase activity, characteristic of slow-twitch fibers (Johnston, Ward and Goldspink, 1975; Mosse and Hudson, 1976) and appear to be the equivalent of slow-oxidative or type I fibers described in mammalian systems (Brooke and Kaiser, 1970; Peter, Barnard, Edgerton, Gillespie and Stempel, 1972). Electromyographic studies have shown that at low swimming speeds, less than 0.5 body lengths per sec (L/sec), only these 'red' fibers are active (Hudson, 1973; Johnston, Davison and Goldspink, 1977).

In some fish, 'pink' or intermediate fiber types have been identified. These fibers have reduced myoglobin, mitochondrial and lipid contents and increased glycolytic enzyme activities relative to the slow, 'red' fibers. Furthermore, their staining for myofibrillar ATPase activity at alkaline pH is intermediate between 'red' and 'white' fibers (Johnston *et al.*, 1975; Mosse, 1979). These fibers are considered aerobic, 'fast' fibers (Johnston, 1982), perhaps the equivalent of fast, oxidative or type IIA fibers in mammals (Brooke

and Kaiser, 1970; Peter et al. , 1972). Their distribution is quite variable; for example, they may be restricted to a few cells scattered between the 'red' and 'white' fibers (as in rainbow trout, Salmo gairdneri) (Johnston et al. , 1975) or they may form an extensive layer between the 'red' and 'white' fibers (as in carp, Carassius carassius) (Johnston, Patterson, Ward and Goldspink, 1974). In carp, these fibers are recruited at intermediate swimming speeds of about 1.5 L/sec (Johnston et al. , 1974).

The 'white' fibers are by far the most abundant, constituting up to 95% of the myotomal mass (Nag and Nursall, 1972; Greer-Walker and Pull, 1975). In general, fish 'white' muscle is poorly vascularized, has few mitochondria, little myoglobin, possesses high glycolytic enzyme activities, and thus is geared towards glycolytic metabolism (Crabtree and Newsholme, 1972; Hamoir et al. , 1972; Bilinski, 1974). These fibers possess alkaline-stable, acid- labile myofibrillar ATPase activity and fatigue quite rapidly (Bone, 1978) and therefore, are similar in nature to the fast-glycolytic or type IIB fibers described in mammals (Brooke and Kaiser, 1970; Peter et al. , 1972). During bursts of vigorous exercise, the majority of the propulsive force is provided by contractions of these 'white' fibers. 'Pink' and 'red' fibers are also active at these swimming speeds, but their overall contribution is small in comparison to the 'white' fibers (Johnston and Goldspink, 1973; Bone, 1978). The threshold speed for recruitment of 'white' fibers differs between species. In carp, the white fibers are active at very low speeds, 0.5 - 1.0 L/sec (Bone, 1978), whereas in trout, they are recruited at

intermediate swimming speeds, 1.4 - 2.5 L/sec (Hudson, 1973; Bone, Kiceniuk and Jones, 1978; Johnston and Moon, 1980) and in striped bass (Morone saxitilis) and bluefish (Pomatomus sultatrix), they become active only at very high speeds, 3.2 and 4.5 L/sec (Freadman, 1979). Such species differences may be related to differences in the pattern of innervation of the muscle fibers (see below).

In salmonids, it has long been recognized that the 'white' muscle mass is really a mosaic, consisting of smaller (10 - 25 um) and larger (50 - 100 um) diameter fibers (Boddeke, Slijper and van der Shelt, 1959). These smaller fibers have higher mitochondrial densities, but do not appear to differ in lipid content from the surrounding larger fibers (Johnston et al. , 1975). This mosaic nature of the muscle mass was originally interpreted as a mixture of small diameter 'red' and large diameter 'white' fibers (Webb, 1971; Hudson, 1973). In rainbow trout (Johnston et al. , 1975) and brook trout (Salvelinus fontinalis) (Johnston and Moon, 1980), the small and large diameter fibers showed identical staining reactions for alkaline - stable myofibrillar ATPase and succinate dehydrogenase (a marker enzyme for aerobic capacity) activities, which differed markedly from the small diameter 'red' fibers of the superficial lateral muscle. These observations led Johnston (1982) to suggest that these smaller fibers represent growth stages of the larger ones, rather than distinct fiber types.

> In this thesis, the terms 'white' and 'red' are used to describe the muscle mass composed of mainly 'white', glycolytic, fast fibers or 'red', aerobic, slow fibers, and do not imply that the tissues are homogeneous from a histological point of view.

Muscle Fiber Capillary Supply

The number of capillaries of the red and white fibers varies between species and appears to be correlated with swimming performance. For example, in active pelagic teleosts, such as mackerel (Scomber australasicus) and Australian salmon (Arripis trutta), the average fiber areas per peripheral capillary are 153 um^2 and 200 um^2 for red fibers and 2040 um^2 and 9873 um^2 for white fibers, respectively (Mosse, 1979). In contrast, in the benthic, sluggish flathead (Platycephalus bassenius), the figures are 320 um^2 and 10,470 um^2 for red and white fibers, respectively (Mosse, 1978). The considerable interspecies heterogeneity in the capillary supply to the white fibers appears to be correlated with the type of innervation (see below) and pattern of fiber recruitment. Those white fibers with high capillary densities (i.e. low area per peripheral capillary) are recruited at slower swimming speeds (Johnston, Bernard and Maloiy, 1983).

Less is known about the capillary supply to the intermediate, 'pink', fibers, but the few data available show an average fiber area per peripheral capillary intermediate to the red and white fibers- e.g. 1070 um^2 in the grass whiting (Haletta semifasciata) (Mosse, 1978).

Muscle Fiber Innervation

The pattern of innervation of fish skeletal muscle fibers is quite different from that characteristic of mammalian skeletal muscle, where the fibers are innervated by a single motoneuron terminating at

a single motor endplate (Burke, Levine and Zajac, 1971).

In all fish groups, the slow, red, fibers are multiply innervated. Typically, each fiber receives a dense network of innervation which terminates on numerous endplates distributed along the fiber. In most species studied to date, more than 2 axons probably innervate each slow fiber (Bone, 1978).

The innervation pattern of fast, white, fibers is more diverse. In elasmobranchs and some teleosts, fast fibers are focally innervated, usually at one end of the fiber; each white fiber is innervated by two separate axons which contribute to the formation of a single motor endplate. There is usually no recognizable difference between the two motor terminals; thus the significance of this dual innervation is uncertain (Bone, 1978). In most teleosts, fast, white fibers and pink fibers are multiply innervated, showing a pattern not unlike that described for slow, red fibers. The axons innervating these fibers are derived from several motor neurons (Bone, 1978). For example, in the sculpin (Cottus scorpius), fast, white, fibers are innervated by up to 22 different axons, each of which forms its own endplate (Hudson, 1969).

This inter-specific difference in innervation of white fibers is believed to be related to the pattern of fiber recruitment. In species with focally innervated fast fibers (eg. spiny dogfish, Scyliorhinus canicula , and Pacific herring, Clupea harengus), the fibers are not recruited until very fast swimming speeds, greater than 5 L/sec (Bone, 1978; Bone et al. , 1978). In contrast, in species with multiply innervated fast fibers (eg. carp and rainbow trout),

fibers are recruited at much lower swimming speeds, 0.5 - 1.4 L/sec (Hudson, 1973; Johnston et al. , 1975).

The number of muscle fibers innervated by a single axon (i.e. the size of a motor unit) is generally not well known in fish. In dogfish, which have focally innervated fast fibers, Bone (1978) estimated that the fast motor unit consisted of 50 - 100 fibers.

Metabolism and H^+ Production During Burst Exercise

In fish, as in most vertebrates, exhaustive, 'burst-type' activity results in a pronounced reduction in blood pH (Black, Chiu, Forbes and Hanslip, 1959; Piiper, Meyer and Drees, 1972; Wood, McMahon and McDonald, 1977; Holeton and Heisler, 1983; Turner, Wood and Clark, 1983; Turner, Wood and Hobe, 1983; Schwalme and Mackay, 1985a). The acidosis is a consequence of the efflux of aerobically produced CO_2 and glycolytically produced H^+ from the working muscle mass.

The source of H^+ is basically the same in all vertebrates, resulting from a complex interplay between proton producing and consuming reactions within the muscle. In the very early stages of burst exercise (i.e. within the first few seconds), the main fuel source is creatine phosphate (CrP). The hydrolysis of CrP to yield adenosine triphosphate (ATP) proceeds via the following reaction, catalyzed by the enzyme phosphocreatine kinase:

$$CrP^{2-} + ADP^{3-} + H^+ \longrightarrow ATP^{4-} + H_2O + Cr \qquad (1.1)$$

(Gevers, 1977; Hochachka and Mommsen, 1983). This reaction is proton consuming, the exact stoichiometry dependent upon such factors as pH and $[Mg^{2+}]$. Thus, during the early stages of activity, an

alkalosis in the working muscle would be expected. In confirmation, Dawson, Gadain and Wilkie (1977), using ^{31}P - nuclear magnetic resonance (^{31}P - NMR) observed a transient alkalosis immediately following stimulation of the toad gastrocnemius muscle, which was associated with a depletion of creatine phosphate. In contrast, hydrolysis of the ATP formed from CrP is an H^+ producing reaction (see below), so overall, this will negate the alkalosis created by CrP hydrolysis.

Creatine phosphate levels are rapidly depleted, within 1-2 seconds of the onset of exercise (Hochachka and Somero, 1984), and consequently, ADP (adenosine diphosphate) levels increase as ATP is hydrolyzed. As ADP accumulates, additional ATP and AMP (adenosine monophosphate) are formed by the adenylate kinase reaction

$$2ADP^{3-} \longrightarrow ATP^{4-} + AMP^{2-} \qquad (1.2)$$

due simply to a mass action effect. The increase in AMP levels and overall decrease in ATP stimulate the activity of the enzyme AMP deaminase, which catalyzes the formation of inosine monophosphate (IMP) and ammonia:

$$H_2O + AMP^{2-} \longrightarrow IMP^{2-} + NH_3 \qquad (1.3)$$

Ammonia may also be produced by the action of the enzyme adenosine deaminase, which catalyzes the reaction:

$$\text{Adenosine} + H_2O \longrightarrow \text{inosine} + NH_3 \qquad (1.4)$$

However, in fish muscle, the activity of this enzyme is quite low, so the above reaction does not contribute significantly to ammonia production during exercise (Driedzic and Hochachka, 1978). In essence, ammonia is produced as NH_3 (Atkinson and Camien, 1982);

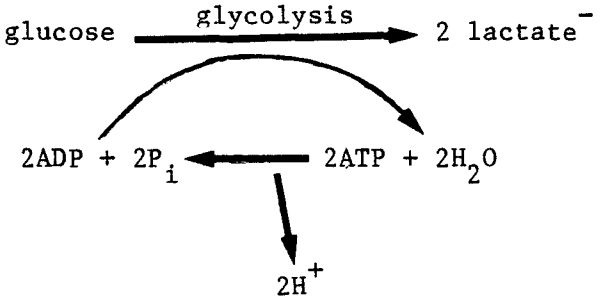
since the pK of ammonia is quite high (about 9.5, Cameron and Heisler, 1983), at cytoplasmic pH (7.0 - 7.6) ammonia will have an alkalinizing effect. However, as ammonia production in fish white muscle during exercise is quite small (< 1 mmol/kg wet weight; Driedzic and Hochachka, 1976), its effect on intracellular acid-base status is likely minimal.

The aforementioned reactions (1.1 and 1.2) are only 'stop-gap' measures, supplying ATP during the very early stages of intense activity, after which glycolysis becomes the chief source of ATP. Glycogen is the major, if not sole, substrate for glycolysis in fish white muscle. Black, Robertson, Lam and Chiu (1962) and Stevens and Black (1966) reported a 50% reduction in muscle glycogen levels within the first minute of vigorous activity in the rainbow trout. Furthermore, since hexokinase activity is low in fish white muscle, relative to other glycolytic enzymes, blood-borne glucose is probably not a significant substrate for glycolysis (Crabtree and Newsholme, 1972; Johnston and Moon, 1981).

Since the early 1900's, glycolytic degradation of glucose and glycogen has been associated with the accumulation of lactate and protons, with 'lactic acid' the assumed endproduct. In support of this traditional view, Wilkie (1979) and Hultman and Sahlin (1980) argue that H^+ accumulates as a consequence of the dissociation of lactic acid, which, as a weak acid (pK less than 4), will be fully (greater than 99%) ionized at cytoplasmic pH. In addition, the formation and accumulation of hexose-phosphates (eg. glucose -6-phosphate; fructose -1,6- disphosphate) will result in H^+

production, the exact stoichiometry dependent upon pH (Hultman and Sahlin, 1980). Furthermore, these authors argue that since ATP levels do not change appreciably in mammalian muscle after glycolytically fueled exercise, H^+ produced by ATP hydrolysis does not contribute significantly to muscle acidification.

Krebs, Woods and Alberti (1975), Gevers (1977) and more recently, Hochachka and Mommsen (1983) offer an opposing view, arguing that glycolysis per se does not result in the 1:1 stoichiometry between lactate and H^+ production, but rather that this stoichiometry is a result of the coupling of ATP hydrolysis and glycolysis, according to the general equation:



(1.5)

If glycogen, rather than glucose, is used as the substrate, as it is in fish white muscle, then 3 ATP molecules are produced per mole of glucosyl unit fermented because the first ATP consuming step of glycolysis,

$$\text{glucose} + \text{ATP} \xrightarrow{\text{hexokinse}} \text{glucose-6-phosphate} + \text{ADP} \qquad (1.6)$$

is bypassed (Newsholme and Leech, 1983). However, as some H^+ are consumed during the glycolytic fermentation, the net H^+ yield when coupled to ATP hydrolysis is still 2 H^+ (Hochachka and Mommsen, 1983). Furthermore, because the pH dependence of H^+ production by

ATP hydrolysis is opposite that of glycolytic fermentation, this stoichiometry holds true regardless of the endproduct produced: lactate, succinate or proprionate (Hochachka and Mommsen, 1983).

Clearly, this is a controversial issue, which awaits both theoretical and experimental verification. Nonetheless, it follows from both arguments that H^+ and lactate need not accumulate in equimolar quantities. In the argument put forth by Wilkie (1979) and Hultman and Sahlin (1980), accumulation of hexose-phosphates would lead to an excess of H^+ over lactate. In humans after high intensity exercise, glucose-6-phosphate and fructose-1,6-diphosphate levels increased by about 4.6 and 1.6 mmol/kg, respectivley (Jones, McCartney, Graham, Spreit, Kowlachuk, Heigenhauser and Sutton, 1985). In carp (Cyprinus carpio) muscle after strenuous exercise, glucose-6-phosphate and fructose-1,6-diphosphate increased by, at most, 1 mmol/kg (Driedzic and Hochachka, 1976).

According to Krebs et al. (1975), Gevers (1977) and Hochachka and Mommsen (1983), if ATP hydrolysis and glycolysis become uncoupled, i.e. ATP demand exceeds supply, then H^+ will accumulate in excess of lactate, as net ATP levels decline. In humans, after high intensity work, muscle ATP levels fall by about 2 mmol/kg (Sutton, Jones and Toews, 1981). In cod (Gadus callarias) (Jones and Murray, 1960) and carp (Driedzic and Hochachka, 1976), white muscle ATP levels fell by about 2 and 5 mmol/kg after strenuous exercise, respectively, illustrating a certain degree of uncoupling between ATP utilization and replenishment.

Lactate and H^+ Dynamics in Fish

In the context of this thesis, H^+ refers to fixed, non-volatile acid produced by metabolism (as opposed to CO_2). The terms 'metabolic acid' (ΔH^+_m) and 'acidic equivalents' may be used interchangably for 'H^+'.

In fish white muscle, lactate is the major endproduct of glycolysis, and a number of studies have focussed on the generation of lactate within the muscle, its movement between compartments and its fate (Black, Robertson, Hanslip and Chiu, 1960; Black et al. , 1962; Stevens and Black, 1966; Wardle, 1978; Batty and Wardle, 1979; Turner, Wood and Clark, 1983; Turner, Wood and Hōbe, 1983; Schwalme and Mackay, 1985b). The general pattern emerging from these studies is that lactate is slow to accumulate in the blood space, not reaching its peak until 2-4 h into recovery. The disappearance of lactate from the muscle mass is also slow, requiring up to 12-24 hour. Similarly, restoration of the depleted glycogen store is slow, not complete until 12-24 h.

H^+ appearance in the bloodspace follows a different time course than that of lactate and the two do not appear in equal quantities (Piiper et al. , 1972; Wood et al. , 1977; Holeton and Heisler, 1983; Holeton, Neumann and Heilser, 1983; Turner, Wood and Clark, 1983; Turner, Wood and Hōbe, 1983). Typically, the blood H^+ load peaks by the time of exhaustion or shortly thereafter, slowly declining to rest levels by 12h (Wood and Perry, 1985).

These patterns contrast sharply with those generally observed in man. After exercise to exhaustion, blood lactate and H^+ appear in equal quantities which reach a peak within a few minutes of

recovery, and then return to rest levels within 60 min (Hultman and Sahlin, 1980).

Initially, the slow appearance of lactate in the blood was attributed to a slow diffusion rate at low temperature (10 - 15°C; Black et al. , 1959). However, Black et al. (1962) later suggested that a reduction in circulation to the white muscle mass may be a more important factor and quoted Hayashi's unpublished results which showed that muscle circulation was impaired after exercise. However, more recent work suggests that in trout exercised to exhaustion by electrical stimulation, white muscle blood flow increases substantially (Neumann, Holeton and Heilser, 1983). Turner and Wood (1983) have suggested that lactate is retained within the muscle by active mechanisms at the cell membrane level and that this accounts for the slow appearance of lactate in the blood space in exercised trout. Wardle (1978) and Batty and Wardle (1979) presented a similar argument for flatfish, and provided evidence for catecholamine involvement in the lactate retention.

The discrepancy between lactate and H^+ appearance in the bloodspace after exhaustive exercise is somewhat more complex. While the protracted time course of lactate appearance in the blood is common to all fish species examined to date, the pattern of H^+ and lactate accumulation varies between species (Wood and Perry, 1985). In dogfish (Scyliorhinus stellaris L.) exercised to exhaustion by electrical stimulation, lactate accumulated in the blood in excess of H^+ by about 16 meq/l (Piiper et al. , 1972; Holeton and Heisler, 1983). A similar pattern has been described in rainbow trout (Holeton

et al. , 1983; Turner, Wood and Clark, 1983) and skipjack tuna (Katsuwonus pelamis) (Perry, Daxboeck, Emmet, Hochachka and Brill, 1985) exercised to exhaustion by manual chasing. The opposite pattern, H^+ appearing in excess of lactate by 4 - 10 meq/l, has been observed in starry flounder (Platichthys stellatus) (Wood et al. , 1977), flathead sole (Hippoglossoides elassodon) (Turner, Wood and Hōbe, 1983), sea raven (Hemitripterus americanus) (Milligan and Farrell, 1986, see Appendix I) and Atlantic skate (Raja ocellata) (Wood and Graham, unpublished, cited in Wood and Perry, 1985) swum to exhaustion.

Some progress has been made in elucidating the mechanism(s) leading to this inequality between H^+ and lactate levels in the bloodstream. Both gills and muscle are potential control sites, although it remains controversial whether differential release from the muscle or differential excretion into the water, or a combination of these and other factors, are involved (Piiper et al. , 1972; Wardle, 1978; Heisler, 1982; Holeton and Heisler, 1983; Holeton et al. , 1983; Turner, Wood and Clark, 1983; Turner, Wood and Hōbe, 1983; Turner and Wood, 1983). Furthermore, nothing is known about changes in acid-base status within the intracellular compartment of fish white muscle after exhaustive exercise. Finally, little is known about the possible controlling relationships between acid-base status and metabolism in either the intracellular or extracellular compartments in vivo , though in vitro studies suggest that important interactions should occur (Hochachka and Somero, 1984).

To date, the only tissue in which intracellular pH (pHi) regulation after exercise has been examined is the red blood cells. Following exercise to exhaustion, red cell pHi remained virtually constant in striped bass (Nikinmaa, Cech and McEnroe, 1984) and rainbow trout (Primmett, Randall, Mazeaud and Boutilier, 1986), despite the pronounced extracellular acidosis. Pharmacological experiments have indicated a role for plasma catecholamines in this pHi regulation (Nikinmaa, 1982, 1983; Heming, 1984; Cossins and Richardson, 1985). These results raise a number of interesting questions about blood oxygen transport after exercise, in view of the fact that numerous in vitro studies have shown that hemoglobin - oxygen affinity is adversely affected by reductions in extracellular and red cell pHi in the absence of catecholamines (eg. Wood, Johansen and Weber, 1975; Albers, Götz and Welbers, 1981).

Aims of the Investigation

There are therefore a number of intriguing problems concerning the physiology of extracellular and intracellular acid - base regulation and metabolism after exhaustive exercise in teleost fish. Among these are:

1) The factors contributing to the H^+ - lactate discrepancy in blood.

2) The consequence(s) of metabolic acid production for the intracellular acid - base status of white muscle and other tissues.

3) The fate of lactate and H^+ produced during exercise.

4) The interactions between acidosis and O_2 transport by the red cells.

To address these problems, the following studies were carried out:

1) The contribution of H^+ excretion to the environment in correcting the post-exercise acidosis was quantified and its possible role in the development of the H^+ - lactate discrepancy in blood was assessed.

2) The intracellular acid-base and metabolite disturbances incurred by the white muscle as a consequence of exhaustive, burst-type exercise were measured.

3) The fate(s) of H^+ and lactate produced during burst exercise were examined.

4) The effects of the exercise-induced extracellular acid-base and metabolite disturbance on other tissues were investigated.

5) The effect of the extracellular acidosis on blood oxygen transport and the possible involvment of catecholamines in ameliorating the effect were examined.

Experimental Approach

(1) Experimental Animals

These investigations were carried out on two teleost fish species: rainbow trout (Salmo gairdneri), and starry flounder (Platichthys stellatus). These two fish species were chosen because they differ greatly in their habitats, lifestyles and their ability to

burst exercise as well as their physiology of recovery from exercise (eg. Wood et al. , 1977; Turner, Wood and Clark, 1983).

Rainbow trout are active, pelagic fish, found in both marine and freshwater environments. Trout are well adapted for their swimming lifestyles, as they possess the classic streamlined round body that reduces drag (Webb, 1971). In trout more than 10% of the muscle mass consists of red fibers, which allow the fish to swim almost indefinitely at speeds of up to 2 L/sec (Webb, 1971; Hudson, 1973). Salmonids in general, are highly aerobic fish, capable of a maximal O_2 consumption of about 30 mmol/kg/h (Brett, 1972). This capacity for sustained aerobic swimming is important for survival, for salmonids often migrate long distances in short periods of time during their spawning run (eg. 1100 km in 11 days; Lindsey, 1978). Furthermore, trout white muscle has a relatively high glycolytic potential, which enables this species to swim at high burst velocities (up to 15 L/sec), for short periods (Webb, 1971; Mosse, 1979; Johnston, 1982). This capacity for short-term burst performance allows trout to readily escape predation, capture fast - swimming prey, and negotiate rapid currents which may be encountered in rivers during spawning migrations (Beamish, 1978).

Starry flounder, in contrast, are more sluggish animals. These benthic, euryhaline flatfish spend much of their time buried in the substratum or foraging slowly along the bottom, making occasional forays into the water column. Flatfish are morphologically specialized for bottom dwelling and burrowing; they are not well adapted for a swimming lifestyle, possessing little red muscle for

sustained swimming, less than 8% of the total muscle mass (Mannan, Fraser and Dyer, 1961; Greer-Walker and Pull, 1975). Furthermore, maximal rates of O_2 consumption in flatfish are low, 6-8 mmol/kg/h (Duthie, 1982), in comparison to salmonids. Although flatfish can migrate long distances (Hart, 1973), they rely heavily on tidal transport (Greer-Walker, Harden-Jones and Arnold, 1978). The flounder's capacity for glycolytic burst exercise is also limited, because their white muscle mass has a relatively low glycolytic potential (Castellini and Somero, 1981). Consequently, these fish rarely attain swimming speeds greater than 2 L/sec (Priede and Holliday, 1980).

The studies on trout were somewhat more extensive and detailed than those of flounder, because the former were studied in the home laboratory, while the latter could only be studied at a marine station under field conditions.

(2) DMO Method

A necessary pre-requisite for studying intracellular acid-base status after exhaustive exercise is the ability to measure reliably the associated intracellular pH transients. There are several different techniques for measuring intracellular pH, either by direct (microelectrodes) or indirect means (^{31}P - NMR, distribution of weak acids/bases). The relative merits of these techniques have been discussed in detail by Roos and Boron (1981) and Nuccitelli and Deamer (1982). The technique best suited for measurement of pHi <u>in vivo</u> is that based upon the distribution of weak acids/bases, as it involves a minimum of disturbance to the animal prior to terminal sampling.

The weak acid most widely used for the estimation of pHi is 5,5- dimethyl -2,4- oxazolidinenione (DMO), introduced by Waddell and Butler (1959). The DMO distribution method has not been used previously to measure pHi in a dynamic situation, such as that likely to occur in muscle after exercise. The first objective of this thesis was to assess the reliability of the DMO method to detect pHi transients. Two *in vitro* preparations were employed for this study: trout whole blood in a tonometer and an isolated-perfused trout trunk preparation. Once this technique had been validated, it was then utilized to measure pHi transients *in vivo* associated with exercise in trout and flounder.

(3) Investigations

Three parallel studies were undertaken using rainbow trout and starry flounder to probe the strategies adopted by these two different species in dealing with the acid-base and metabolite disturbances of exhaustive exercise.

1) The first series of experiments examined the role of transcompartmental fluxes of H^+ and lactate in the recovery process and the possible role of differential excretion of lactate and H^+ in the development of the reported discrepancies between H^+ and lactate in the blood. In addition, the behaviour of the markers used in the DMO technique, ^{14}C-DMO and ^{3}H-mannitol, in long-term experiments was assessed. Also, the impact of arterial *versus* venous sampling sites on the calculation of pHi was assessed.

2) A second series of experiments examined in detail the intracellular acid-base and metabolite disturbances incurred by the

white muscle mass. In addition, the consequences of the extracellular acid-base and metabolite disturbance for other tissues (eg. heart, liver, red blood cell) and the fate of lactate were examined.

3) The third series of experiments were designed to examine the consequences of the extracellular acid-base disturbance on red blood cell acid-base status and blood oxygen transport, and the presence of possible regulatory mechanisms.

CHAPTER II

In vitro ASSESSMENT OF DMO FOR MEASUREMENTS OF INTRACELLULAR pH TRANSIENTS *In vivo*

INTRODUCTION

DMO (5,5-dimethyl-2,4-oxazolidinenione), a weak organic acid, is the most commonly used marker for intracellular pH *in vivo* (Roos and Boron, 1981; Nuccitelli and Deamer, 1982). DMO has a pK of about 6; hence it will readily dissociate at physiological pH (7-8). The degree of dissociation is dependent upon the pH. The fundamental assumption underlying the use of the DMO distribution method is that the non-ionized species, HDMO, is much more permeable to the cell membrane than is the ionized species, DMO^-. With these conditions prevailing, HDMO will come into equilibrium between the intracellular and extracellular compartments, with $[DMO^-]$ dependent upon the pH of each compartment. Therefore, if there is a pH differential across the extracellular/intracellular interface, then there will be a corresponding difference in $[DMO^-]$, and hence in total [DMO] between the two compartments. By employing ^{14}C-labelled DMO, the total concentration in each compartment can readily be determined once the relative extracellular and intracellular volumes are known. The former can be determined by the simultaneous use of an extracellular fluid volume (ECFV) marker. This, in addition to measurement of extracellular pH (pHe) and a knowledge of pK, allows calculation of

intracellular pH (pHi).

The DMO distribution method has been shown, repeatedly, to produce reliable estimates of pHi under steady-state conditions in a variety of tissues from a wide range of animals (Boron and Roos, 1976; Hinke and Menard, 1978; Roos and Boron, 1981; Nuccitelli and Deamer, 1982). Furthermore, comparative studies in a number of tissues (eg. rat liver, barnacle muscle fibers, snail neurons) have shown pHi estimated from DMO distribution is in good agreement with pHi measured directly with either microelectrodes or ^{31}P - NMR (Roos and Boron, 1981; Cohen, Henderson, Iles and Smith, 1982).

In fish, the DMO technique has been employed to describe steady-state differences in intracellular acid-base status associated with acclimation temperature (Heisler, Weitz and Weitz, 1976; Cameron and Kormanik, 1982; Walsh and Moon, 1982) and exogenously or endogenously induced hypercapnia (Cameron, 1980; Hõbe, Wood and Wheatly, 1984). However, the pHi transients associated with these treatments remain unknown, as do those suspected to occur after exercise (Holeton et al ., 1983; Turner, Wood and Clark, 1983).

To assess the reliability of the DMO method for detecting pHi transients, two in vitro preparations were employed: rainbow trout whole blood in tonometers and an isolated-perfused trout trunk. The former was used to provide optimal conditions for rapid re-distribution of DMO across the intracellular/extracellular interface and to permit independent validation of the DMO distribution estimate of pHi by direct measurement on red cell lysates. This technique has been extensively used and is considered to yield

reliable estimates of erythrocytic pHi, at least in mammalian blood (Bromberg, Theodore, Robin and Jensen, 1965; Roos and Boron, 1981). The isolated perfused trunk preparation was selected to allow examination of the behaviour of the marker in the tissue of principal interest (white muscle) in the planned exercise studies. This preparation represented a situation more closely resembling the conditions in skeletal muscle *in vivo* which may be sub-optimal for DMO re-distribution across the cellular boundary, i.e. where tissue perfusion may be limiting. In both preparations, pHi transients were induced by altering extracellular P_{CO_2} as CO_2 is known to readily penetrate cell membranes, in contrast to H^+ or HCO_3^- (Roos and Boron, 1981).

MATERIALS AND METHODS

Experimental Animals

Adult rainbow trout (*Salmo gairdneri*), weighing 200-600g, from Spring Valley Trout Farm, Petersburg, Ontario were held in large (600 l) fiberglass tanks supplied with a continuous flow of dechlorinated Hamilton tap water (4-20°C; seasonal fluctuations) and fed twice weekly with commercial trout pellets. At least one week prior to experimentation, fish were acclimated to the experimental temperature (15°C), during which period they were starved.

To facilitate collection of blood for *in vitro* tonometry, 12 fish were cannulated in the dorsal aorta while under MS:222 (1:10,000; Sigma) anaesthesia as described by Soivio, Westman and Nyhom (1972). The catheters were filled with Cortland salmonid saline (Wolf, 1963)

heparinized at 50 i.u./ml (Na-heparin; Sigma). Fish were allowed to recover in darkened plexiglass boxes supplied with a continuous flow of fresh water at 15°C for 24-48 hr.

In Vitro Experiments

(1) Whole Blood

Whole blood was drawn from the dorsal aortic cannulae of several fish, pooled, heparinized (5,000 i.u./ml) and 0.03 uCi/ml ^{14}C-DMO (New England Nuclear; specific activity: 50 mCi/mmol) was added. Blood was equilibrated in shaking tonometer flasks (15°C) to a typical resting arterial P_{CO_2} of 2 torr, balance air (eg. Fig. 3.1; also Hōbe et al ., 1984) and sampled (600 ul) at 5 min intervals for measurement of pHe and red cell pHi. Once these parameters had stabilized (40-75 min), four control measurements were taken over a 1h period (-60, -45, -30, and 0 min). P_{CO_2} was then increased (time 0) to 8 torr, a typical post-exercise arterial value (eg. Fig. 3.1; also Turner, Wood and Clark, 1983) and samples drawn at 5 min intervals for the first hour, with further samples at 75, 90, and 120 min. The P_{CO_2} was then returned to 2 torr to further test the reliability of the DMO method, with samples drawn on the same schedule over the next 90 min. Humidified gas mixtures were supplied with Wosthoff gas mixing pumps.

To determine intra- and extracellular [DMO], several replicates of 80 ul of whole blood were centrifuged in Radiometer hematocrit tubes for 5 min at 5,000g. Plasma and red cell pellets were counted separately and counts were corrected for trapped extracellular fluid in the red cell pellet, as described below. Red

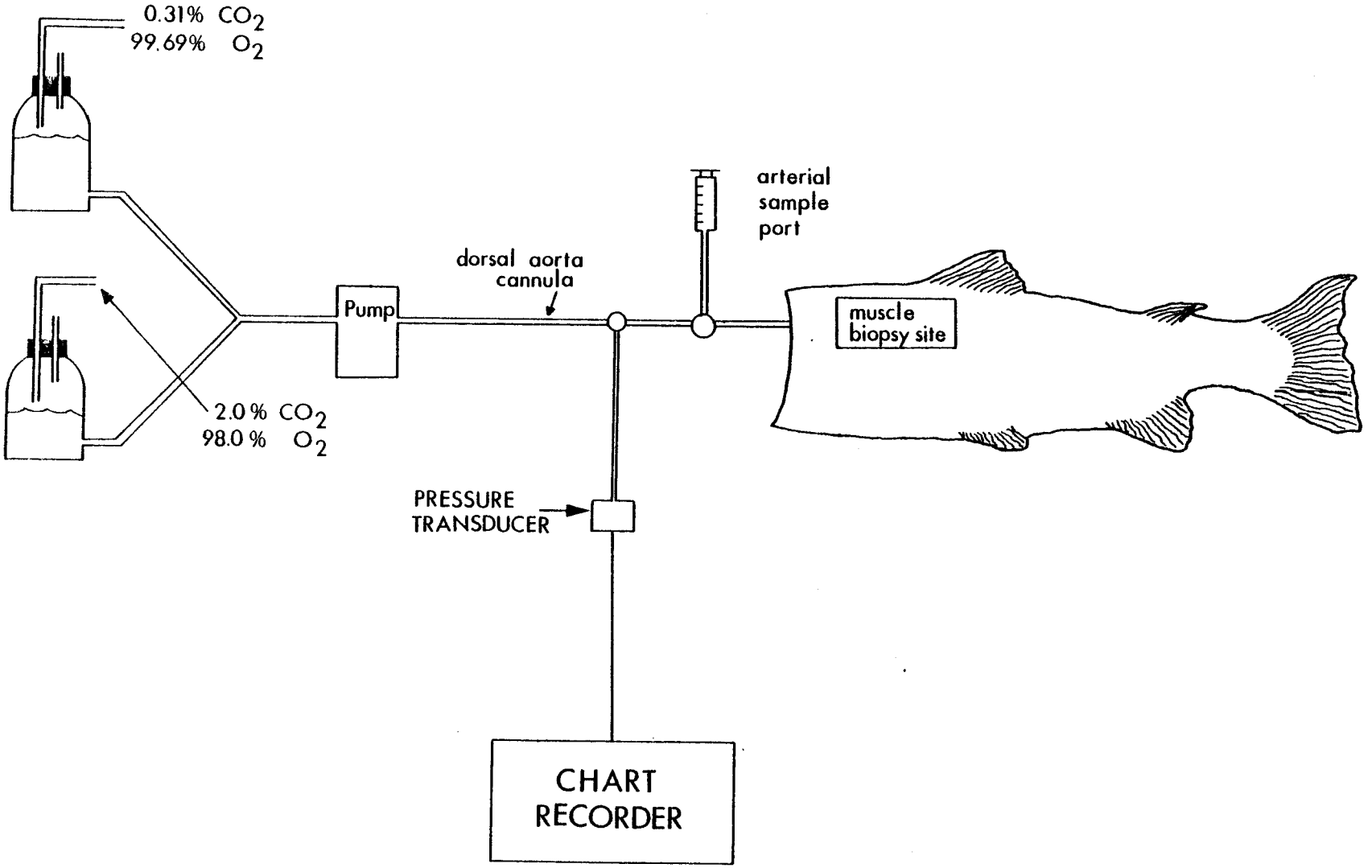
cell pHi was also measured directly on red cell lysates.

(2) Trunk Preparation

An isolated-perfused trunk preparation very similar to that described by Turner and Wood (1983) and shown in Fig. 2.1, was employed. Temperature was maintained at 15°C. Trout were quickly killed by a cephalic blow, the head severed posterior to the cleithrum and the trunk eviscerated, leaving the kidney intact. The dorsal aorta was cannulated at the cut surface with PE 50 polyethylene tubing (Clay-Adams), through which the trunk was perfused. Inflow pressure was maintained at physiological levels (20-35 cm H_2O) and was monitored using a Narco pressure transducer attached to a Gilson chart recorder. Dorsal aortic flow was maintained at 6.0-7.0 ml/kg/min, using Buchler or Gilson peristaltic pumps and a windkessel to reduce pulse pressure to the normal range (4-8 cm H_2O).

Thus flow is probably close to resting in vivo values in fish. Cardiac output at rest has been estimated, with the Fick principle, at about 18 ml/kg/min (Cameron and Davis, 1970; Davis and Cameron, 1971; Kiceniuk and Jones, 1977; Jones and Randall, 1978). The Fick principle, however, neglects gill oxygen consumption and as a result, tends to overestimate cardiac output (Daxboeck, Davie, Perry and Randall, 1982), which in turn is somewhat reduced before it reaches the posterior dorsal aorta by the flow to the head and by venous return from the gills (Jones, Langille, Randall and Shelton, 1974). In addition, blood flow to resting skeletal muscle is estimated at 5-10 ml/kg in trout in vivo (Randall and Daxboeck, 1982). Thus, the flow rates in the present study are within the physiological range.

Figure 2.1. Schematic diagram of the isolated-perfused trunk preparation (not drawn to scale). Both perfusates were maintained at 15°C by immersion in water baths, and a cooling jacket was placed around the trunk for temperature control


0.31% CO_2
99.69% O_2
2.0% CO_2
98.0% O_2
Pump
dorsal aorta cannula
arterial sample port
muscle biopsy site
PRESSURE TRANSDUCER
CHART RECORDER

The perfusion medium consisted of a basic Cortland saline to which 40 g/l polyvinylpyrrolidone (PVP; MW=40,000; Sigma); 55 g/l bovine hemoglobin (Sigma); 30 uCi/l ^{14}C-DMO and 100 uCi/l ^{3}H-mannitol (New England Nuclear; specific activity: 27.4 mCi/mmol) were added, the latter as an ECFV marker. When equilibrated at P_{CO_2} =2 torr, balance O_2, this perfusate closely resembled trout whole blood with respect to O_2 content (2.3 mmol/l), acid-base status (pH=7.8, $[HCO_3^-]$ = 5.5 mmol/l) and buffer capacity (8-9 mmol/pH/l) (eg. Fig. 3.1; also Turner and Wood, 1983).

Perfusate (100 ul) was sampled from the arterial sample port approximately 10 cm from point of entry of the cannula into the trunk. White muscle samples (100 mg) were taken from the epaxial muscle mass, anterior to the dorsal fin by punching biopsy needles through the trunk. Biopsy needles were stainless steel trocars with an internal diameter of 4.78 mm (Arnold-Nasco Ltd., Guelph, Ontario). Samples were immediately frozen in liquid nitrogen until they were counted, as described below. In pilot experiments, initial mixing time for the markers was determined to be about 90 min, so in all following experiments trunks were perfused for 90 min at a P_{CO_2} of 2 torr (balance O_2) prior to experimentation. Control samples for muscle pHi, total water, ECFV and perfusate pH (pHe) were taken at 15 min intervals for the next 30-45 min. The perfusate was then quickly switched by means of a stopcock to one equilibrated to a P_{CO_2} of 15 torr (balance O_2). A P_{CO_2} of 15 torr rather than 8 torr (used in blood studies) was used to ensure that a large pHi

change would occur, as muscle (see Table 4.1) has a much greater buffer capacity than blood. At the new perfusate P_{CO_2}, muscle and perfusate samples were taken every 15 min for 60 min. In 4 additional experiments, the protocol was reversed, with initial perfusion at a P_{CO_2} of 15 torr, followed by a decrease to 2 torr for 60 min.

Analytical Techniques and Calculations

Samples of perfusate from the isolated perfused trunk were treated as whole blood. Blood pH (pHe) was measured on 40 ul samples with a Radiometer pH microelectrode maintained at experimental temperature (15°C) and linked to a Radiometer PHM 71 or 72 acid-base analyzer. For measurement of pHi of red cell lysates, a red cell pellet, obtained by centrifuging 400 ul of whole blood at 9,000 g for 3 min, was separated from plasma by cutting the polyethylene centrifuge tube immediately below the plasma/red cell interface after quick freezing. The pellet was sealed with plasticene and then repeatedly frozen and thawed under anaerobic conditions in dry ice/ethanol or liquid nitrogen and warm water, respectively (cf. Zeidler and Kim, 1977). The pH was then measured directly on 40 ul of lysate as described.

For determination of pHi of epaxial muscle and red cells by the DMO distribution method, duplicate samples of perfusate (50 ul) or plasma (50 ul) and muscle (80 - 100 mg) or packed red cells (50 - 100 ul) were digested in 2 ml tissue solubilizer (NCS; Amersham) until a clear solution was obtained (7 - 10 days). The solution was neutralized with 60 ul of glacial acetic acid, then 10 ml fluor (OCS;

Amersham) was added. Samples were stored overnight in the dark to reduce chemiluminescence and counted on a Beckman LS-250 scintillation counter. Single (blood samples) or dual (perfusate and muscle samples) label quench correction was performed using the external standard ratio method as described by Kobayashi and Maudsley (1974). Water contents of plasma, red cell pellets, perfusate and muscle samples were determined by drying duplicate samples to a constant weight at 85°C.

Corrections for trapped ECFV in red cell pellets were determined in a separate series of experiments in which whole blood was equilibrated to a P_{CO_2} of either 2 torr or 8 torr in the presence of the extracellular marker ^{14}C-mannitol (30 uCi/l; New England Nuclear, specific activity: 50 mCi/mmol) alone. The percentage trapped ECFV, determined by counting samples of packed red cells as described, ranged from 0.8-1.5%, depending upon hematocrit.

Muscle ECFV was calculated from the distribution of ^{3}H-mannitol according to the equation:

$$\text{ECFV (ml/g)} = \frac{\text{Tissue}\left[^{3}\text{H-mannitol}\right]\ \text{(dpm/g)}}{\text{Plasma}\left[^{3}\text{H-mannitol}\right]\ \text{(dpm/ml)}/\text{Plasma } H_2O\ \text{(ml/ml)}} \qquad (2.1)$$

Tissue intracellular fluid volume (ICFV) was calculated as the difference between total water and ECFV.

Tissue pHi was calculated from the distribution of ^{14}C-DMO according to the equation:

$$pHi = pK_{DMO} + \log\left\{\frac{[DMO]i}{[DMO]e} \times \left(10^{(pHe - pK_{DMO})} + 1\right) - 1\right\} \qquad (2.2)$$

where pK_{DMO} at 15° was taken from Malan, Wilson and Reeves (1976), pHe was plasma or perfusate pH, and [DMO]e and [DMO]i were extra- and intracellular DMO concentrations, respectively. These were calculated as follows:

$$[DMO]e \text{ (dpm/ml)} = \frac{\text{Plasma } [^{14}\text{C-DMO}] \text{ (dpm/ml)}}{\text{Plasma } H_2O \text{ (ml/ml)}} \qquad (2.3)$$

and

$$[DMO]i \text{ (dpm/ml)} = \frac{\text{Tissue } [^{14}\text{C-DMO}] \text{ (dpm/g)} - (\text{ECFV (ml/g)} \times [DMO]e \text{ (dpm/ml)})}{\text{Tissue ICFV. (ml/g)}} \qquad (2.4)$$

The transmembrane distribution ratio for H^+, (r_{H^+}), was calculated as:

$$r_{H^+} = [H^+]e/[H^+]i \qquad (2.5)$$

where $[H^+]e$ and $[H^+]i$ were based on extracellular and intracellular pH measurements.

Statistical Analyses

Means ± S.E.M. are reported throughout, unless otherwise stated. Significant differences within each group were tested ($p<0.05$) with Student's two-tailed t-test (paired design). Lines were fitted using the method of least squares linear regression, and the significance of simple Pearson's correlation coefficients was assessed.

RESULTS

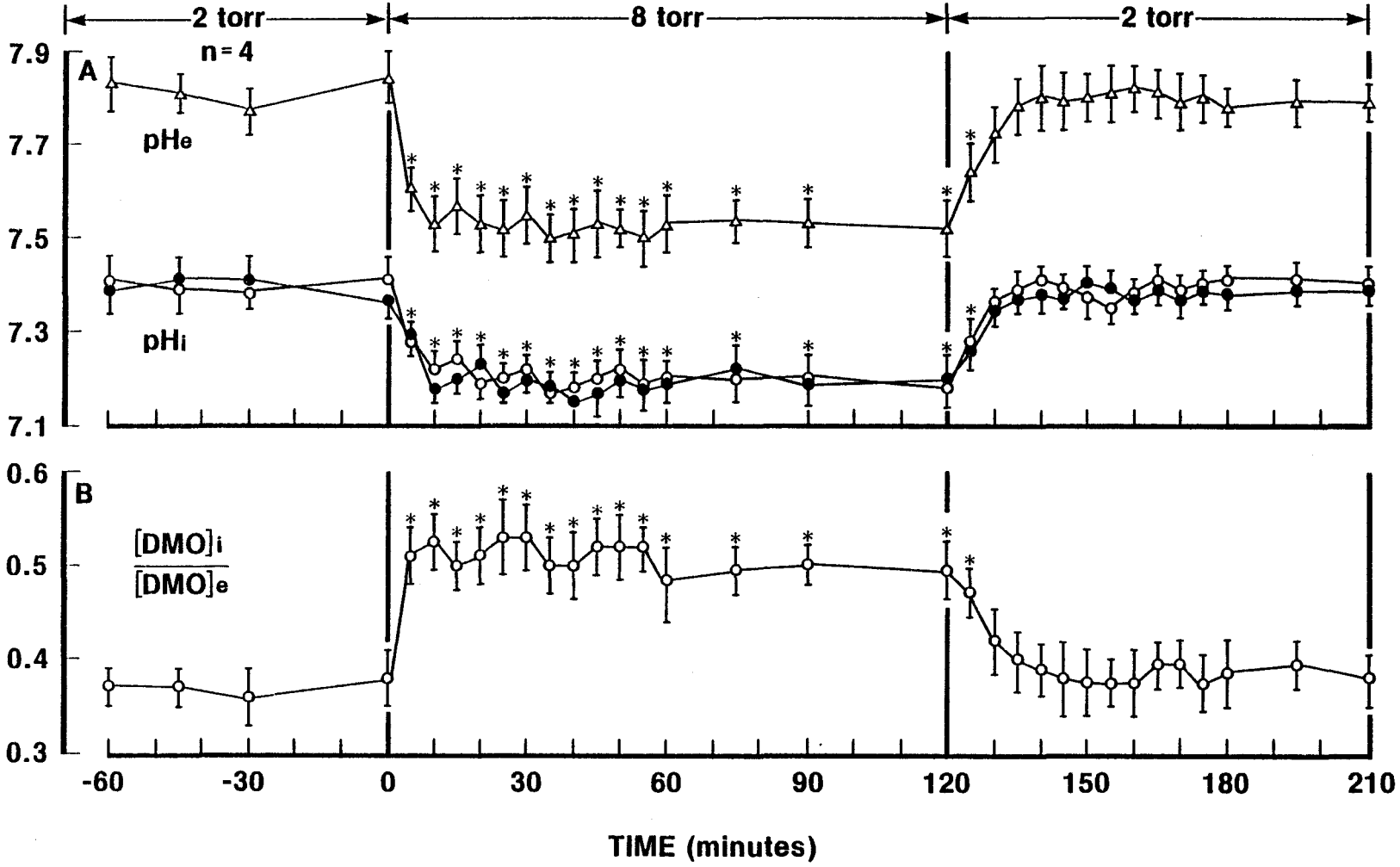
Whole Blood

The whole blood experiments in which the complete sequence of P_{CO_2} = 2 torr, 8 torr and 2 torr was imposed are shown in Fig. 2.2. The hematocrits ranged from 14-26%, with a mean of 18.3 ± 2.0%. Water contents of plasma and red cells at P_{CO_2} = 2 torr were 962.5 ± 0.4 ml/kg (n=6) and 664.3 ± 1.1 ml/kg (n=5), respectively and at 8 torr were 953.5 ± 1.0 ml/kg (n=6) and 696.3 ± 2.0 ml/kg (n=4). This significant change in water distribution reflected red cell swelling at the higher P_{CO_2}.

At P_{CO_2} = 2 torr, pHe = 7.81, the mean [DMO]i/[DMO]e ratio was 0.37 ± 0.02, corresponding to a mean pHi of 7.39 ± 0.02 (n=6), which was not different than that measured directly on red cell lysates (7.40 ± 0.02; Fig. 2.2A,B). Upon increasing P_{CO_2} to 8 torr, pHe fell and the [DMO]i/[DMO]e ratio increased significantly to 0.50 ± 0.03, resulting in a significant drop in pHi to 7.19 ± 0.03. Again, red cell pHi measured directly on cell lysates (7.19 ± 0.02) did not differ significantly. Red cell pHi measured on cell lysates and by DMO distribution reached a plateau within 15 min of the P_{CO_2} change and remained stable throughout the 120 min equilibration period. Upon returning P_{CO_2} to 2 torr, both pHe and pHi, as measured by both methods, returned to initial levels within 15 min.

The time courses of pHe and pHi changes were identical, for changes in both the acid and alkaline direction. At no point during the experimental period, either at a P_{CO_2} of 2 or 8 torr, did

Figure 2.2. Responses of (A) plasma pHe and red cell pHi and (B) the DMO distribution ratio in whole blood to changes of P_{CO_2} *in vitro*. Δ-pHe; ●-pHi measured on cell lysates; O-pHi measured by DMO distribution. Means ± 1 S.E.M. * indicates significant difference from initial values at P_{CO_2} = 2 torr. ($p<0.05$)


2 torr
8 torr
2 torr
n = 4
A
pHe
pHi
7.9
7.7
7.5
7.3
7.1
B
[DMO]i / [DMO]e
0.6
0.5
0.4
0.3
-60
-30
0
30
60
90
120
150
180
210
TIME (minutes)

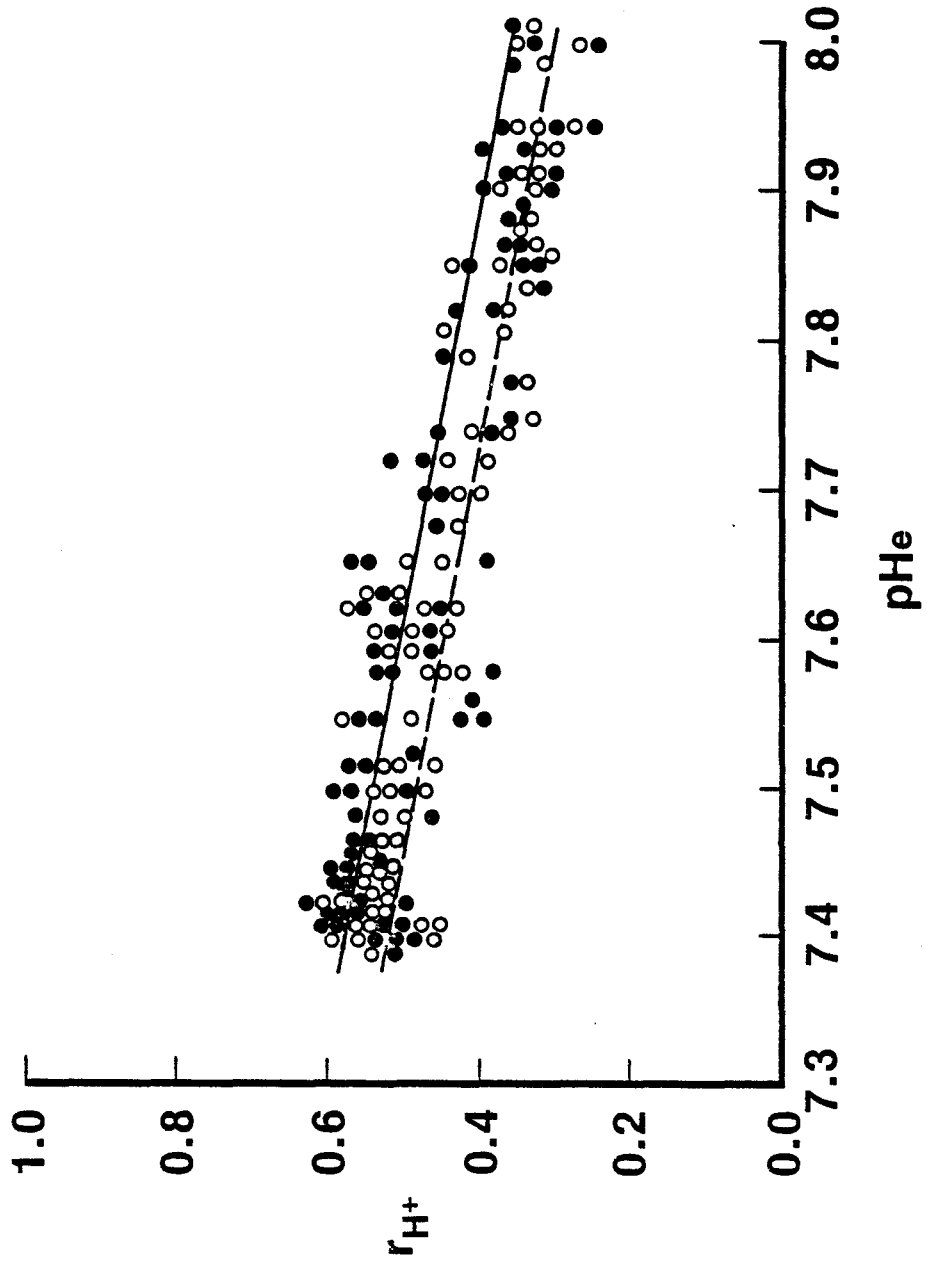
red cell pHi measured by DMO distribution differ significantly from that measured directly on red cell lysates. Red cell pHi, as measured by both techniques, responded as quickly to the P_{CO_2} change as did pHe. The limiting factor for all three measurements, therefore, appeared to be the time for complete gas changeover and equilibration (about 15 min); the actual DMO redistribution occurred within about 5 min as shown by the rapid change in the [DMO]i/[DMO]e ratio and the correspondence between pHi_{DMO} and pHi_{lysate} values.

The transmembrane distribution ratio for H^+ (r_{H^+}) was calculated separately from pHi_{DMO} and pHi_{lysate} values and plotted against pHe in Fig. 2.3. The regression lines describing the relationship between pHe and r_{H^+} DMO (r_{H^+} DMO = -0.37 x pHe + 3.26) and r_{H^+} lysate (r_{H^+} lysate = -0.36 x pHe + 3.24) are not significantly different, with r_{H^+} significantly correlated with pHe ($r= -0.73$; $p<0.001$).

Trunk Preparation

In initial experiments, duplicate muscle samples were taken throughout the perfusion period for determination of muscle water. However, total muscle water did not change during the perfusion period, so in subsequent experiments, muscle samples for total water were taken only at the beginning (P_{CO_2} = 2 torr; 768.6 $\pm$ 5.1 ml/kg; n=6) and end (P_{CO_2} = 15 torr; 770.4 $\pm$ 3.2 ml/kg; n=6) of the perfusion period. The mean of these two values was used for calculation of muscle ICFV. Muscle ECFV was also stable for the duration of the perfusion period, yielding mean values of 94.1 $\pm$ 5.0 ml/kg (n=8) at 2 torr and 97.6 $\pm$ 3.1 ml/kg (n=8) at 15 torr.

Figure 2.3. The relationship between pHe and the transmembrane distribution ratio for H^+ (r_H+) distribution across the red cell membrane in the blood of rainbow trout *in vitro*. O- r_H+ determined by pHi_{DMO}; ●- r_H+ determined from pHi_{lysate}. The regression lines are: $r_{H^+\ DMO} = -0.37 \times pHe + 3.26$; $r = -0.74$, $n=88$, $p<0.001$ (broken line); $r_{H+\ lysate} = -0.36 \times pHe + 3.24$; $r=-0.73$, $n=88$, $p<0.001$ (solid line)


1.0
0.8
0.6
0.4
0.2
0.0
r_{H^+}
7.3
7.4
7.5
7.6
7.7
7.8
7.9
8.0
pHe

After 90 min of perfusion at P_{CO_2} = 2 torr and pHe=7.79 ± 0.04 (n=6), the mean DMO distribution ratio for white muscle was 0.38 ± 0.03, corresponding to a mean pHi of 7.35 ± 0.04, which was not significantly different at 120 min (Fig. 2.4A). Switching to a perfusate equilibrated to a P_{CO_2} of 15 torr and pHe = 7.34 ± 0.02 resulted in a rapid, significant decrease in muscle pHi to 7.11 ± 0.03 (Fig. 2.4A). The change in [DMO]i/[DMO]e to 0.58 ± 0.03 was complete within 15 min and this ratio remained stable up to 60 min (Fig. 2.4B). To further test the reliability of the DMO distribution method, the reciprocal experiment was performed, where trunks were initially perfused with the acidic perfusate (P_{CO_2} = 15 torr, pHe = 7.37 ± 0.01 (n=4)) then switched to a more alkaline perfusate (P_{CO_2} = 2 torr, pHe = 7.81 ± 0.02). Muscle pHi increased significantly, from a mean of 7.00 ± 0.02, to a mean of 7.29 ± 0.03 (n=4; Fig. 2.4C). The DMO distribution ratio decreased significantly (Fig. 2.4D), again emphasizing that DMO was indeed redistributing in response to the pH change.

As expected, both white muscle pHi and red cell pHi, were significantly correlated ($P<0.01$) with pHe (Fig. 2.5A,B). The slope of the white muscle pHi vs. pHe relationship (0.53) was significantly less than the slope of the red cell pHi vs. pHe relationship (0.73 - 0.75), presumably reflecting a difference in buffer capacity of the two cell types.

Figure 2.4. Response of pHe, [DMO]i/[DMO]e and white muscle pHi to step changes in P_{CO_2} from 2 torr to 15 torr (n=6; A & B) and from 15 torr to 2 torr (n=4; C & D). * indicates a significant difference from values at time 0 ($p<0.05$).

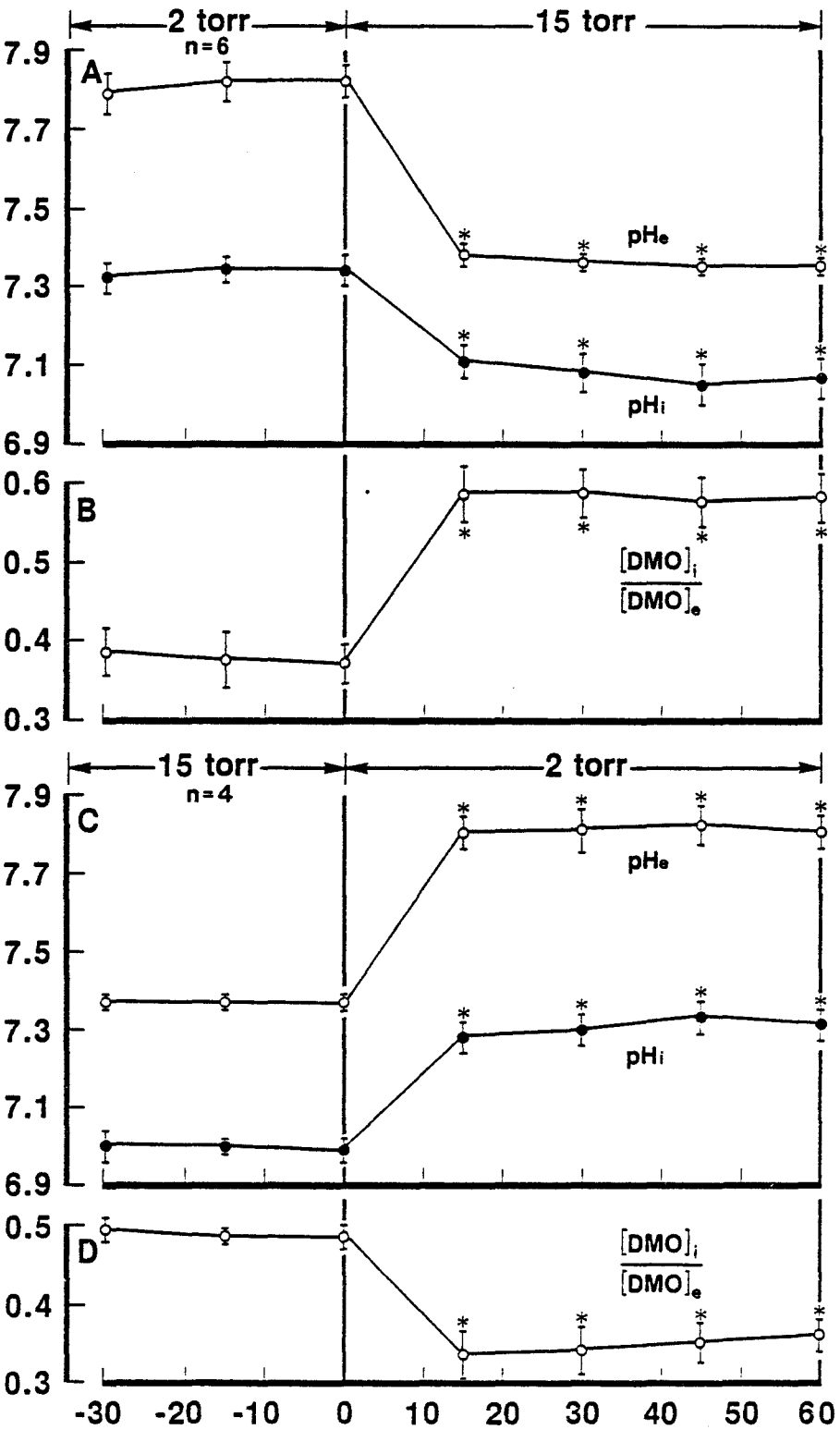
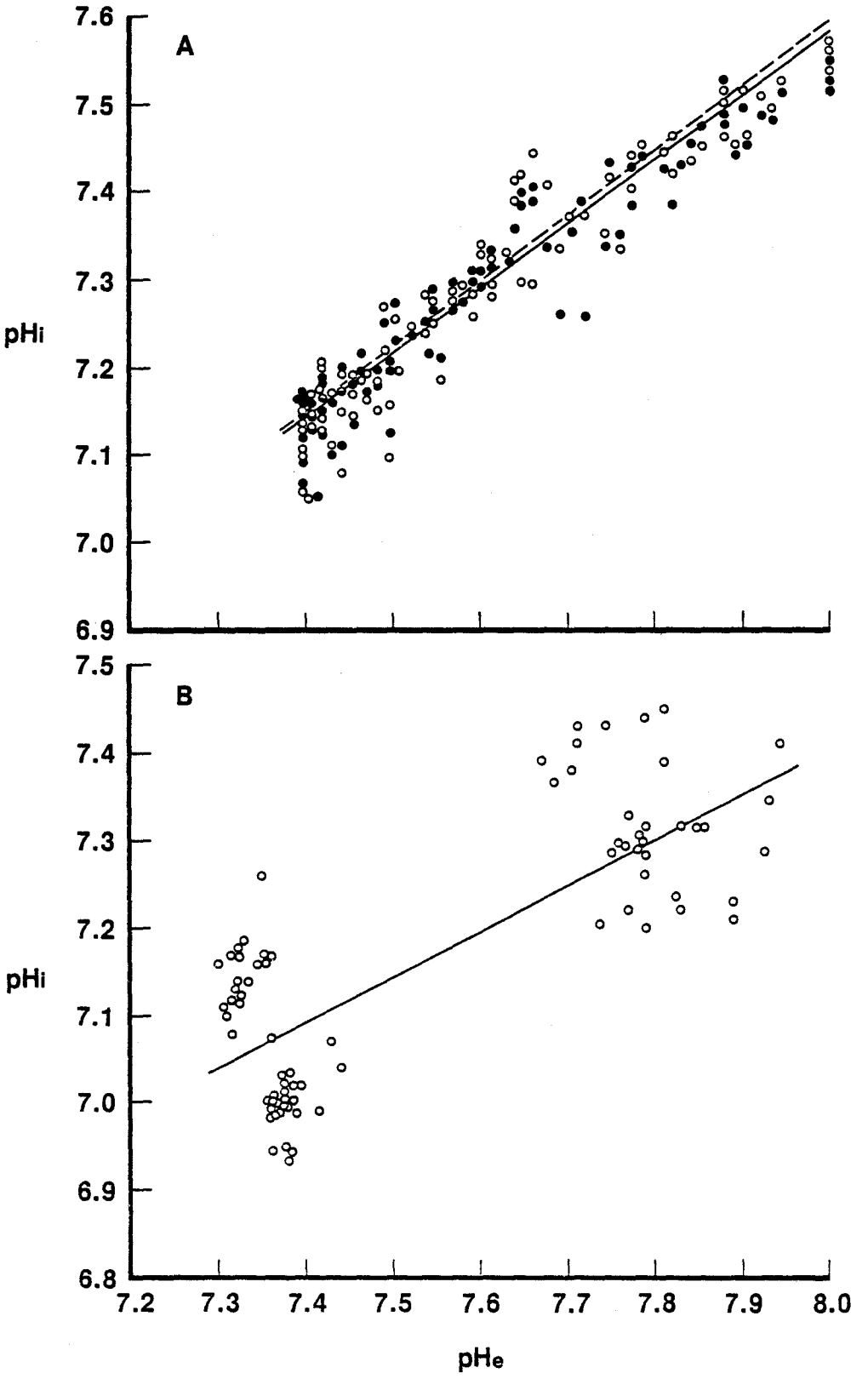

2 torr
n=6
15 torr
A
pHe
pHi
B
[DMO]i / [DMO]e
15 torr
n=4
2 torr
C
pHe
pHi
D
[DMO]i / [DMO]e
7.9
7.7
7.5
7.3
7.1
6.9
0.6
0.5
0.4
0.3
-30 -20 -10 0 10 20 30 40 50 60
TIME (minutes)

Figure 2.5. Relationships between pHe and (A) red cell pHi and (B) white muscle pHi. O-pHi_{DMO}; ●-pHi_{lysate}. The regression lines: red cell pHi_{DMO} = 0.75 x pHe + 1.59; r= 0.86, n=88, ($p<0.001$) (broken line); red cell pHi_{lysate} = 0.73 x pHe + 1.74; r= 0.90, n=88, ($p<0.001$) (solid line); muscle pHi = 0.53 x pHe + 3.00; n=68, r=0.75, ($p<0.001$).


A
B
pHi
pHi
pHe
7.6
7.5
7.4
7.3
7.2
7.1
7.0
6.9
6.8
7.2
7.3
7.4
7.5
7.6
7.7
7.8
7.9
8.0

DISCUSSION

Values of pHi

In trout blood *in vitro*, there was good agreement between DMO and direct lysate measurements of erythrocytic pHi over a wide range of pHe and pHi (Fig. 2.2, 2.5A). This agrees with numerous studies on mammalian erythrocytes (see Roos and Boron, 1981 for a review) and indicates that at least in this one fish tissue, DMO provides reliable estimates of pHi. It must be pointed out, however, that fish erythrocytes are nucleated, thus their intracellular compartment heterogeneous. Whatever the effect of this heterogeneity on intracellular H^+ distribution, both the cell homogenate and DMO method measured the same pHi.

The value (7.39 $\pm$ 0.02) for red cell pHi in the present study at pHe = 7.81 agreed well with that of 7.34 found for trout erythrocytes *in vivo* (see Fig. 3.3; Chapter III) and as well as for trout red cells incubated in saline at a similar pHe (Nikinmaa, 1982, 1983). The observed regression relationships between r_{H^+} and pHe (Fig. 2.3) are very similar to those described for $r_{HCO_3^-}$ in trout erythrocytes by Wood, McDonald and McMahon (1982), and r_{Cl^-} and $r_{HCO_3^-}$ in human erythrocytes (Bromberg *et al.*, 1965; Funder and Wieth, 1966). In the latter, this relationship was interpreted as a passive Gibbs-Donnan distribution of these ions. According to the Gibbs-Donnan equilibrium, the higher concentration of impermeant anions (eg. hemoglobin) within the red cell than in plasma will impose a concentration gradient upon freely permeant anions (eg. Cl^-, HCO_3^-) and cations (eg. H^+) (Woodbury, 1974).

If the permeant ions are passively distributed across the membrane, then the intracellular/extracellular distribution ratio (Donnan ratio) will be dependent upon the net charges on the impermeant anions. As the intracellular compartment is acidified, titration of the negative charges on hemoglobin by H^+ will reduce the net charge of impermeant anions, and the Donnan ratio will increase (extracellular/intracellular for cations; intracellular/extracellular for anions). The linear relationship between r_{Cl^-} and pHe observed by Haswell, Zeilder and Kim (1978) in tilapia (Oreochromis mossambica) blood and by Heming (1984) in trout blood also suggests that this is true of fish erythrocytes. However, definitive proof of Gibbs-Donnan distribution awaits simultaneous determination of the membrane potential.

Once P_{CO_2} was changed, red cell pHi changed in concert with pHe, and then was stable over time in the present study, as indicated by the constancy in the [DMO]i/[DMO]e ratio. This suggests that apart from the pHi regulation attributable to a passive physico-chemical buffering, trout erythrocytes in vitro , under the particular conditions of these experiments, were unable to regulate pHi back towards control values. However, recent evidence suggests that in vivo , catecholamine mobilization during the post-exercise acidosis permits pHi regulation by net H^+ extrusion (Nikinimaa, 1983; Cossins and Richardson, 1985). This topic is dealt with in greater detail in Chapter VII.

At a perfusate P_{CO_2} = 2 torr and pHe = 7.79, typical resting arterial levels in fish, values for white muscle pHi from the

perfused trunk (7.29 - 7.35) are in good agreement with those measured *in vivo* at 15 °C with the DMO distribution method in trout, (7.21, see Fig. 4.3A; 7.30-7.32, Hōbe *et al.*, 1984), eels (*Anguilla rostrata*) (7.34, Walsh and Moon, 1982) and channel catfish (*Ictalurus punctatus*) (7.36, Cameron and Kormanik, 1982).

The only pHi regulation observed during high P_{CO_2} perfusion of the trunk was that attributable to passive physico-chemical buffering reflected in the decreased pHe - pHi gradient (Fig. 2.4A). This apparent lack of recovery of muscle pHi over the 90 min perfusion period may have been due to an inhibition of the pHi regulatory mechanism(s) caused by the persistent depression of pHe (Roos and Boron, 1981). It is also possible that some rapid recovery occurred within the first 15 min of high P_{CO_2} so that the observed values are 'partially recovered'. However in intact fish *in vivo*, recoveries of both pHe and pHi in the face of maintained high blood P_{CO_2} are lengthy processes with time courses well beyond the present experimental period (Cameron, 1980; Hōbe *et al.*, 1984).

pHi Transients

The results from both the whole blood and perfused trunk experiments demonstrated that the DMO distribution method could reliably detect pHi transients within 15 min of the extracellular P_{CO_2} change, as indicated by the rapid, significant, and stable changes in the DMO distribution ratio (Figs. 2.2, 2.4). It therefore should be capable of detecting the pHi changes which are suspected to occur over such a time course after exhaustive exercise in fish (Holeton *et al.*, 1983; Turner, Wood and Clark, 1983). These

results therefore justify the use of this technique for measuring such changes in the remainder of the present study.

Traditionally, the DMO distribution method has not been used to measure pHi transients, as it was thought that DMO equilibration time would be limiting (Roos and Boron, 1978, 1981). There is, however, some ambiguity over the interpretation of equilibration time. The initial equilibration period, i.e. the time requirement for convective mixing, can, indeed, be quite lengthy: 4-8 h in fish (Cameron and Kormanik, 1982; Walsh and Moon, 1982; Hōbe et al. , 1984) and 1-2h in humans (Manfredi, 1963). Indeed, the mixing time for mannitol alone throughout the ECFV in intact trout is about 3h (Milligan and Wood, 1982). The in vitro trunk preparation of the present study required 90 min for initial distribution of markers. However, once DMO had distributed throughout the system, equilibration of DMO across the intracellular/extracellular interface occurred quickly, as indicated by the rapid response of the DMO distribution ratio to changes in P_{CO_2} observed in both whole blood and muscle (Fig. 2.2, 2.4). This is in agreement with several previous studies. In isolated eel hepatocytes (Walsh and Moon, 1983) and isolated barnacle muscle fibers (Hinke and Menard, 1978), DMO diffusion across the cell membrane was similarly rapid (less than 15 min). In a study of isolated rat diaphragms, Roos and Boron (1978) successfully demonstrated pHi transients with the DMO technique, and furthermore, showed that their results agreed reasonably well with a parallel study using pH microelectrodes. However, the temporal resolution of the transients was only 30 min, which may have reflected

the slower nature of the true pHi change compared to the present study. In addition, DMO equilibration in their study may have been limited by lack of perfusion, as the extracellular fluid was bathing, rather than perfusing, the diaphragm. The DMO technique was also successfully employed in isolated perfused rat hearts to detect pHi transients in response to ischemia and anoxia (Neely, Whitman and Rovetto, 1975). These authors were able to detect pHi changes within 5 min of ischemia; the faster resolution time probably reflected the faster pHi change.

In conclusion, the DMO technique appears suitable for measurement of pHi transients over approximately 15 min in fish tissues in vivo , provided the markers have already distributed throughout the system.

CHAPTER III

INTRACELLULAR AND EXTRACELLULAR ACID-BASE STATUS AND H^+ EXCHANGE WITH THE ENVIRONMENT AFTER EXHAUSTIVE EXERCISE IN THE RAINBOW TROUT

INTRODUCTION

In the previous chapter, it was shown that the DMO technique (Waddell and Butler, 1959) for measuring mean tissue intracellular pH (pHi) can reliably detect pHi transients over a time course similar to that likely to occur after exhaustive exercise in fish. In the present study, this method was applied to examine changes in mean intracellular acid-base status of the whole body after exhaustive exercise in the rainbow trout.

This chapter focuses on acid-base changes in the whole body intracellular fluid (ICF), extracellular fluid (ECF) (arterial and venous blood) and environmental water after strenuous exercise in the rainbow trout, with particular emphasis on transcompartmental H^+ (metabolic acid) and lactate exchanges. This study also assesses the behaviour of the radiolabels used in the DMO technique (^{3}H-mannitol, ^{14}C-DMO) during long term experiments <u>in vivo</u>, the possible influence of arterial <u>versus</u> venous sites of measurement, and changes in erythrocyte pHi after exhaustive exercise.

MATERIALS AND METHODS

Experimental Animals

Adult rainbow trout (Salmo gairdneri) of both sexes (280-1200 g) were purchased from Spring Valley Trout Farm, Petersburg, Ontario at various times of the year and held as described in the previous chapter. Acclimation and experimental water had the following typical composition: $[Na^+]$ = 0.6; $[Cl^-]$ = 0.8; $[Ca^{2+}]$ = 1.6; $[Mg^{2+}]$ = 0.3; $[K^+]$ = 0.05 and titration alkalinity = 2.0 meq/l; total hardness = 140 mg/l as $CaCO_3$; pH = 8.0.

Trout were anaesthetized with MS 222 (1:10,000; Sigma) and surgically fitted with dorsal aorta cannulae (Soivio et al .,1972) and, in some cases, ventral aortic cannulae (Kiceniuk and Jones, 1977). Fish were allowed to recover at least 48h prior to experimentation in 10 l darkened Lucite flux boxes of the design described by McDonald (1983). During recovery, the boxes were flushed with fresh water at about 0.5 l/min. In this box, the fish was confined within an inner chamber through which water was continuously recirculated at about 0.3 l/min by an air-lift pump at the rear. The air lift also maintained oxygenation of the water. For measurements of acidic equivalent, ammonia, and lactate excretion, inflow to the box was stopped; it was then operated as a low volume (3-6 l) recirculating system. Temperature was controlled at 15 $\pm$ 1 ^{o}C by bathing the boxes with chilled water.

Experimental Protocols

(1) Series I

The first series focused on post-exercise changes in arterial acid-base and lactate status, whole body pHi and ECFV, acidic equivalent exchanges with the environment, and excretion of ^{3}H-mannitol and ^{14}C-DMO. The fish (mean weight = 346.3 $\pm$ 43.5 g; n = 18) were fitted with arterial catheters only. Parallel experiments were performed on two separate groups, only one of which was subjected to exhaustive exercise (n = 8). The other (n = 10) served as a control for handling and sampling effects; these fish were left at rest throughout but otherwise treated identically to the experimental group.

Twenty-four hours prior to exercise, the inflow to the flux box was closed, the volume standardized to 1 liter per 100g body weight, and thereafter, water recirculated within the box by means of aeration. The box was thoroughly flushed with fresh water at experimental temperature (15 $\pm$ 1 ^{o}C) for approximately 20 min at 12h prior to exercise, at the time of exercise, then again at 12 and 24h post-exercise, at which point the experiment was terminated. Thus two 12h control periods at rest were followed by two 12h periods post-exercise. The water was flushed at 12h intervals to prevent ammonia levels in the box from exceeding 500 umol/l, for experience suggests that this represents a threshold above which branchial ion and/or acid-base exchanges may be inhibited (C.M.Wood, personal communication). At the beginning of the 12h flux period prior to exercise, fish were infused with a precisely measured dose (1 ml/kg)

of 5 uCi/ml ^{14}C-DMO plus 20 uCi/ml ^{3}H-mannitol in Cortland saline followed by an equal volume of saline through the arterial cannula. This allowed 12h for marker distribution before the first blood sample was taken. Water samples were taken for analysis of ammonia and titratable acidity flux at the start and end of each 12h period prior to exercise and at 0,1,2,4,8,12 and 24h post exercise. Thus, net fluxes of ammonia and acidic equivalents, as well as losses of markers to the environmental water, were followed before and after exercise.

Fish were exercised by vigorously chasing them around a large circular tank (500 l) for 6 min (cf. Turner, Wood and Clark, 1983), by which point they were completely incapable of further burst exercise, but were able to keep swimming slowly. This probably represents a situation of fatigue of the white muscle (see Chapter IV) with any continued activity dependent on aerobic red muscle. The exhausted trout were then immediately returned to the experimental chambers.

Fluxes of titratable acidity and ammonia and loss of markers to the water could not be measured during the brief exercise period. However, even if such fluxes had occurred at up to five times the immediately post-exercise rate during the 6 min exercise period, their influence on all calculated parameters would have been negligible.

Blood samples (500 ul) were drawn prior to ('rest'), immediately after (time 0) exercise, as well as at 0.25, 0.5, 1, 2, 4, 8, 12 and 24 hours post-exercise. Samples were analyzed for pH, total CO_2 (in both whole blood and plasma), hematocrit, [hemoglobin], whole blood [lactate] and [glucose], and plasma levels of ^{3}H and ^{14}C

radioactivity. The volume of blood sampled was replaced with saline.

(2) Series II

The second series employed much larger trout (mean weight = 806.4 $\pm$ 172 g; n= 5) fitted with both arterial and venous catheters. This series of experiments were designed to compare post-exercise changes in venous versus arterial acid-base and lactate status, to assess possible differences in ^{14}C-DMO and ^{3}H-mannitol distribution between arterial and venous blood and their impact on pHi calculations, and to directly measure changes in red cell (RBC) pHi after exercise. With the following exceptions, the experimental protocol was identical to that of the exercised group in the first series. The larger size of the fish necessitated running the flux boxes on a continual flow-through basis, thereby precluding measurement of acidic equivalent exchange with the water or radiolabel excretion, and therefore calculation of whole body ECFV or pHi. Simultaneous arterial and venous blood samples were drawn at each time, and these were of larger volume (900 ul) to facilitate the RBC pHi determinations. Samples were analyzed for pH, total CO_2 (in both whole blood and plasma), hematocrit, whole blood [lactate], RBC pHi, and plasma levels of ^{3}H and ^{14}C radioactivity.

(3) Series III

The third series employed non-cannulated fish (mean weight = 216.4 $\pm$ 8.8 g; n = 4) to assess lactate excretion into the water after exercise in comparison to net acidic equivalent exchange during the period of maximum blood lactacidosis. The flux boxes were operated as closed low volume systems, and water samples taken for analysis of

lactate, ammonia and titratable acidity flux at 0, 2 and 4h post-exercise.

Analytical Techniques and Calculations

Blood pH was determined on 40 ul samples injected into a Radiometer pH microelectrode (type E5021) maintained at 15 ± 1 °C and linked to a Radiometer PHM 71 or 72 acid-base analyzer. Total CO_2 in both whole blood and plasma was measured on 50 ul samples by the method of Cameron (1971) using Radiometer CO_2 electrodes. P_{CO_2} and $[HCO_3^-]$ in blood and plasma were calculated by rearranging the Henderson-Hasselbalch equation for the bicarbonate/carbonic acid system:

$$Pa_{CO_2}\ (torr) = \frac{Ca_{CO_2}\ (mmol/l)}{\alpha CO_2\ (mmol/l/torr) \times (1 + 10^{(pHa - pK')})} \qquad (3.1)$$

and

$$\left[HCO_3^-\right]\ (mmol/l) = Ca_{CO_2}\ (mmol/l) - (\alpha CO_2\ (mmol/l/torr) \times Pa_{CO_2}\ (torr)) \qquad (3.2)$$

using αCO_2 and pK' values determined for rainbow trout plasma at 15 °C by Boutilier, Heming and Iwama (1984).

Whole blood metabolic acid load ($\Delta H^+ m_{WB}$) was calculated in the cumulative fashion described by Turner, Wood and Clark (1983), using the equation derived by McDonald, Boutilier and Toews (1980) for each time interval:

$$\Delta H^+ m_{WB} = \left[HCO_3^-\right]_1 - \left[HCO_3^-\right]_2 - \beta(pH_1 - pH_2) \qquad (3.3)$$

and summing (taking account of the sign) for each period from the rest sample onwards. In this equation, $[HCO_3^-]$ was the value for whole blood (in meq/l) because ΔH^+m_{WB} was compared to whole blood lactate, and β, the non-bicarbonate buffer capacity of whole blood, was estimated from the blood hemoglobin concentration at each time using the regression relationship derived by Wood et al . (1982) from in vitro tonometry of rainbow trout blood at 15°C:

$$\beta \text{ (mmol/pH/l)} = -1.073 \times [\text{hemoglobin}] \text{ (g/100 ml)} - 2.48 \qquad (3.4)$$

Hemoglobin is the major component of non-bicarbonate buffering in fish blood (Albers, 1970).

Hematocrit was determined by centrifuging 80 ul of whole blood in heparinized capillary tubes (Radiometer) for 5 min at 5000 g. [Hemoglobin] was determined on 20 ul blood by the cyanmethemoglobin method (Blaxhall and Daisley, 1973) using Sigma reagents. Mean cell hemoglobin concentration (MCHC) was calculated as [hemoglobin]/hematocrit. Whole blood [lactate] was measured on 100 ul whole blood deproteinized with 200 ul ice-cold 8% perchloric acid and then centrifuged at 9000 g for 3 min. The supernatant was analyzed enzymatically (l-lactate dehydrogenase method; Loomis, 1961) for lactate using Sigma reagents. Whole blood [glucose] was analyzed on 100 ul samples deproteinized in 900 ul ice-cold trichloro-acetic acid, using the O-toluidine method of Hyvarinon and Nikkita (1962) and Sigma reagents.

For determination of ^{3}H-mannitol and ^{14}C-DMO activity in the extracellular fluid, 50 ul of plasma was added to 10 ml

scintillation fluid (ACS; Amersham) and counted on a Beckman LS-250 scintillation counter. The injected stock was similarly assayed. Radiotracer loss to the water was determined by counting 5 ml water in 10 ml ACS fluor. Dual label quench correction was performed using the external standard ratio method as described by Kobayashi and Maudsley (1974).

Red cell pHi was measured on a pellet of cells, as described in Chapter II.

Whole body extracellular fluid volume (ECFV) at each time was calculated as:

$$\text{ECFV (ml)} = \frac{^{3}\text{H-mannitol injected (dpm)} - \Sigma\text{excreted (dpm)} - \Sigma\text{sampled (dpm)}}{\text{Plasma}\left[^{3}\text{H-mannitol}\right]\ \text{(dpm/ml)} \ /\text{Plasma } H_2O\ \text{(ml/ml)}} \qquad (3.5)$$

where Σ sampled refers to loss of marker via blood sampling and was calculated as

$$\Sigma\text{sampled (dpm)} = \frac{(1 - \text{Ht}) \times \text{vol (ml)} \times \text{Plasma}\left[^{3}\text{H-mannitol}\right]\ \text{(dpm/ml)}}{\text{Plasma } H_2O\ \ \text{(ml/ml)}} \qquad (3.6)$$

where Ht was hematocrit as a decimal and vol was the volume of each blood sample. Σ excreted at each time was calculated as:

$$\Sigma\text{excreted (dpm)} = \text{Water}\left[^{3}\text{H-mannitol}\right]\ \text{(dpm/ml)} \times \text{Water volume (ml)} \qquad (3.7)$$

taking into account the exchange of water during flushes. Intracellular fluid volume (ICFV) was calculated as the difference between total body water and ECFV. Plasma water was calculated from the mean refractive index at each time from the experiments in Chapter IV.

Mean whole body pHi at each time was calculated according to equation 2.2, where [DMO]e was calculated according to equation 2.3 and

$$[DMO]i \text{ (dpm/ml)} = \frac{^{14}\text{C-DMO injected (dpm)} - \Sigma\text{excreted (dpm)} - \Sigma\text{sampled (dpm)} - (\text{ECFV (ml)} \times [DMO]e \text{ (dmp/ml)})}{\text{ICFV (ml/ml)}} \quad (3.8)$$

where Σ sampled and Σ excreted were calculated analogously to equations 3.6 and 3.7, respectively. The importance of accounting for marker excretion and loss due to sampling in whole body pHi calculations has been demonstrated by Hobe *et al*. (1984) and Wood and Cameron (1985).

Total metabolic acid load to the whole body at each time was calculated as

$$\Delta H^+m \text{ (meq/kg)} = \Delta H^+m_{ECFV} \text{ (meq/kg)} + \Delta H^+m_{ICFV} \text{ (meq/kg)} \quad (3.9)$$

where

$$\Delta H^+m_{ECFV} \text{ (meq/kg)} = BV \text{ (l/kg)} \times \Delta H^+m_{WB} \text{ (meq/l)} + \{(ECFV - PV) \text{ (l/kg)} \times \Delta H^+m_{ISF} \text{ (meq/l)}\} \quad (3.10)$$

BV and PV were blood and plasma volumes, respectively, using values measured in rainbow trout at 15 ^{o}C by Milligan and Wood (1982) and ΔH^+m_{WB} was calculated by equation (3.3). To estimate ΔH^+m_{ISF}, the metabolic acid load to the interstitial fluid (ISF), the β value of the ISF was taken to be the same as that for separated plasma of rainbow trout at 15 ^{o}C: -2.59 mmol/pH/l (Wood *et al*.,1982) and interstitial [HCO_3^-] was assumed to be the same as that of true plasma. For details concerning calculation of ΔH^+m_{ICFV}, metabolic acid load to the intracellular compartment, see Discussion.

The exchange of acidic equivalents (H^+) with the environmental water was calculated as the sum of titratable acidity flux and ammonia flux, signs considered. Titratable acidity flux was calculated from titratable alkalinity measurements in which continuously aerated 10 ml water samples, maintained at experimental temperature (15 ^{o}C), were titrated to to an end point pH of 4.0 with 0.02 N HCl, as outlined in McDonald and Wood (1981). Water [ammonia] was determined using a micro-modification of the phenol-hypochlorite method of Solorzano (1969). The methods used do not distinguish between acidic equivalent (H^+) excretion and basic equivalent uptake, or vice-versa . Fortunately, this does not matter with respect to the acid-base status of the animal. When H^+ excretion or uptake is referred to, it could also be interpreted as basic equivalent uptake or excretion, respectively.

Since lactate levels in the water were quite low (0.5-2.2 umol/l), 500 ml water samples were freeze-concentrated 100-fold (Birchard, 1977), then assayed for lactate as described. In addition recovery tests, this method was found to yield 60 $\pm$ 1.3% (n=8) recovery, and experimental values were corrected accordingly.

Statistical Analysis

Means $\pm$ 1 SEM are reported throughout. Student's two-tailed t-test (paired design) was used to assess significant differences ($p<0.05$) within groups (eg. arterial versus venous, post-exercise versus rest) using each fish as its own control. A non-paired t-test was used for between group comparisons.

RESULTS

Behaviour of ^{14}C-DMO and ^{3}H-mannitol

The concentrations of both ^{14}C-DMO and ^{3}H-mannitol in arterial and venous blood plasma were identical at rest (Table 3.1). Immediately after exercise (0h), arterial concentrations of both radiolabels were significantly elevated over venous values by about 5%. There were no significant differences at any other time. The impact of arterial versus venous measurement sites on whole body ECFV and pHi calculations (see below) turned out to be negligible.

Significant excretion of both radiolabels occurred into the environmental water (Table 3.2). Excretion rates were highly variable between individual fish, but were more or less linear over time and of about the same absolute size for ^{14}C-DMO and ^{3}H-mannitol. There were no significant differences at any time between the control and experimental groups, indicating that exercise did not influence the rate of marker loss. Cumulative losses due to repetitive blood sampling amounted to only about 10% of losses due to excretion. By time 0 (i.e. 12h post-infusion), only about 85-90% of the injected ^{14}C-DMO and ^{3}H-mannitol doses remained in the animals, and by 24h (i.e. 36h post-infusion) this was reduced to about 70% (Table 3.2). Failure to take these losses into account would have led to overestimates of mean whole body pHi by 0.13 (time 0) to 0.37 units (24h) and overestimates of ECFV by 28 (time 0) to 107 ml/kg (24h).

ECFV estimates were also complicated by the length of time the marker was in the fish. At the rest sample, ^{3}H-mannitol had been in the animal for about 12h, and by the end of the experiment, 36h had

Table 3.1. Ratio of arterial to venous plasma concentrations of ^{14}C-DMO and ^{3}H-mannitol.

	Time after exercise (hours)								
REST	0	0.25	0.5	1	2	4	8	12	24
^{14}C-DMO arterial/venous									
1.007 ± 0.016 (5)	1.032 ± 0.008 (5) *	1.061 ± 0.033 (5)	1.022 ± 0.021 (5)	1.015 ± 0.011 (5)	1.016 ± 0.010 (5)	1.019 ± 0.011 (5)	0.994 ± 0.016 (5)	1.021 ± 0.021 (4)	1.012 ± 0.008 (3)
^{3}H-mannitol arterial/venous									
1.009 ± 0.011 (5)	1.052 ± 0.011 (5) *	1.024 ± 0.019 (5)	1.017 ± 0.023 (5)	1.049 ± 0.029 (5)	1.038 ± 0.023 (5)	1.034 ± 0.020 (5)	1.005 ± 0.023 (5)	1.023 ± 0.013 (5)	1.012 ± 0.016 (5)

Means ± 1 SEM (n)

* indicates value is significantly different ($P < 0.05$) from 1.000.

Table 3.2. Percent of injected marker doses excreted to the environment in exercised and non-exercised rainbow trout.

Time after injection (hours)	12	14	16	20	24	36
Experimental time (hours)	0	2	4	8	12	24
Control group						
^{14}C-DMO	11.61 ± 4.36% (10)	14.03 ± 4.29% (10)	16.44 ± 4.48% (10)	18.99 ± 4.59% (10)	24.16 ± 5.21% (10)	39.17 ± 9.68% (4)
^{3}H-mannitol	14.00 ± 3.23% (10)	17.00 ± 4.02% (10)	18.78 ± 4.46% (10)	21.48 ± 4.51% (10)	22.99 ± 4.47% (10)	40.44 ± 11.91% (4)
Exercise Group						
^{14}C-DMO	10.45 ± 2.03% (8)	13.45 ± 2.19% (8)	15.13 ± 2.28% (8)	17.51 ± 2.28% (8)	22.06 ± 1.33% (8)	29.20 ± 0.83% (4)
^{13}H-mannitol	13.27 ± 3.16% (8)	16.47 ± 2.95% (8)	17.42 ± 2.92% (8)	21.77 ± 4.19% (8)	23.54 ± 4.61% (8)	39.00 ± 6.86% (8)

Means ± 1 SEM (n)

There were no significant differences at any time between the control and experimental group.

elapsed since marker infusion. At rest (12h post-infusion), the calculated ECFV in both groups was about 250 ml/kg (Table 3.3), which is in close agreement with two previous determinations of ECFV in freshwater rainbow trout obtained by entirely different methods (Milligan and Wood, 1982; M.V.E. Attygalle, G. Shelton and P.C. Croghan, unpublished observations). However during the experimental period (12-36h post-infusion), the control group exhibited a gradual rise in ECFV, so that by 2h, the rise was significant, and by 24h, ECFV had increased by nearly 30% (Table 3.3). This apparent expansion of the extracellular space was due to gradual ^{3}H-mannitol penetration into the intracellular space of the liver, and occasionally heart, after lengthy equilibration; other tissues (brain, white muscle, red muscle) were unaffected (Chapter IV; C.M. Wood and S. Munger, unpublished observations). The calculated ECFV in the exercise group showed a significant decline for the first 2h after exercise, but thereafter exhibited a gradual rise similar to that in the control group (Table 3.3). Clearly the true post-exercise changes were masked by this artifact. In order to 'unmask' these changes, rest values (at 12h post-infusion), which agreed with previous ECFV determinations (see above), were assumed to be correct. The mean increase over rest values in the control group (Table 3.3) was then subtracted from the exercise value at each respective time to yield the corrected ECFV values of Fig. 3.4A. These corrected values were then used in the calculation of mean whole body pHi (Fig. 3.5).

Table 3.3. Whole body extracellular fluid volume (uncorrected) in exercised and non-exercised rainbow trout.

	REST	0h	0.25h	0.5h	1h	2h	4h	8h	12h	24h
Control										
	253.2 ± 10.8 (10)	246.5 ± 12.1 (10)	254.2 ± 10.2 (10)	246.0 ± 6.8 (10)	257.2 ± 10.9 (10)	258.9 ± 8.3 (10) *	277.8 ± 13.9 (10) *	285.1 ± 10.5 (10) *	299.9 ± 16.5 (10) *	303.0 ± 28.4 (4) *
Exercise										
	253.5 ± 11.6 (8)	210.4 ± 14.1 (8) *	201.9 ± 12.2 (8) *	209.1 ± 9.6 (8) *	231.8 ± 15.2 (8) *	230.7 ± 7.2 (8)	249.9 ± 13.2 (8)	303.2 ± 19.3 (6) *	315.2 ± 32.9 (6) *	355.3 ± 18.6 (4) *

Means ± 1 SEM. (n). All values in ml/kg body weight.

*-significantly different ($P<0.05$) from corresponding rest value.

Blood Acid-Base and Metabolite Status

In the first series, exhaustive exercise resulted in a pronounced arterial blood acidosis, with pHa maximally depressed immediately post-exercise (Fig. 3.1A). Blood acid-base status recovered relatively quickly, showing an alkalosis at 8 and 12h post-exercise, and by 24h, recovery was complete. The initial acidosis was of mixed respiratory and metabolic origin, as indicated by the rapid elevation of Pa_{CO_2} (Fig. 3.1C) and slower depression of $[HCO_3^-]$ (Fig. 3.1B). Analysis based upon the principles outlined by Wood et al. (1977) revealed that immediately post-exercise, the respiratory and metabolic components contributed equally to the acidosis. The respiratory component dissipated quickly, as Pa_{CO_2} returned to rest levels (Fig. 3.1C), so that by 2h the metabolic component prevailed, as reflected in the continuing depression of plasma $[HCO_3^-]$ (Fig. 3.1B). However, at 8 and 12h, $[HCO_3^-]$ was elevated above the resting level, causing the observed alkalosis at these times. Blood acid-base status in the control group was unaffected by sampling (Fig. 3.1A,B,C).

Blood lactate followed an entirely different pattern, rising slowly after exercise and peaking at about 17.5 mmol/l at 2-4h, a time when blood pH was beginning to recover. Lactate levels then fell slowly, returning to rest values by 24h (Fig. 3.1D). In the control group, [lactate] did not deviate significantly from resting levels.

Whole blood [glucose] rose by about 50% during the first hour after exercise and remained significantly elevated for 12h (Fig. 3.2A). There were very much smaller elevations in the control group.

Figure 3.1. Blood acid-base status in exercised (●) and non-exercised (O) rainbow trout: (A) pHa; (B) arterial plasma bicarbonate concentration, $[HCO_3^-]$; (C) arterial CO_2 tension, Pa_{CO_2}; (D) whole blood [lactate]. Means $\pm$ 1 SEM. Control group: n=10, except at 24h, n=4. Exercise group: n=8, except at 8 and 12h, n=6 and at 24h, n=4. R = rest, bar indicates 6 min of exhaustive exercise, 0 = immediately after exercise. * indicates a significant difference ($p<0.05$) from corresponding rest value.

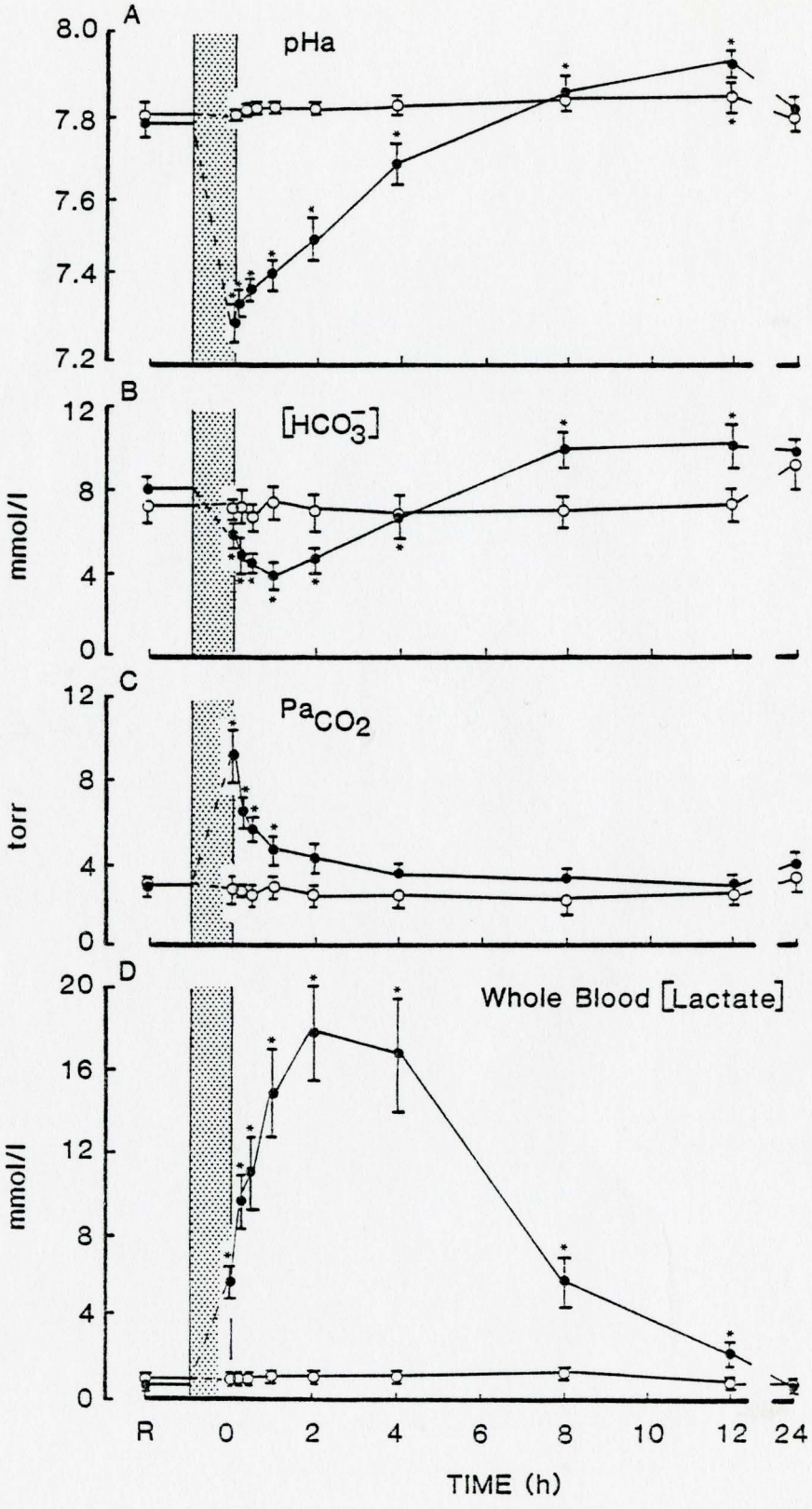
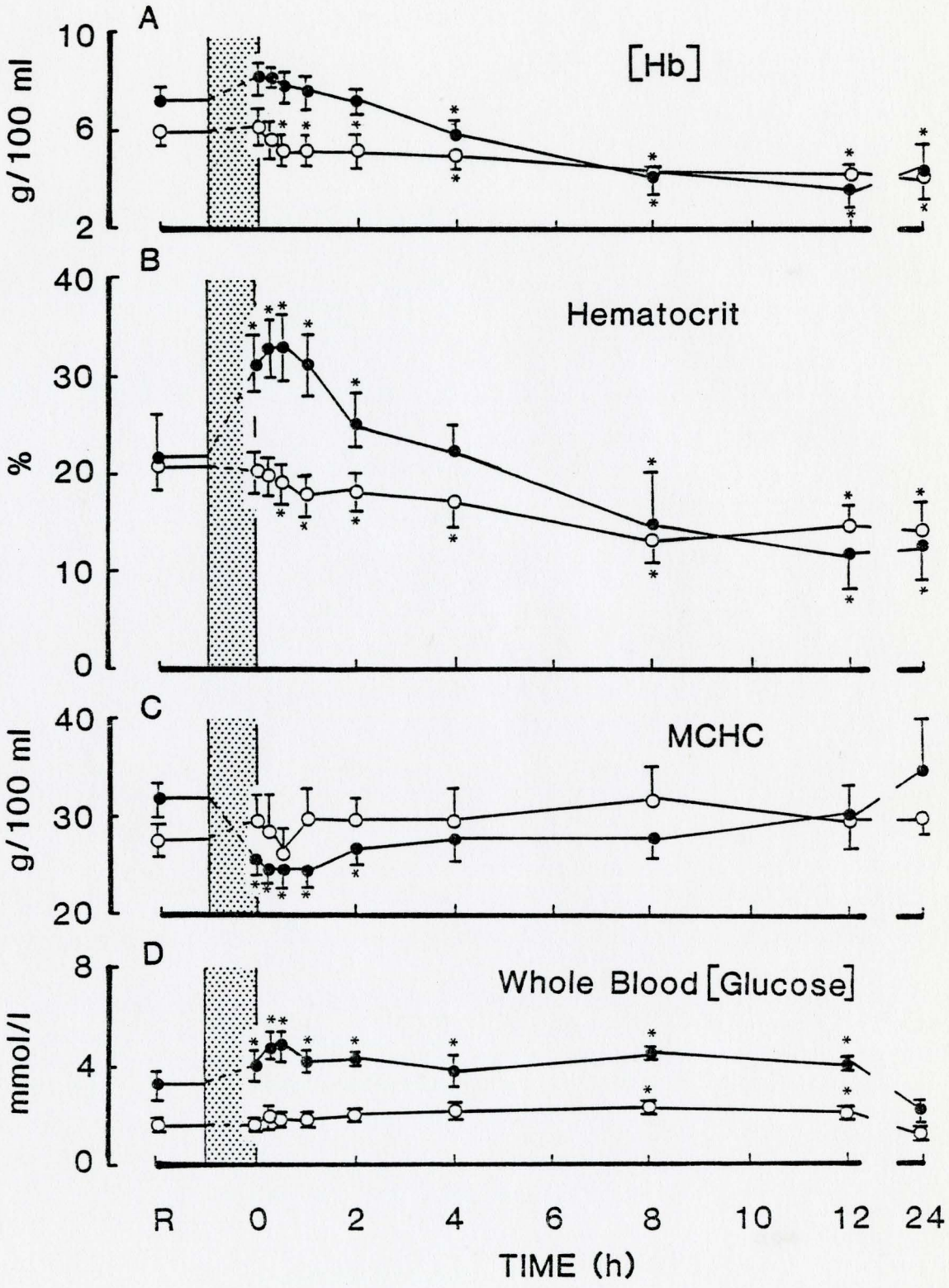

A
pHa
8.0
7.8
7.6
7.4
7.2
B
$[HCO_3^-]$
mmol/l
12
8
4
0
C
Pa_{CO_2}
torr
12
8
4
0
D
Whole Blood [Lactate]
mmol/l
20
16
12
8
4
0
R
0
2
4
6
8
10
12
24
TIME (h)

Figure 3.2. (A) [Hemoglobin] ([Hb]), (B) hematocrit, (C) whole blood [glucose], and (D) mean cell [hemoglobin] (MCHC) in exercised (●) and unexercised (O) fish. Means $\pm$ 1 SEM. Control group: n=10, except at 24h, n=4. Exercise group: n=8, except at 8 and 12h, n=6 and at 24h n=4. R = rest, bar indicates 6 min of exhaustive exercise, 0 = immediately after exercise. * indicates a significant difference ($p<0.05$) from corresponding rest value.


A
[Hb]
g/100 ml
10
6
2
B
Hematocrit
%
40
30
20
10
0
C
MCHC
g/100 ml
40
30
20
D
Whole Blood [Glucose]
mmol/l
8
4
0
R
0
2
4
6
8
10
12
24
TIME (h)

[Hemoglobin] was elevated slightly up to 2h post-exercise, despite the dilution due to sampling which, in the control group, caused [hemoglobin] to decline significantly over this period (Fig. 3.2B). The sampling effect in the exercise group became evident at 4h, when [hemoglobin] began to decline. Hematocrit initially rose by more than 50% after exercise, again despite the diluting effect evident in the control group (Fig. 3.2C). By 8h hematocrit fell as a consequence of continual sampling. Mean cell [hemoglobin] (MCHC), calculated as the ratio of [hemoglobin]/hematocrit, fell significantly after exercise, remaining depressed up to 2h, (Fig. 3.2D) which suggests swelling of the red cell and/or recruitment of hemoglobin-poor reticulocytes. MCHC was unaffected by sampling, as it did not change in the control group (Fig. 3.2D).

In the second series which compared arterial and venous responses (Table 3.4, Fig. 3.3) post-exercise changes in all arterial parameters followed the same trends as in the first series (Fig. 3.1). Minor quantitative differences (eg. slightly smaller pHa depression and Pa_{CO_2} elevation, larger hematocrit declines) could have resulted from the larger fish size, greater blood sample volume, and the use of flow-through versus closed systems. At rest, arterial and venous pH, P_{CO_2}, and lactate were virtually identical, and the arterial-venous (a-v) difference in total CO_2 (reflected in HCO_3^-; Fig. 3.3B) was less than 1 mmol/l. After exercise, pHv closely followed pHa and was only significantly lower at 0h (Fig. 3.3A). The a-v total CO_2 and $[HCO_3^-]$ differences increased to about 2 mmol/l for several hours after exercise (Fig.

Table 3.4. Simultaneously measured arterial and venous whole blood lactate prior to and following exhaustive exercise in rainbow trout.

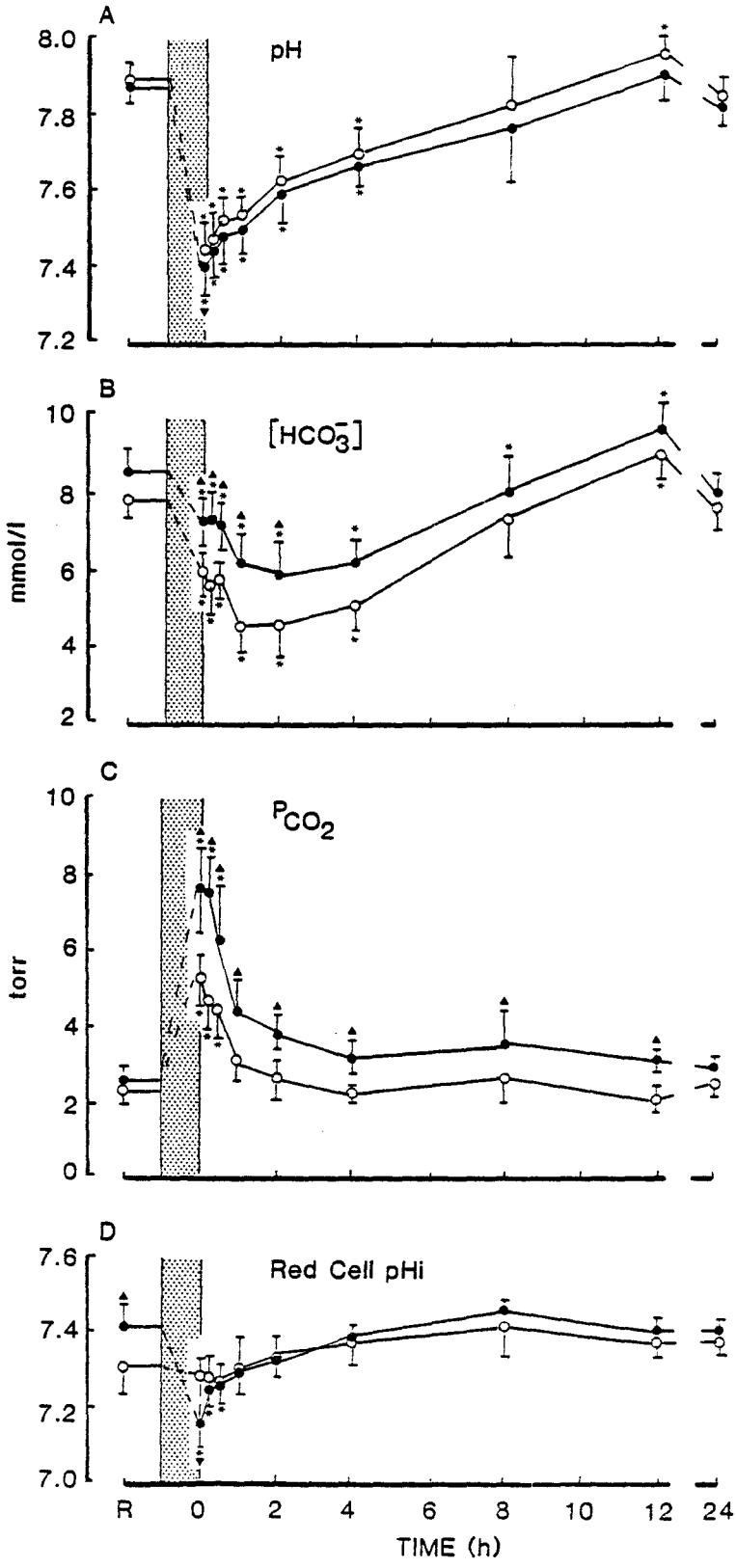
	Time after exercise (hours)								
Rest	0	0.25	0.5	1	2	4	8	12	24
Arterial									
2.29 ± 1.65 (5)	6.76 ± 1.44 (5) *	10.00 ± 2.26 (5) *	12.50 ± 2.14 (5) *	13.85 ± 2.00 (5) *	17.68 ± 2.01 (5) *	17.11 ± 2.58 (5) *	8.30 ± 1.66 (5) *	3.96 ± 1.22 (4) *	0.90 ± 0.40 (3)
Venous									
1.98 ± 1.32 (5)	6.32 ± 1.59 (5) *	9.47 ± 1.67 (5) *	12.14 ± 1.96 (5) *	15.46 ± 1.90 (5) *	17.66 ± 2.31 (5) *	17.42 ± 2.83 (5) *	7.67 ± 1.39 (5) *	3.98 ± 1.05 (4) *	0.92 ± 0.44 (3)

Means ± 1 SEM (n). All values in mmol/l whole blood.

* indicates a significant difference ($P<0.05$) from corresponding rest value.

There were no significant differences at any time between arterial and venous levels.

Figure 3.3. Arterial (O) and venous (●) blood acid-base status prior to and after exhaustive exercise in rainbow trout. (A) pH; (B) plasma bicarbonate concentration, $[HCO_3^-]$; (C) CO_2 tension, Pa_{CO_2}; (D) red blood cell pHi. Means $\pm$ 1 SEM, n = 5, except at 12h, n=4 and at 24h, n=3. R = rest, bar indicates 6 min of exhaustive exercise, 0 = immediately after exercise. ▲ indicates venous values significantly different ($p<0.05$) from simultaneous arterial values. * indicates a significant difference ($p<0.05$) from corresponding rest value.


A
pH
8.0
7.8
7.6
7.4
7.2
B
[HCO3-]
mmol/l
10
8
6
4
2
C
PCO2
torr
10
8
6
4
2
0
D
Red Cell pHi
7.6
7.4
7.2
7.0
R
0
2
4
6
8
10
12
24
TIME (h)

3.3C). There were no significant differences at any time between arterial and venous lactate levels after exercise (Table 3.4).

At rest, RBC pHi values were 0.5 units below plasma pH (Fig. 3.3D). Despite the similarity of plasma pHa and pHv at rest (Fig. 3.3A), RBC pHi was significantly higher on the venous side. After exercise, which caused large drops in plasma pH, arterial RBC pHi did not fall and indeed tended to rise slightly, though non-significantly, later in recovery (Fig. 3.3D). In contrast, venous RBC pHi fell precipitously by approximately 0.25 units at 0h followed by recovery to resting levels over a time course rather similar to that of the Pv_{CO_2} changes (Fig. 3.3C).

Whole Body Fluid Volumes and Intracellular pH

Whole body ECFV (corrected as described on page 61) fell by approximately 28%, or about 70 ml/kg, after exhaustive exercise, reaching a minimum at 0.25h and remaining significantly depressed until 8h (Fig. 3.4A). As total body water did not vary significantly at any sample time (see Chapter IV), this reflected a direct shift of fluid out of the extracellular into the intracellular compartment. The latter was therefore reciprocally expanded until 8h (Fig. 3.4B).

Whole body intracellular pH at each time in the exercise group was calculated using the corrected fluid volumes. In the control group, pHi at each time was calculated using the rest fluid volumes for each animal.

At rest, whole body pHi in the experimental group of the first series did not differ from that of the control group, averaging about 7.25 (Fig. 3.5A). In the control group, pHi remained stable

Figure 3.4. (A) Whole body extracellular fluid volume (ECFV) and (B) intracellular fluid volume (ICFV) prior to and following exhaustive exercise in the rainbow trout. Data corrected as described in text. Dashed line indicates the mean of the rest value. Means $\pm$ 1 SEM. n=8, except at 8 and 12h, n=6 and at 24h n=4. R = rest, bar indicates 6 min of exhaustive exercise, 0 = immediately after exercise. * indicates a significant difference ($p<0.05$) from corresponding rest value.

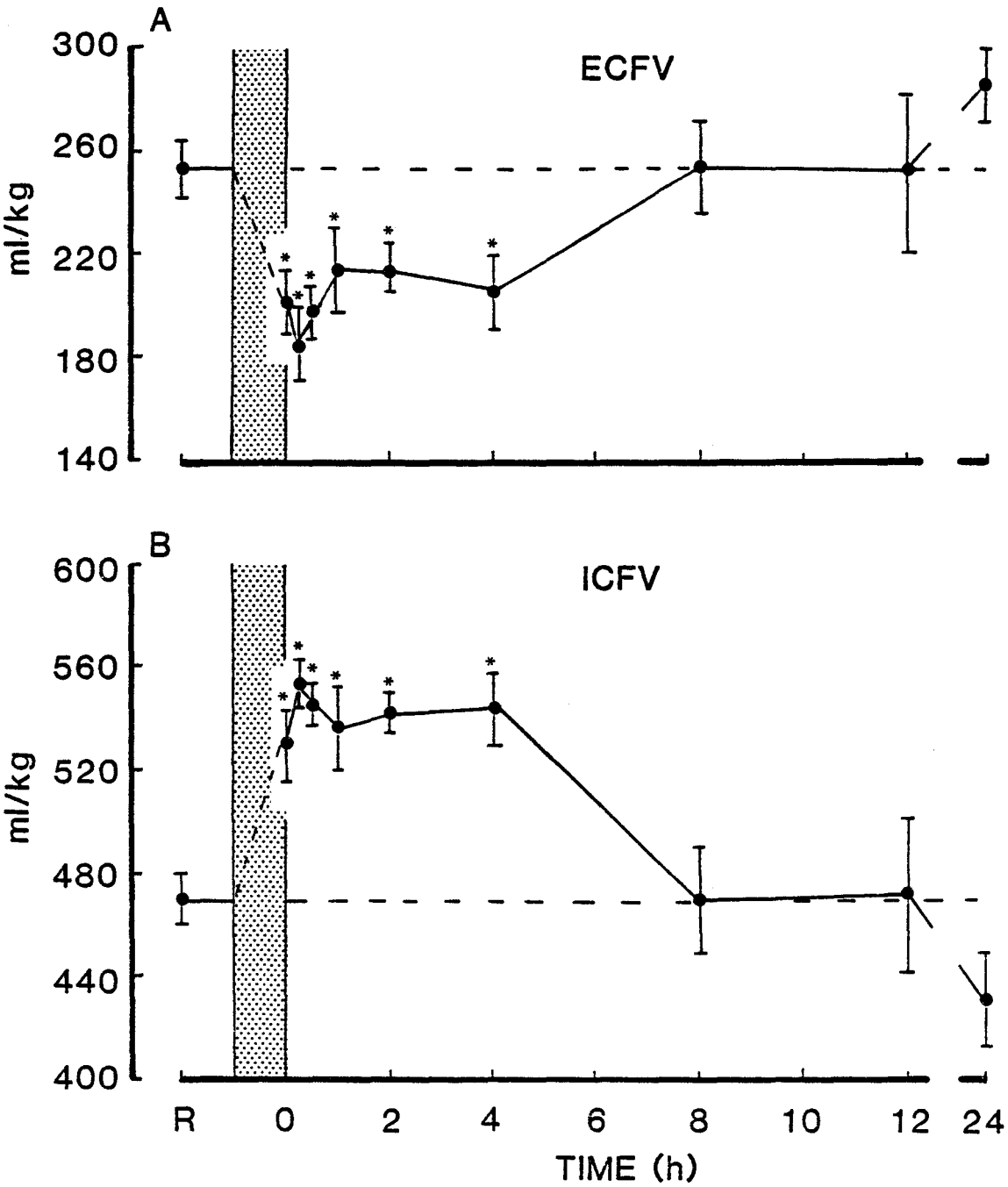
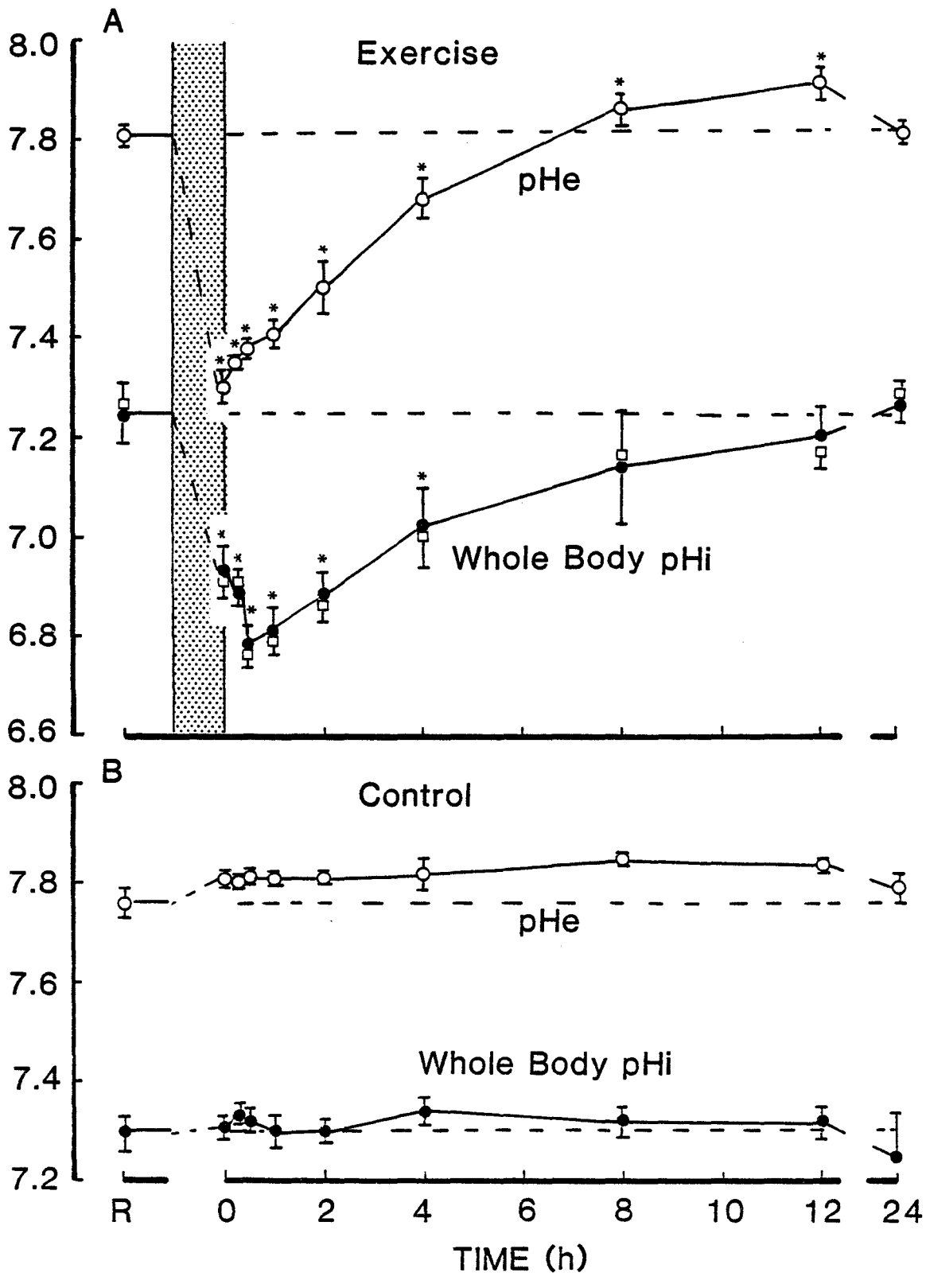

A
ECFV
300
260
220
180
140
ml/kg
B
ICFV
600
560
520
480
440
400
ml/kg
R
0
2
4
6
8
10
12
24
TIME (h)

Figure 3.5. Whole body intracellular (pHi-●) and extracellular (pHe-○) pH in (A) exercised and (B) unexercised rainbow trout. □ are pHi values calculated from venous data: see text for details. Means ± 1 SEM. Control group: n=10, except at 24h, n=4. Exercise group: n=8, except at 8 and 12h, n=6 and at 24h n=4. R = rest, bar indicates 6 min of exhaustive exercise, 0 = immediately after exercise. * indicates a significant difference ($p<0.05$) from corresponding rest value.


A
Exercise
pHe
Whole Body pHi
B
Control
pHe
Whole Body pHi
8.0
7.8
7.6
7.4
7.2
7.0
6.8
6.6
R
0
2
4
6
8
10
12
24
TIME (h)

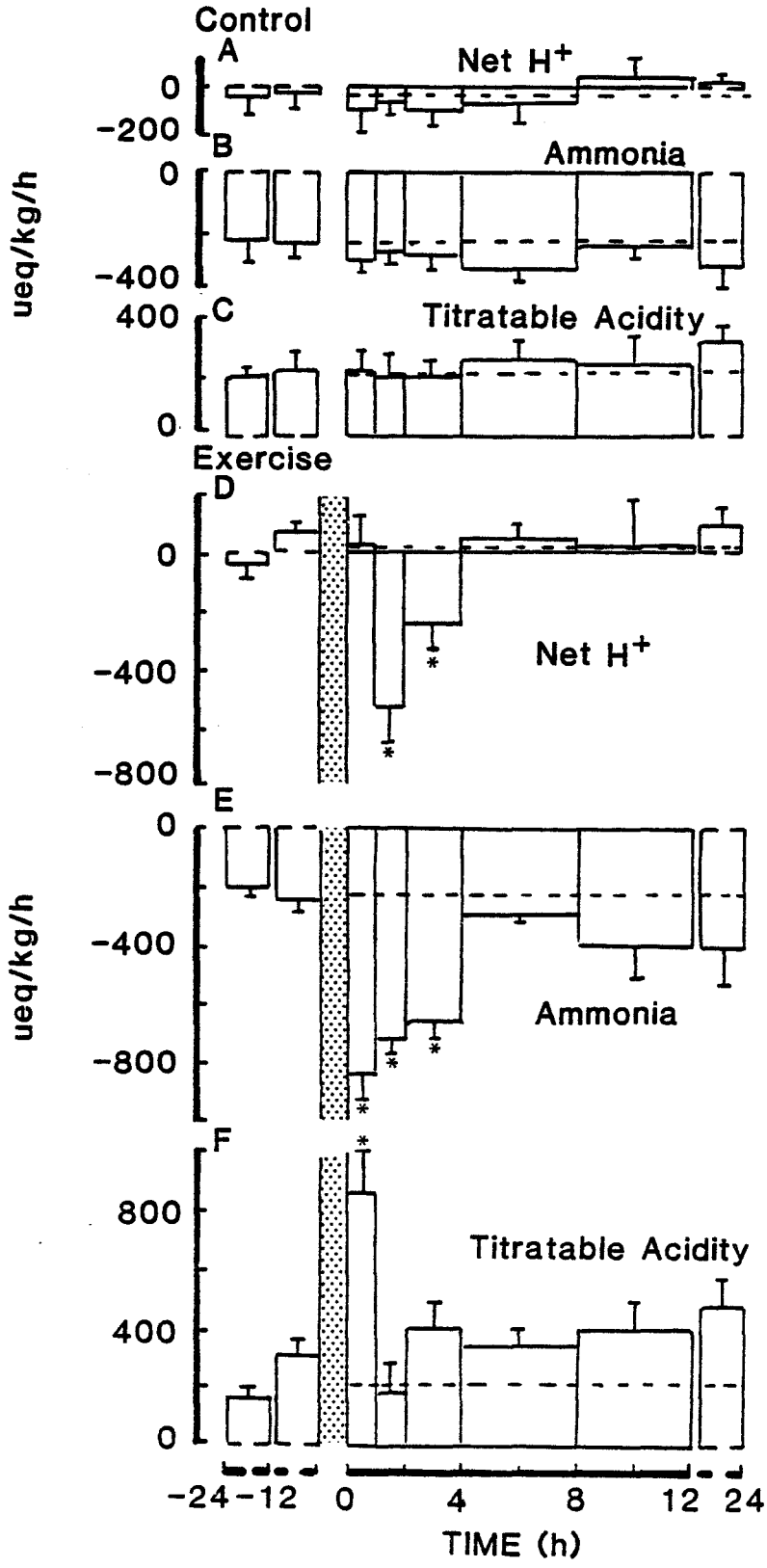
throughout the experimental period (Fig. 3.5B). However, exercise resulted in a rapid, severe decline in pHi, to a low of 6.78 $\pm$ 0.05 at 0.5h (Fig. 3.5A). Recovery of pHi was slower than that of pHe, for pHi had just recovered at 8-12h, a time when pHe was overcompensated. Such an alkalosis was not observed in pHi.

In theory, calculations of pHi should be based on interstitial fluid (ISF) as the representative ECF (Roos and Boron, 1981), though in practice this is impossible. The composition of ISF is probably intermediate to that of arterial and venous blood plasma. An index of the possible impact of this on the pHi measurements was obtained by recalculating the whole body pHi data of the first series using estimated venous values for ECF based on the mean a-v pH (Fig. 3.3A) and radiolabel differences (Table 3.2) of the second series. These differences tended to be self-compensating, with the result that mean whole pHi estimated from venous data was identical to that from arterial data at all times (Fig. 3.5A).

H^+ and Lactate Exchange with the Environment

During the two 12h periods prior to the experimental period, net H^+ exchange with the environment was similar in both the control and exercise group (Fig. 3.6A and D). Resting ammonia excretion just exceeded the titratable acidity uptake, resulting in a net H^+ flux of about -30 ueq/kg/h. This situation did not change appreciably during the experimental period in the control group (Fig. 3.6 A,B,C). However, in the first hour following exercise, both titratable acidity uptake and ammonia excretion increased approximately 4 fold; consequently, there was no change in net H^+

Figure 3.6. Net H^+, ammonia and titratable acidity fluxes in control (A,B,C) and exercised (D,E,F) rainbow trout, respectively. -24 h, -12 h refer to two-12 h periods prior to the experimental period. Dashed line indicates mean of the two pre-experimental flux periods. Means $\pm$ 1 SEM. Control group: n=10, except at 24h, n=4. Exercise group: n=8, except at 8 and 12h, n=6 and at 24h n=4. R = rest, vertical bar indicates 6 min of exhaustive exercise, 0 = immediately after exercise. * indicates a significant difference ($p<0.05$) from corresponding rest value.


Control
A
Net H+
0
-200
B
Ammonia
0
-400
ueq/kg/h
400
C
Titratable Acidity
0
Exercise
D
0
Net H+
-400
-800
E
0
-400
Ammonia
-800
ueq/kg/h
F
800
Titratable Acidity
400
0
-24 -12 0 4 8 12 24
TIME (h)

excretion (Fig. 3.6 D,E,F) However, over the next 3 hours, the situation changed; ammonia excretion remained significantly elevated while titratable acidity uptake fell to control levels, resulting in a significant increase in net H^+ efflux. During the remainder of the experimental period, ammonia excretion returned to resting values. Titratable acidity uptake tended to increase leading to a trend towards net H^+ uptake from the environmental water, though the changes were not significant.

In the third series, excretion of lactate to the environment over the first 4h after exercise was less than 1% of the simultaneously measured net H^+ efflux (Table 3.5), despite the fact that these four fish showed rather higher net H^+ excretion rates than the general average (see Fig. 3.6D). Therefore excretion did not contribute significantly to the clearance of lactate from the blood.

DISCUSSION

Blood Acid-Base Changes

Exhaustive exercise led to a pronounced acidosis in arterial blood of mixed respiratory and metabolic origin (Fig. 3.1). These effects were very similar to those previously described by Turner, Wood and Clark (1983) and the same explanations probably apply. The elevation in Pa_{CO_2} was due, in part, to the efflux of aerobically produced CO_2 from the muscle and titration of extracellular and intracellular HCO_3^- by glycolytically produced H^+. Efflux of the latter from the white muscle resulted

Table 3.5. Lactate and net H^+ excretion in the 4 hours following exhaustive exercise in the rainbow trout.

	0-2h	2-4h
Lactate ueq/kg/h	6.08 ± 2.99 (4)	4.42 ± 1.08 (4)
Net H^+ ueq/kg/h	865.8 ± 223.8 (4)	693.2 ± 216.6 (4)

Means ± 1 SEM.

in the metabolic component of the acidosis. It is now possible, however, to account more fully for the Pa_{CO_2} elevation. A short-lived (about 1h) respiratory acidosis following strenuous exercise is a phenomenon observed in all fish species examined to date (see Wood and Perry, 1985). This rise in P_{CO_2} has been considered a paradox, given that fish gills are thought to be hyper-ventilated with respect to CO_2 excretion. The rate limiting step in CO_2 excretion in fish is thought to be HCO_3^- flux through the red cell (Perry, Davie, Daxboeck and Randall, 1982). Recently, it has been demonstrated *in vitro* that β-adrenergic stimulation inhibits HCO_3^- flux through trout red cells, thus leading to a reduction or inhibition of CO_2 excretion *in vivo* (Heming, 1984). Certainly, following exercise the levels of circulating catecholamines increase (eg. Fig. 7.2; also Mazeaud and Mazeaud, 1982; Butler, 1985; Ristori and Laurent, 1985; Primmett *et al.*, 1986). Furthermore, in rainbow trout *in vivo*, arterial P_{CO_2} increased in response to adrenaline infusions (Perry and Vermette, 1986). But how does the fish benefit from CO_2 retention? Since CO_2 is carried in the plasma mainly as HCO_3^- which is the major non-protein buffer, Heming (1984) has proposed that CO_2 retention allows the benefits of HCO_3^- buffering to be shared by other tissues (eg. heart and brain). It has also been suggested that the rise in P_{CO2} and/or the associated fall in pHa may stimulate the post-exercise hyperventilation at a time when stimuli from other sources (eg. arterial O_2 sensors, proprioceptors) would be minimal, thus allowing correction of the

O_2 debt associated with anaerobic exercise (Wood and Perry, 1985).

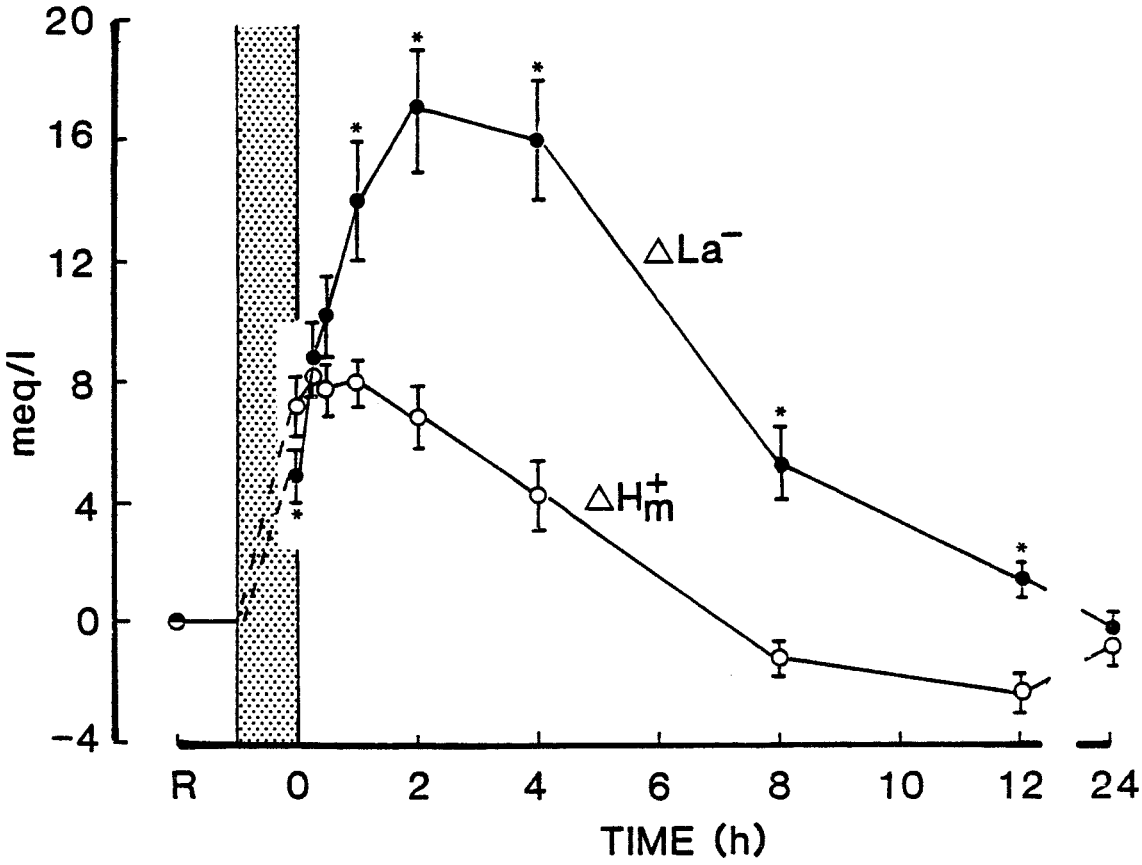
While some information is available on venous acid-base changes associated with exhaustive exercise (Wood et al. ,1977; Turner, Wood and Hōbe, 1983; Schwalme and Mackay, 1985a), the present study is the first to compare simultaneous arterial and venous measurements in detail. In general, the data (Fig. 3.3) indicate rather close similarity between arterial and venous changes, though with the expected larger P_{CO_2} elevations and pH depressions, and smaller HCO_3^- depressions, on the venous side. These differences undoubtedly reflected a greater CO_2 addition to the blood by the systemic tissues due to both aerobic metabolism and the titration of HCO_3^- reserves via metabolic acid production, and the subsequent removal of this CO_2 at the gills. The slightly higher ^{14}C-DMO concentration in the arterial than venous plasma immediately after exercise (Table 3.2) was in accord with the slightly higher arterial pH, though it is not clear why ^{3}H-mannitol levels were similarly elevated. In any event, the data indicate that arterial versus venous measurement sites have negligible impact on the mean whole body pHi estimated by the DMO technique (Fig. 3.5A). Finally, the data (Table 3.4) did not substantiate the claim of Driedzic and Kiceniuk (1976) that lactate is removed from the blood by the gills, though these workers were similarly unable to demonstrate any significant a-v lactate difference. Certainly, there was very little excretion of lactate into the water (Table 3.5).

Blood Lactate and Metabolic Acid Loads

The blood buffer capacity used to calculate metabolic acid load to the blood (equation 3.3) was estimated from oxygen-saturated blood and does not take into account changes in buffer capacity which may have resulted from deoxygenation of hemoglobin after exercise (Davenport, 1974). Therefore, the estimate of ΔH^+m at time 0, 0.5h and 1h after exercise, when hemoglobin-oxygen saturation may be reduced (see Fig. 7.4; Chapter VII), may slightly underestimate the true blood metabolic acid load.

After exercise, there was a severe acidosis in the whole body intracellular compartment (Fig. 3.5), which reflected, to a large extent, changes occurring in the white muscle mass (Fig. 4.4A, Chapter IV). Both H^+ and lactate, the major end products of anaerobic metabolism, are released from the muscle after exercise, the former causing the metabolic acidosis. Lactate and H^+ efflux appear to be governed by different factors, for the metabolic acid load in the blood peaked immediately post-exercise, whereas the lactate load continued to rise, not peaking until about 2h into recovery (Fig. 3.7). In addition, the lactate load appeared in excess of the H^+ load from about 1 hour onwards, reflecting differential rates of release and/or removal. However up until this time (1 hour post-exercise), net H^+ excretion into the environmental water remained at resting levels (essentially zero; Fig 3.6D) so the results tend to support the hypothesis of differential release of H^+ and lactate anion from the muscle, as earlier proposed (Turner, Wood and Clark, 1983; Turner and Wood, 1983). Turner, Wood and Clark (1983)

Figure 3.7. Changes in blood lactate (ΔLa^-,●) and blood metabolic acid load (ΔH^+m,O) during recovery from exercise; see text for details of calculation. Means ± 1 SEM. Control group: n=10, except at 24h, n=4. Exercise group: n=8, except at 8 and 12h, n=6 and at 24h n=4. R = rest, bar indicates 6 min of exhaustive exercise, 0 = immediately after exercise. * indicates a significant difference ($p<0.05$) between ΔLa^- and ΔH^+m at the same time.


20
16
12
8
4
0
−4
meq/l
△La⁻
△H⁺m
R
0
2
4
6
8
10
12
24
TIME (h)

have discounted differential uptake of H^+ over lactate by tissues as a contributing factor to the H^+ versus lactate differential in blood, for following lactic acid infusion the rates of lactate and H^+ removal were equal. Furthermore, evidence is presented in Chapter IV which suggests that lactate is taken up by tissues (heart and liver) without an accompanying H^+. If anything, this would tend to cause an excess of H^+ over lactate in the blood, the reverse of the observed pattern. Neither ΔH^+m nor ΔLa^- deviated significantly from zero in the control group.

The efflux of H^+ is thought to be a passive process, subject to 'equilibrium limitations', which include such factors as pH gradient between the intra- and extracellular compartment and extracellular buffer capacity (Holeton and Heisler, 1983; Turner and Wood, 1983). The transfer of lactate is somewhat more complex, for the relative movements of the dissociated and undissociated forms are unknown. If lactate moved as the free acid, the efflux would be subject to the same limitations as H^+ (eg. pH, Mainwood and Worsley-Brown, 1975). If, as appears to be the case, lactate moves as the free anion, perhaps with the involvement of a carrier (Hochachka and Mommsen, 1983; Turner and Wood, 1983), then its movement may be independent of the movement of H^+. This is an intriguing problem that deserves further inquiry.

Red Blood Cell pHi

The RBC pHi results cast some new light on the functional role of the red cell (Fig. 3.3D). At rest, the pHi of venous erythrocytes was considerably higher than that of arterial cells, despite similar

plasma pHe values. This was probably due to the Haldane effect, whereby relative deoxygenation of venous blood raised the H^+ binding capacity of the hemoglobin. The phenomenon has been observed both *in vivo* in tench (*Tinca tinca*) (Jensen, 1986) and *in vitro* in tench (Jensen, 1986) and sheatfish (*Silurus glanis*) (Albers *et al.* , 1981) blood. After exhaustive exercise, arterial RBC pHi remained stable in the face of a large plasma acidosis. Similar results have recently been reported in both striped bass (Nikinmaa *et al.* , 1984) and rainbow trout (Primmett *et al.* , 1986) and have been attributed to a β-adrenergic influence of catecholamines mobilized into the blood by exercise stress. The role of circulating catecholamines in maintenance of red cell pHi is discussed in Chapter VII.

It is not clear why venous pHi was not regulated to the same extent as arterial, but it may be related to the much larger elevations in P_{CO_2} on the venous side (Fig. 3.3C) which surpassed some regulatory threshold. This is not to say that there was no pHi regulation in the venous RBC. Based on the *in vitro* relationship for trout blood equilibrated to varying P_{CO_2} at 15°C established in Fig. 2.5 of Chapter II (pHi = 0.73 x pHe + 1.74), venous pHi, which fell by 0.25 units *in vivo* at 0h (Fig. 3.3C), would have declined by 0.36 units had there been only passive physico-chemical buffering. Arterial pHi, which did not change *in vivo* , would have fallen by 0.32 units.

Whole Body pHi

In fish, whole body pHi reflects to a large extent, that of the white muscle, though is biased upwards by tissues with higher pHi,

such as liver, brain and heart (Cameron and Kormanik, 1982) The resting whole body pHi estimates in the present study (Fig. 3.5), were similar to those measured in rainbow trout by Hobe et al. (1984). After exercise, the drop in pHi was of similar magnitude to that observed in white muscle and followed a similar time course of recovery (see Chapter IV), though again, the absolute values were biased upwards.

The accuracy of the estimate of pHi in a dynamic situation is dependent upon full equilibration of DMO between the intracellular and extracellular compartments. In trout white muscle perfused in vitro , full equilibration was attained within 15 min of a step change in pH (Fig. 2.4, Chapter II). Thus the reliability of the present 0h pHi estimate, obtained immediately after the 6 min exercise period, cannot be assessed though some qualitative conclusions can be drawn. The [DMO]i/[DMO]e distribution ratio (of equation 2.2) significantly increased after exercise (from 0.57 to 0.62). Thus there was a net movement of DMO from extracellular to intracellular compartments. If, at time 0, this re-equilibration was not complete, then the pHi estimate at this time would have been too low, and the magnitude of the true pHi change would have been overestimated . The disappearance of this disequilibrium during longer recovery periods (1-2h) would be expected to increase calculated pHi substantially from the time 0 value. The fact that this did not occur (Fig. 3.5) suggests that disequilibrium, if it occurred at all, was not a serious problem. All other sample periods met the 15 min criterion. It seems likely that true pHi undergoes continuous rather than step changes in vivo , so

some blurring of the transients is likely with the DMO method.

Fluid Volume Distribution

Resting ECFV in both the control and exercise groups (Fig. 3.4) was similar to values previously reported for the rainbow trout (Milligan and Wood, 1982; M.V.E. Attygalle, G. Shelton and P.C. Croghan, unpublished observations). Exercise resulted in a relatively long-lived (about 4h) contraction of the extracellular space, and as there was no change in total body water (Chapter IV), the intracellular space exhibited a reciprocal expansion. The breakdown of glycogen to the osmotically more active lactate in addition to accumulation of other osmolytes (eg. inorganic phosphate, glycolytic intermediates) within the muscle were undoubtedly important contributors to the water shift. A significant reduction in white muscle ECFV and expansion of white muscle ICFV after exhaustive exercise has been demonstrated in Chapter IV (Fig. 4.7).

The reduction in ECFV resulted in a pronounced hemoconcentration, as indicated by the increases in [hemoglobin] and hematocrit (Fig. 3.2). Other factors which probably also contributed to the rise in hematocrit include red cell swelling in response to P_{CO_2} and catecholamine elevations (Heming, 1984) and mobilization of red cells from the spleen in response to adrenergic stimulation (Yamamoto, Itzawa and Kobayashi, 1980). The latter would also contribute to the rise in [hemoglobin].

Whole blood [glucose] followed a different pattern from [hemoglobin] or hematocrit after exercise, suggesting that factors other than hemoconcentration were involved, such as mobilization of

glucose from glycogen reserves in the liver (Driedzic and Hochachka, 1975).

Mechanisms of H^+ Exchange with the Environment

In freshwater teleost fish, H^+ excretion by the gills is achieved mainly by manipulation of Na^+ versus acidic equivalent (NH_4+, H^+) and/or Cl^- versus basic equivalent (HCO_3^-, OH^-) exchanges between the internal and external environment (Cameron, 1976; Wood, Wheatly and Hõbe, 1984; Heisler, 1984). There is evidence to suggest that these exchanges are dynamically manipulated to regulate extracellular acid-base status during an acid-base disturbance. Extracellular acidosis was shown to stimulate Na^+ influx and reduce Cl^- influx (Cameron, 1976; Wood et al. , 1984) while during an extracellular alkalosis, the opposite was observed: Na^+ influx was reduced and Cl^- influx was stimulated (Wood et al. , 1984). However, Wood et al. (1984) have pointed out that since Na^+ and Cl^- efflux rates as well as influx rates are manipulated during periods of H^+ excretion, it is not simply the rate of influx that will determine the rate of net acidic equivalent excretion. Rather the net H^+ flux will be a function of the difference between net Na^+ and Cl^- flux rates, which is in accord with the strong ion difference concept of acid-base regulation (Stewart, 1978). Correlations between net H^+ excretion and the difference between net Na^+ and Cl^- flux rates have been reported during recovery from exhaustive exercise in rainbow trout subjected to a regime very similar to that of the present study (C.M. Wood, unpublished observations cited in Wood and Perry, 1985).

In the present study, Na^+ and Cl^- fluxes were not measured, but trout exhibited large increases in both ammonia and acidic equivalent excretion to the environmental water (Fig. 3.6) after exercise. By analogy with the results of Holeton et al. (1983), the major portion of both fluxes would have occurred at the gills rather than at the kidney. However it must be noted that there were important differences between the experimental conditions of the two studies (see below).

Whether the animal exploited this increased ammonia production for acid-base regulation is unclear, as relative excretions of NH_3 and NH_4^+ could not be determined with the present methods. Recently, Wright and Wood (1985) have examined ammonia excretion in resting trout under conditions very similar to those of the present study (i.e. same water quality, pH about 8.0, same flux conditions) and have concluded that both NH_3 diffusion and NH_4^+/Na^+ exchange occur, with the latter clearly dominant. They have furthermore suggested that the relative contribution of NH_3 and NH_4^+ to ammonia excretion in rainbow trout is flexible, dependent upon such factors as water pH, [ammonia] and perhaps, acid-base status of the fish. The present data suggest that ammonia was excreted in both forms (Fig. 3.6). In the first hour post-exercise, the elevation in titratable acidity uptake nearly equalled that in ammonia flux, suggesting that NH_3 excretion predominated, while over the next 3 hours, titratable acidity uptake declined with ammonia excretion still elevated, suggesting that NH_4^+ excretion predominated and served as a route of acidic

equivalent excretion. This conclusion differs from that of Holeton et al. (1983) who argued that NH_3 excretion predominated throughout after exhaustive exercise in trout, and that acidic equivalent excretion was achieved largely by H^+/Na^+ exchange. However it must be noted that the lower water pH (about 7.4) in their study would have favoured NH_3 diffusion, and that again the methods used by Holeton et al. (1983) were incapable of absolutely separating NH_3 and NH_4^+ movements.

It has been claimed previously that lactate transfer to the environmental water does not occur after exhaustive exercise in fish (Holeton and Heisler, 1983; Holeton et al. , 1983). However by increasing the sensitivity of the assay 100 - fold through freeze-concentration of water samples, it was possible to demonstrate that a very small lactate transfer to the water (Table 3.5) did occur. This amounted to less than 1% of the simultaneous H^+ transfer and only approximately 2% of the total blood lactate load. Thus it is clear that the excretion of H^+ as free lactic acid was negligible, and that lactate excretion made almost no contribution to the final disposition of the lactate burden. It is likely that at least some of this small loss occurred at the kidney, as the elevated blood lactate levels after exercise would have surpassed the renal lactate threshold (Kobayashi and Wood, 1980).

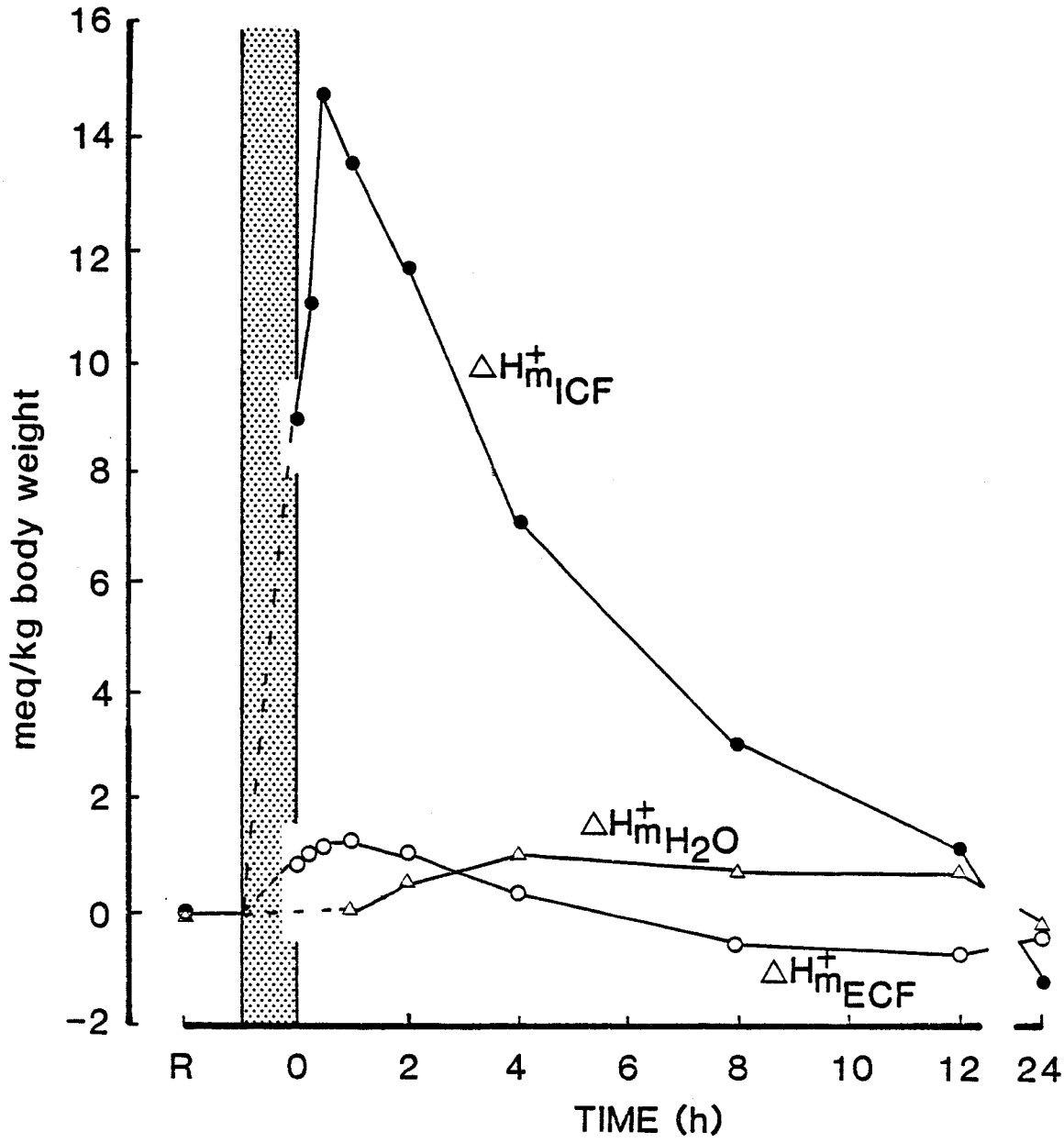
H^+ Distribution between ECF, ICF and the Environment

The data presented allow construction of a metabolic acid load (ΔH^+m) budget to the three compartments: intracellular, extracellular and environment (Fig. 3.8). Analysis of ΔH^+m in the

ECF was calculated as described in METHODS. Calculation of ΔH^+m in the entire ICF was complicated by the fact that the β value for this compartment is unknown. However, ΔH^+m in the ICF was approximated by using the measured β value of white muscle, -51.3 mmol/pH/kg = -73.6 mmol/pH/l ICF (Table 4.1, Chapter IV) and the calculated mean whole body pHi changes of Fig. 3.5 and by assuming that the bulk of intracellular buffer capacity resides in the white muscle, contributions from other tissues being negligible. Muscle was assumed to occupy 60% of the body mass (Stevens, 1968). It was also assumed that β did not change with exercise. This estimate of ΔH^+m in the ICF will tend to err on the conservative side. H^+ loading to the environment was calculated assuming that pre-exercise excretion rates represented basal production. The mean of the two pre-exercise periods was then subtracted from the means of subsequent post-exercise periods to estimate exercise-induced H^+ excretion.

Analysis of ΔH^+m distribution (Fig. 3.8) revealed that the bulk of the H^+ load remained in the intracellular compartment at all times during recovery with only small amounts transferred to the ECFV or transiently 'stored' in the water. Thus, the results do not support the contention that the major portion of the H^+ load is stored in the water during the later stages of recovery, thereby allowing full correction of intracellular acidosis prior to final lactate metabolism (cf. Heisler, 1982; Holeton and Heisler, 1983; Holeton et al. , 1983). However in the studies of Heisler and co-workers, there occurred about a four-fold greater H^+ flux to the environment, despite a post-exercise blood acid-base status

Figure 3.8. Distribution of metabolic acid load between the intracellular compartment ($\Delta H^{+}m_{ICF}$-●), extracellular compartment ($\Delta H^{+}m_{ECF}$-O) and environmental water ($\Delta H^{+}m_{H_2O}$-Δ) following exhaustive exercise in trout. See text for details of calculation. R= rest, bar indicates 6 min of exhaustive exercise, 0=immediately post-exercise.


16
14
12
10
8
6
4
2
0
−2
meq/kg body weight
$\Delta H^+_{m_{ICF}}$
$\Delta H^+_{m_{H_2O}}$
$\Delta H^+_{m_{ECF}}$
R
0
2
4
6
8
10
12
24
TIME (h)

similar to that of the present study. The reason for this difference may be related to the type of exercise, electrical stimulation (Holeton et al. ,1983) versus manual chasing (present study), or water quality. Indeed, less than 10% of the metabolic acid initially produced by exhaustive exercise in the present study was transferred to the ECFV and then subsequently stored in the water. This H^+ excretion to the environment appeared to expedite the recovery of the extracellular compartment and was associated with the ΔH^+m deficit (or metabolic alkalosis) in the ECFV at 8-12h. After 24h of recovery, all H^+ excreted to the water had been taken back up by the fish, thus correcting the extracellular alkalosis. In contrast, clearance of H^+ from the intracellular compartment was not by export, but rather dependent upon aerobic metabolism, a point which is dealt with in detail in the next chapter.

In summary, rainbow trout swum to exhaustion experienced a severe intracellular acidosis which persisted longer than the extracellular acidosis. The bulk of the H^+ load remained in the intracellular compartment at all times during recovery. Lactate appeared in the blood in excess of H^+, apparently due to differential release from the muscle; differential excretion of H^+ to the water could not account for this discrepancy and lactate excretion was minimal. However, H^+ excretion to the environment appeared to expedite the correction of the extracellular acid-base disturbance later in the recovery period. Furthermore, despite the pronounced drop in extracellular pH, red cell pHi in arterial blood remained virtually constant; in venous blood, red cell pHi was

slightly, though, significantly depressed. The implications of the red cell response for blood oxygen transport are dealt with in detail in Chapter VII.

CHAPTER IV

THE EFFECTS OF EXHAUSTIVE EXERCISE ON TISSUE ACID-BASE AND METABOLITE STATUS IN THE RAINBOW TROUT

INTRODUCTION

In the previous chapter, it was shown that exhaustive exercise in the trout resulted in a severe intracellular acidosis in the whole body, with the bulk of this metabolic acid load remaining in the intracellular compartment.

The present study investigates further the intracellular acid-base disturbance associated with exhaustive exercise in trout, with emphasis on the white muscle, the likely source of the major portion of the H^+ load. Since lactate and proton production are inextricably linked, the question of muscle acid-base regulation cannot be considered in isolation from metabolism (Hochachka and Somero, 1984). Therefore, muscle metabolite, as well as acid-base status, was followed after exercise. In addition, the consequences of the exercise-induced disturbances in extracellular acid-base and metabolite status on other tissues (heart, liver, brain and red blood cell) were also examined. This investigation explores not only the question of intracellular acid-base regulation after exercise in fish, but also its relationship to metabolism.

MATERIALS AND METHODS

Experimental Animals

Adult rainbow trout (Salmo gairdneri), weighing 180-420 g, were obtained at various times throughout the year from Spring Valley Trout Farm, Petersburg, Ontario and held as described in Chapter II.

Following catheterization of the dorsal aorta, fish were placed in 5 l darkened fish boxes supplied with a continual flow of fresh dechlorinated Hamilton tap water at 15 $\pm$ 1^{o}C (composition as in Chapter III) and allowed to recover 48h prior to experimentation.

Experimental Protocol

In these experiments, fish were sampled only once rather than serially. Approximately 12h prior to sampling, trout were infused with a 1 ml/kg dose of 5 uCi/ml ^{14}C-DMO plus 20 uCi/ml ^{3}H-mannitol in Cortland saline followed by an equal volume of saline. Individual fish (6-9 at each time) were terminally sampled prior to (rest) and immediately following exercise (0h), as well as at 0.5h, 1, 2, 4, 8, 12, 24h after exercise. A blood sample (2000 ul) was drawn from the arterial catheter, the volume replaced with saline, then the fish was grasped firmly by an assistant, immediately removed from the water, placed on a sponge and wiped dry. Ten 80 - 100 mg muscle samples were taken by punching 10 biopsy needles (ID - 4.78 mm) through the dorsal epaxial muscle, just anterior to the dorsal fin. Samples were immediately frozen in the needles in liquid nitrogen. The fish was quickly killed by a cephalic blow, the liver, heart and brain excised and immediately frozen in liquid nitrogen. Larger samples of muscle and liver (1-2g) were then taken for determination

of total tissue water. The carcass was dried to a constant weight at 85 ^{o}C for determination of total body water.

Data from fish which struggled unduly during the sampling procedure were discarded. Average times from first grasping the fish to freezing the samples were about 10 sec for muscle biopsy and 30-60 sec for the other tissues.

Blood was analyzed for pH, total CO_2 (in both whole blood and plasma), whole blood [lactate], [glucose], [hemoglobin], [pyruvate], hematocrit and plasma levels of protein, ^{3}H and ^{14}C radioactivity. Heart, liver and muscle were analyzed for levels of lactate, pyruvate, ^{3}H and ^{14}C radioactivity and total water content with the additional measurements of glycogen in liver and muscle, and adenosine triphosphate (ATP) and creatine phosphate (CrP) in muscle. Brain was assayed for ^{3}H and ^{14}C radioactivity and water content only. The red blood cell was analyzed only for intracellular pH by direct measurement.

Analytical Techniques, Calculations and Statistical Analysis

Arterial blood pHa, total CO_2, [lactate], [glucose], [hemoglobin], hematocrit, and plasma levels of ^{3}H and ^{14}C radioactivity were measured as described in Chapter III, except that 250 ul of whole blood was fixed in 500 ul of 8% perchloric acid for lactate analysis and scintillation counting was done on a LKB Wallac scintillation counter. Whole blood [pyruvate] was measured enzymatically (l-lactate dehydrogenase method of Segal, Blair and Wyngaarden (1956) using Sigma reagents) on 400 ul of the extract prepared for lactate analysis. Plasma [protein] was measured by

refractometry (American Optical TS meter).

To determine tissue levels of lactate and pyruvate, heart (65-110 mg), muscle (80-120 mg) and liver samples (50-200 mg) were homogenized in 1 ml of 8% ice-cold perchloric acid with a Turmax Tissuemizer for 2 min. The homogenate was then transferred to 1.5 ml centrifuge tubes and centrifuged for 3 min at 9,000 G. The supernatant was neutralized with Trizma buffer (Sigma) and analyzed as described by Turner, Wood and Clark (1983), using the l-lactate dehydrogenase method as described for blood samples. As muscle lactate levels were often quite high (30 -40 mmol/l; Fig. 4.3A), samples were diluted 1:4 with 8 % perchloric acid prior to analysis. Muscle and liver glycogen were measured on tissues of similar weight homogenized in 1.1 ml acetate buffer. The glycogen was broken down with amyloglucosidase (Boehringer-Mannheim) and analyzed enzymatically for glucose using the linked hexokinase/glucose-6-phosphate dehydrogenase (Boehringer-Mannheim) assay described by Bergmeyer (1965). Muscle concentrations of ATP and CrP were measured on 80-100 mg samples homogenized in 2 ml 6% perchloric acid. The homogenate was centrifuged for 10 min at 5,000g, the supernatant withdrawn, neutralized with 10N KOH, re-centrifuged and the resultant supernatant analyzed. The technique employed was that described by Bergmeyer (1965) in which the [NADP] is measured fluorometrically and is directly proportional to the [ATP] as linked by the glucose-6-phosphate dehydrogenase/hexokinase enzyme system. [CrP] was then measured by the same assay after the addition of ADP and phosphocreatine kinase (Boehringer-Mannheim). Tissue water content

was determined by drying to a constant weight at 85 ^{o}C. Tissue levels of all metabolites were eventually expressed on an intracellular basis- i.e per 1 of ICF (see below), with appropriate correction for the metabolite levels trapped in the ECF content of a tissue sample.

Levels of ^{3}H and ^{14}C radioactivity in brain, liver, heart and muscle were measured as described for muscle in Chapter II, except that samples were counted on a LKB Wallac liquid scintillation counter. Dual label quench correction was performed using quench standards prepared from the tissue of interest and the external standard ratio method (Kobayashi and Maudsley, 1974).

The non-bicarbonate buffer capacity (β) of muscle, liver, heart and brain was determined by acid titration of tissue homogenates as described by Cameron and Kormanik (1982). Tissue (1-2 g) was frozen and pulverized with a mortar and pestle under liquid nitrogen, then suspended in 3 ml 0.9% NaCl. The tissue homogenate was titrated to pH 8.0 with 1N NaOH, allowed to stabilize, then back-titrated to pH 6.5 with 0.02N HCl under a nitrogen atmosphere at 15 ^{o}C. A Radiometer G-202 pH electrode and associated acid-base analyzer was used to measure pH. The slope of the curve relating pH versus mmol HCl added over the pHi range observed in vivo for each tissue (Fig. 4.2) was taken as the buffer capacity of the tissue in mmol/pH/kg wet weight, and then converted to mmol/pH/l ICF, as for the tissue metabolites.

Tissue extracellular fluid volume (ECFV) was estimated according to equation 2.1, where plasma water content was calculated from the refractive index. Intracellular fluid volume (ICFV) was

calculated as the difference between total tissue water and ECFV. ^{3}H-mannitol distributions after 12h equilibration proved inadequate for estimating ECFV in liver, producing values often as high as or greater than the total liver water content. This obvious artifact was attributed to permeation of the ICFV by mannitol, and perhaps subsequent metabolism. Also mannitol sometimes yielded suspiciously high estimates for the heart. Control experiments with ^{14}C-inulin indicated that this was a problem only for these two tissues, and not for brain or white muscle. Thus in place of these erroneous values, inulin-derived ECFV's (liver: 193.6 ml/kg; heart: 180.3 ml/kg; C.M. Wood and S. Munger, unpublished results) were used.

Tissue pHi was determined according to equation 2.2, where [DMO]e and [DMO]i were calculated from equations 2.3 and 2.4, respectively and pK_{DMO} was taken from Malan et al. (1976). Pa_{CO_2} and HCO_3^- (in plasma and whole blood) were calculated according to equations 3.1 and 3.2, respectively. Tissue intracellular HCO_3^- was similarly computed from Pa_{CO_2} and pHi, assuming plasma Pa_{CO_2} to be in equilibrium between the ECF and ICF. While the use of venous blood values would yield slightly higher values of intracellular HCO_3^-, this would have negligible effect on the calculated changes in ΔH^+m (see below), which was the purpose of these calculations.

The metabolic acid load (ΔH^+m) after exercise in various tissue compartments and whole blood was estimated according to equation 3.3, using the appropriate values of pH, HCO_3^- and for the compartment in question. As each fish was sampled only once,

ΔH^+m was calculated from the mean values at each time. Similarly, ΔLa^- was calculated as the difference between the means of [lactate] at rest and each time after exercise.

Statistical analysis was performed as described in Chapter II.

RESULTS

Tissue Buffer Capacities

White muscle had the greatest buffer capacity of the tissues examined, followed by, in descending order, liver, heart and brain (Table 4.1). It is recognized that these values comprise total physico-chemical buffer capacity of the tissues (i.e. non-bicarbonate + bicarbonate). However, as P_{CO_2} was kept low during titration, intracellular bicarbonate levels would be quite low (less than 1 mmol/l) and would not contribute significantly to the measured buffer capacity. Thus, in essence, these values were representative of the non-bicarbonate buffer capacity, β, of the tissue.

Extracellular Acid-Base and Metabolite Status

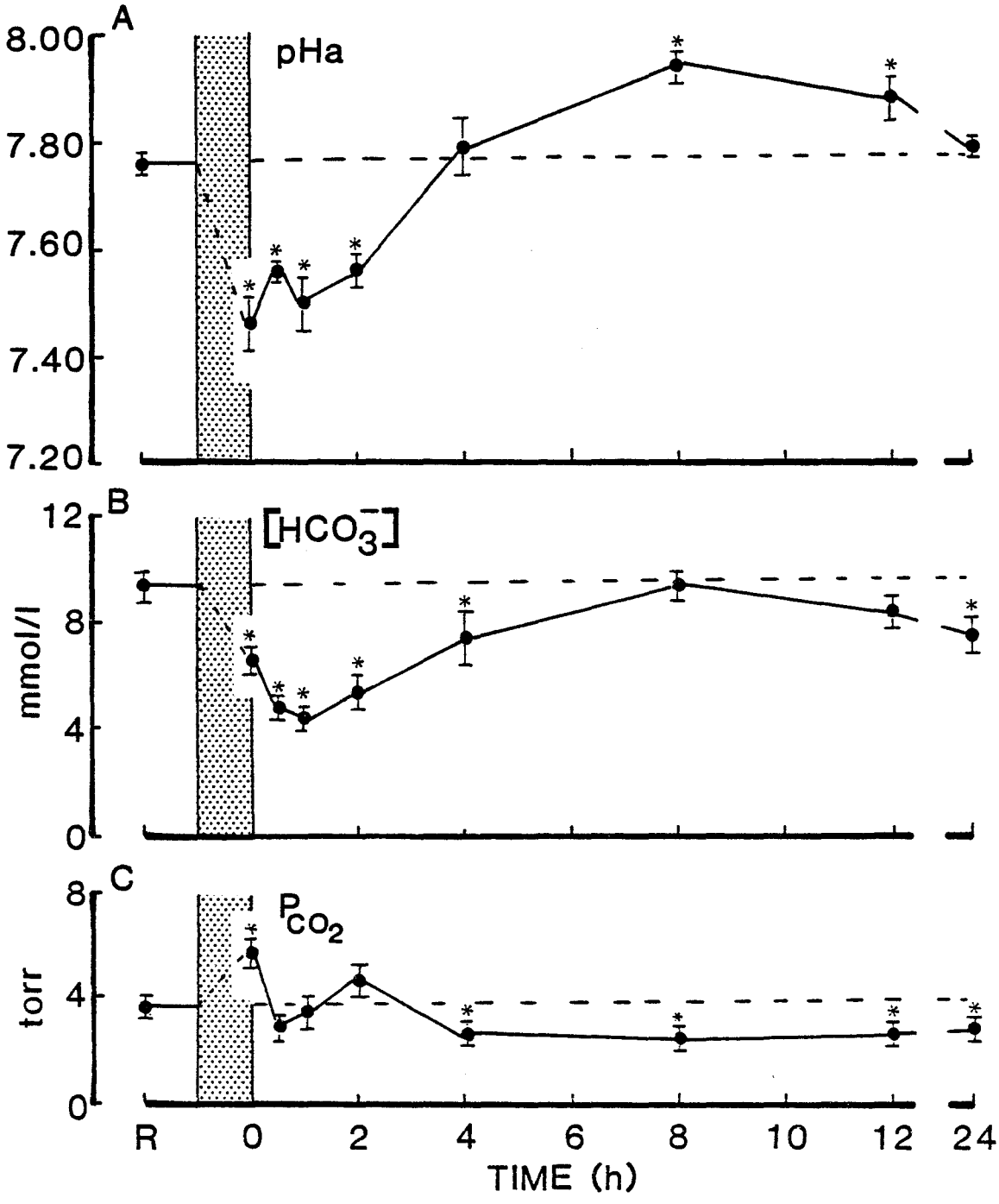
Changes in extracellular (i.e. plasma) acid-base status after 6 min of exercise (Fig. 4.1) were qualitatively similar to those described in Chapter III (Fig. 3.1, 3.3). Note, however, in the present study, that the recovery of pHa was faster and that the alkalosis at 8-12h post-exercise had a partial 'respiratory' origin in these fish due to a significant reduction in Pa_{CO_2} below the resting level. This was not observed in the previous study, and the reason for this difference is unknown. Perhaps serial removal of red cells and saline replacement in some way impedes CO_2 excretion,

Table 4.1. Buffer capacities (β) of brain, heart, liver and white muscle in the rainbow trout.

	β mmol/pH/kg wet weight	β mmol/pH/l ICF
Brain	-17.41 ± 0.34 (5)	-24.83 ± 0.39 (5)
Heart	-20.92 ± 1.04 (6)	-30.39 ± 1.50 (6)
Liver	-25.44 ± 2.88 (6)	-42.76 ± 4.84 (6)
White muscle	-51.32 ± 4.56 (7)	-73.59 ± 4.87 (7)

Means ± 1 SEM (n).

Figure 4.1. Changes in (A) arterial plasma pH, (B) $[HCO_3^-]$ and (C) P_{CO_2} after exhaustive exercise in the rainbow trout as assessed by single terminal samples. Means $\pm$ 1 SEM. N= 9 at rest; 8 at 0h; 6 at 0.5h; 7 at 1h; 6 at 2h; 6 at 4h; 6 at 8h; 7 at 12h and 7 at 24h. Shaded bar indicates 6 minutes of exercise, R indicates rest, and 0 indicates immediately after exercise. Dashed line indicates the mean of the rest value. * indicates a significant ($p<0.05$) difference from corresponding rest value.


A
pHa
8.00
7.80
7.60
7.40
7.20
B
[HCO3−]
12
8
4
0
mmol/l
C
PCO2
8
4
0
torr
R
0
2
4
6
8
10
12
24
TIME (h)

though this was not seen in serially sampled controls (Fig. 3.1). Alternately, it may reflect a slight difference in water P_{CO_2} between closed system (Chapter III) and open system conditions (Chapter IV).

The hematological changes accompanying exercise in the present study (Fig. 4.2) were similar to those reported in Chapter III (Fig. 3.3) but were not complicated by the diluting effect of repetitive blood sampling. Therefore, estimates of changes in blood plasma volume from changes in the suspended constituents were practical. Calculations based on the observed 30-40% increase in both plasma protein and hemoglobin concentrations (Fig. 4.2) suggest that the previously measured 27% decline in whole body ECFV (Fig. 3.4, Chapter III) was fully reflected in a similar decline in blood plasma volume.

As before, whole blood [lactate] reached a peak close to 20 mmol/l at 2h post-exercise, thereafter falling (Fig. 4.3A). At no time after exercise did blood lactate come into equilibrium with muscle lactate (Fig. 4.3A). Whole blood [pyruvate] followed a similar pattern rising slowly and reaching a plateau at 2-4h into recovery, then slowly declining, though the absolute levels and elevations of [pyruvate] were far less than those of [lactate] (Fig. 4.3B). Whole blood [glucose] tended to increase after exercise, though the changes were significant only at 0.5 and 8h (Table 4.2). At 24h, [glucose] declined significantly.

Tissue Intracellular Acid-Base and Metabolite Status

At rest, mean white muscle [lactate] was much higher than that in blood (14 versus 0.6 mmol/l, Fig. 4.3A) and mean muscle pHi was

Figure 4.2 Effects of exhaustive exercise on (A) hematocrit, (B) [hemoglobin] ([Hb]), (C) mean cell [hemoglobin] (MCHC) and (D) plasma [protein] in rainbow trout as assessed by single terminal samples. Means $\pm$ 1 SEM. N= 9 at rest; 8 at 0h; 6 at 0.5h; 7 at 1h; 6 at 2h; 6 at 4h; 6 at 8h; 7 at 12h and 7 at 24h. Shaded bar indicates 6 minutes of exercise, R indicates rest, and 0 indicates immediately after exercise. Dashed line indicates the mean of the rest value. * indicates a significant ($p<0.05$) difference from corresponding rest value.

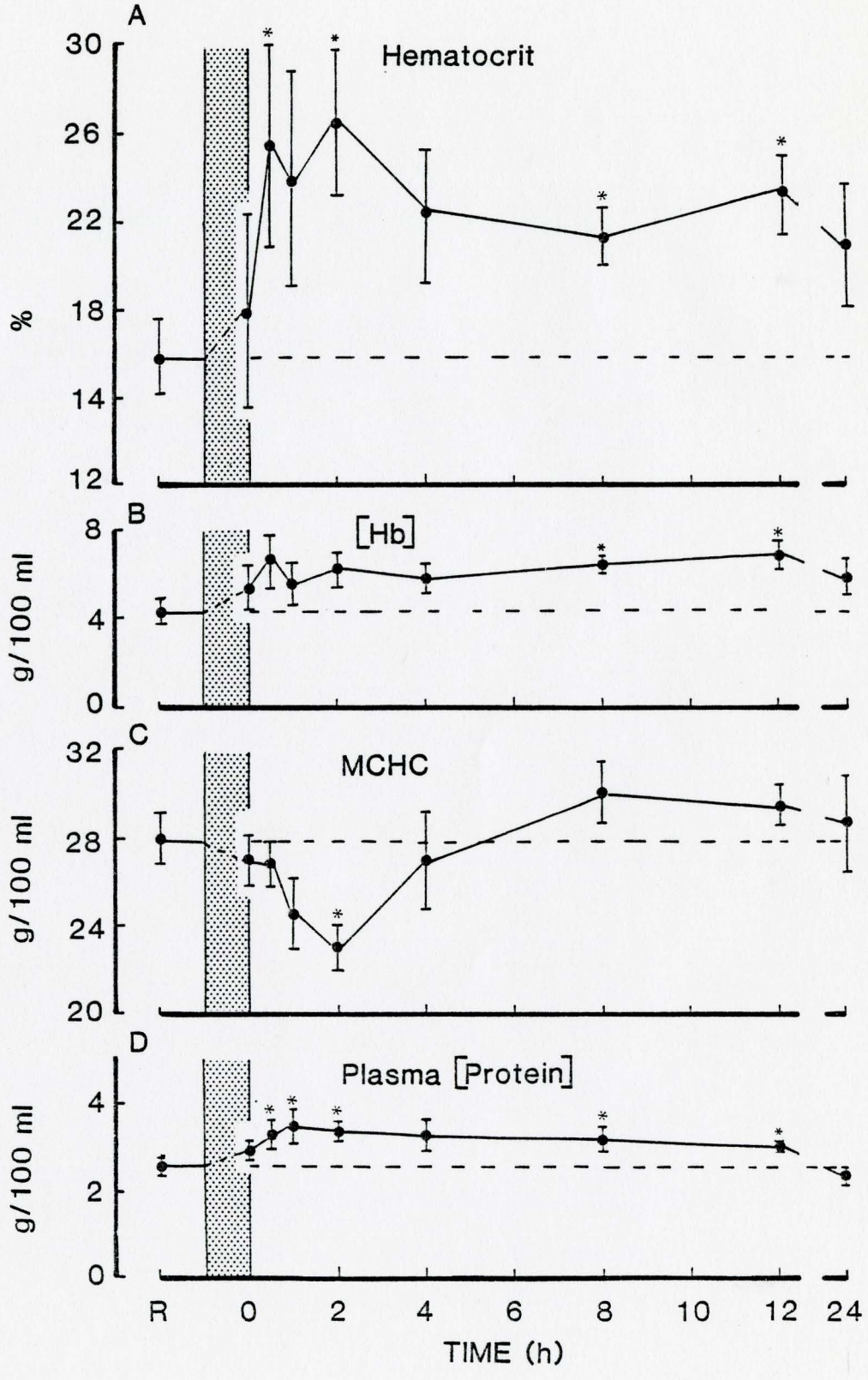

A
Hematocrit
%
30
26
22
18
14
12
B
[Hb]
g/100 ml
8
4
0
C
MCHC
g/100 ml
32
28
24
20
D
Plasma [Protein]
g/100 ml
4
2
0
R
0
2
4
6
8
10
12
24
TIME (h)

Figure 4.3. Concentrations of (A) lactate and (B) pyruvate in white muscle and whole blood prior to and following exhaustive exercise in rainbow trout. Means $\pm$ 1 SEM. N=9 at rest; 8 at 0 h; 6 at 0.5h; 7 at 1 h; 6 at 2 h; 6 at 4 h; 6 at 8 h; 7 at 12 h and 7 at 24 h. Shaded bar indicates 6 minutes of exercise, R indicates rest, and 0 indicates immediately after exercise. Dashed line indicates the mean of the rest value. * indicates a significant difference ($p<0.05$) from corresponding rest value.

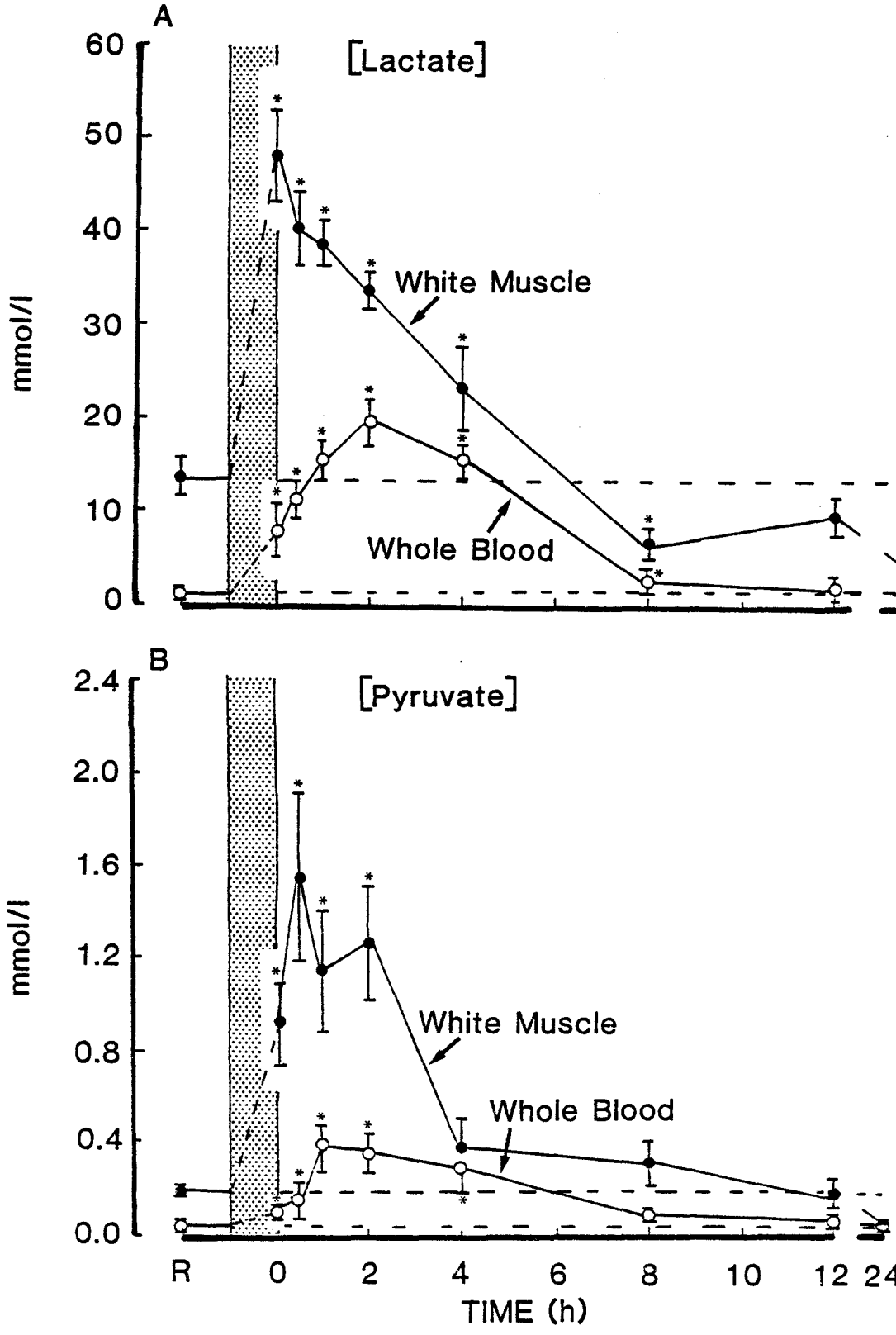

A
[Lactate]
60
50
40
30
20
10
0
mmol/l
White Muscle
Whole Blood
B
[Pyruvate]
2.4
2.0
1.6
1.2
0.8
0.4
0.0
mmol/l
White Muscle
Whole Blood
R
0
2
4
6
8
10
12
24
TIME (h)

Table 4.2. Whole blood levels of glucose prior to and following exhaustive exercise in the rainbow trout.

	Time after exercise (hours)							
REST	0	0.5	1	2	4	8	12	24
3.79 ± 0.11 (8)	4.35 ± 0.19 (7)	4.79 ± 0.26 (6) *	5.47 ± 0.79 (6)	4.78 ± 0.56 (6)	5.45 ± 0.93 (6)	6.07 ± 0.40 (6) *	4.57 ± 0.86 (7)	2.17 ± 0.35 (7)

Means ± 1 SEM. (n). All values are expressed as mmol/l whole blood.

*-significantly different ($p<0.05$) from corresponding rest value

7.21 ± 0.04 (n=9; Fig. 4.4A), about 0.56 units lower than extracellular pH. Exercise to exhaustion drove pHi to a low of 6.62 ± 0.06 (n=6), increasing the pHe-pHi gradient to about 0.8 units (Fig. 4.4A). Muscle pHi did not show any signs of recovery until 2-4h, requiring up to 12 hours for full recovery. Unlike the extracellular compartment, there was no evidence of intracellular alkalosis in the later stages of recovery. A plot of the muscle data on a pH versus $[HCO_3^-]$ diagram (Fig. 4.5A) indicates that the intracellular acidosis was mainly of metabolic origin, with little contribution from P_{CO_2}, except perhaps, immediately after exercise. In plotting these data, it was assumed that arterial P_{CO_2} was representative of intracellular P_{CO_2}. While this may underestimate intracellular P_{CO_2}, it does not appreciably alter the interpretation.

Accompanying this metabolic acidosis was a 4-5-fold increase in muscle [lactate] to almost 50 mmol/l and a slightly larger relative elevation in [pyruvate] to about 1.5 mmol/l (Fig. 4.3A,B) and a near depletion of glycogen, ATP and creatine phosphate stores (Fig. 4.6). The latter changes indicate that exercise was, indeed, exhaustive. The breakdown of muscle glycogen accounts for about 70% of the lactate produced.

Lactate levels in white muscle declined relatively quickly, so that by 8 hours, resting levels were obtained (Fig. 4.3A). However, [lactate] continued to fall, and by 24h, it was only about 30% of that at rest. Glycogen restoration was very slow, requiring up to 24h for full recovery (Fig. 4.6C). Resynthesis of glycogen stores did not

Figure 4.4. (A) Muscle, (B) liver, (C) heart, (D) brain and (E) arterial red cell pHi prior to and after exhaustive exercise. Means $\pm$ 1 SEM. N= 9 at rest; 8 at 0h; 6 at 0.5h; 7 at 1h; 6 at 2h; 6 at 4h; 6 at 8h; 7 at 12h and 7 at 24h. Shaded bar indicates 6 minutes of exercise, R indicates rest, and 0 indicates immediately after exercise. Dashed line indicates the mean of the rest value. * indicates a significant ($p<0.05$) difference from corresponding rest value.

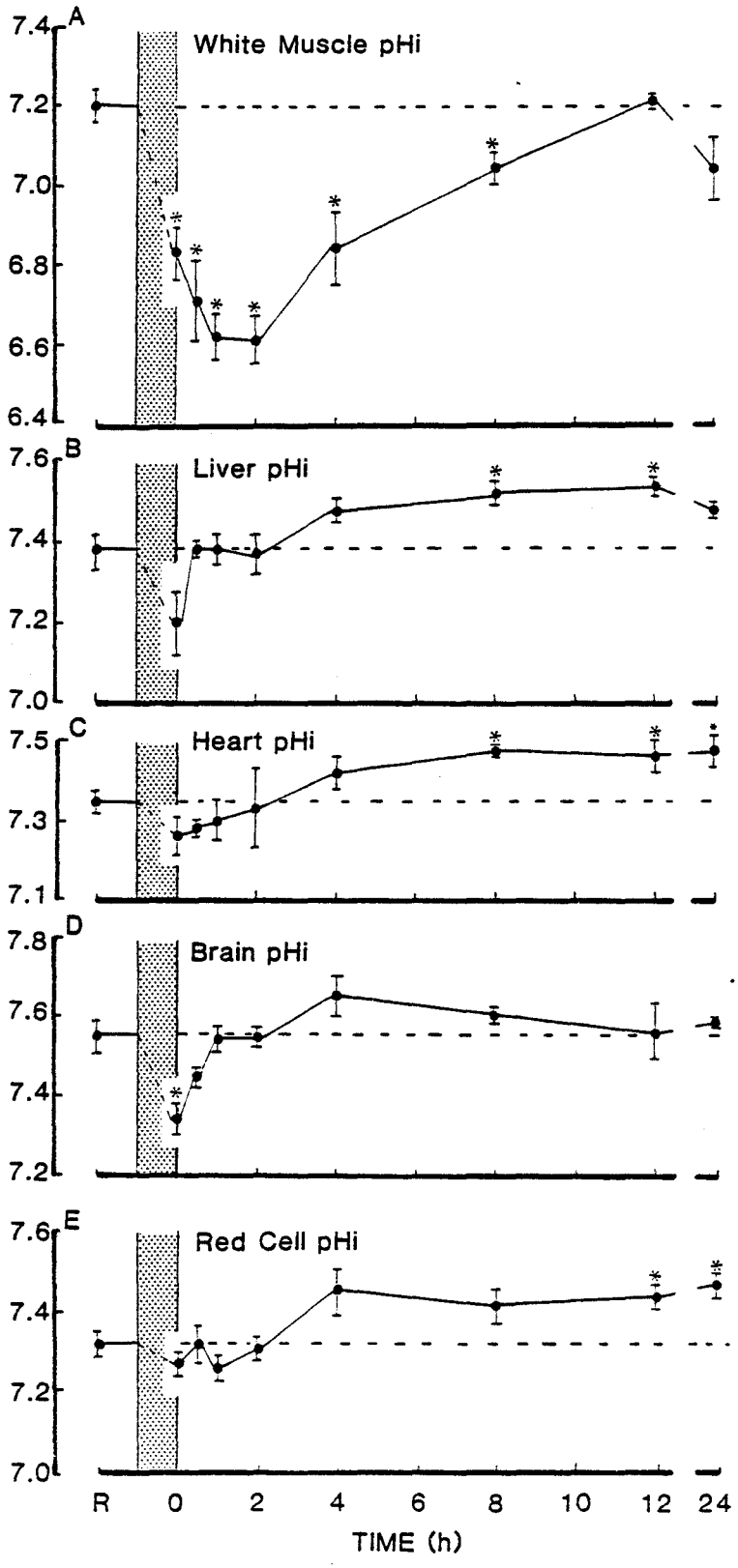

A
White Muscle pHi
7.4
7.2
7.0
6.8
6.6
6.4
B
Liver pHi
7.6
7.4
7.2
7.0
C
Heart pHi
7.5
7.3
7.1
D
Brain pHi
7.8
7.6
7.4
7.2
E
Red Cell pHi
7.6
7.4
7.2
7.0
R
0
2
4
6
8
10
12
24
TIME (h)

Figure 4.5. pHi-[HCO_3^-] diagrams of changes in intracellular acid-base status after 6 min of exhaustive exercise in (A) white muscle, (B) liver, (C) heart, and (D) brain of rainbow trout. Straight lines plotted are tissue <u>in vitro</u> non-bicarbonate buffer capacities from Table 4.1; isopleths are P_{CO_2}. Means are plotted, (n as indicated in Fig. 4.4). R indicates resting value, 0 is immediately after exhaustive exercise, 0.5, 1, 2, 4, 8, 12, and 24 indicate time, in hours, after exercise.

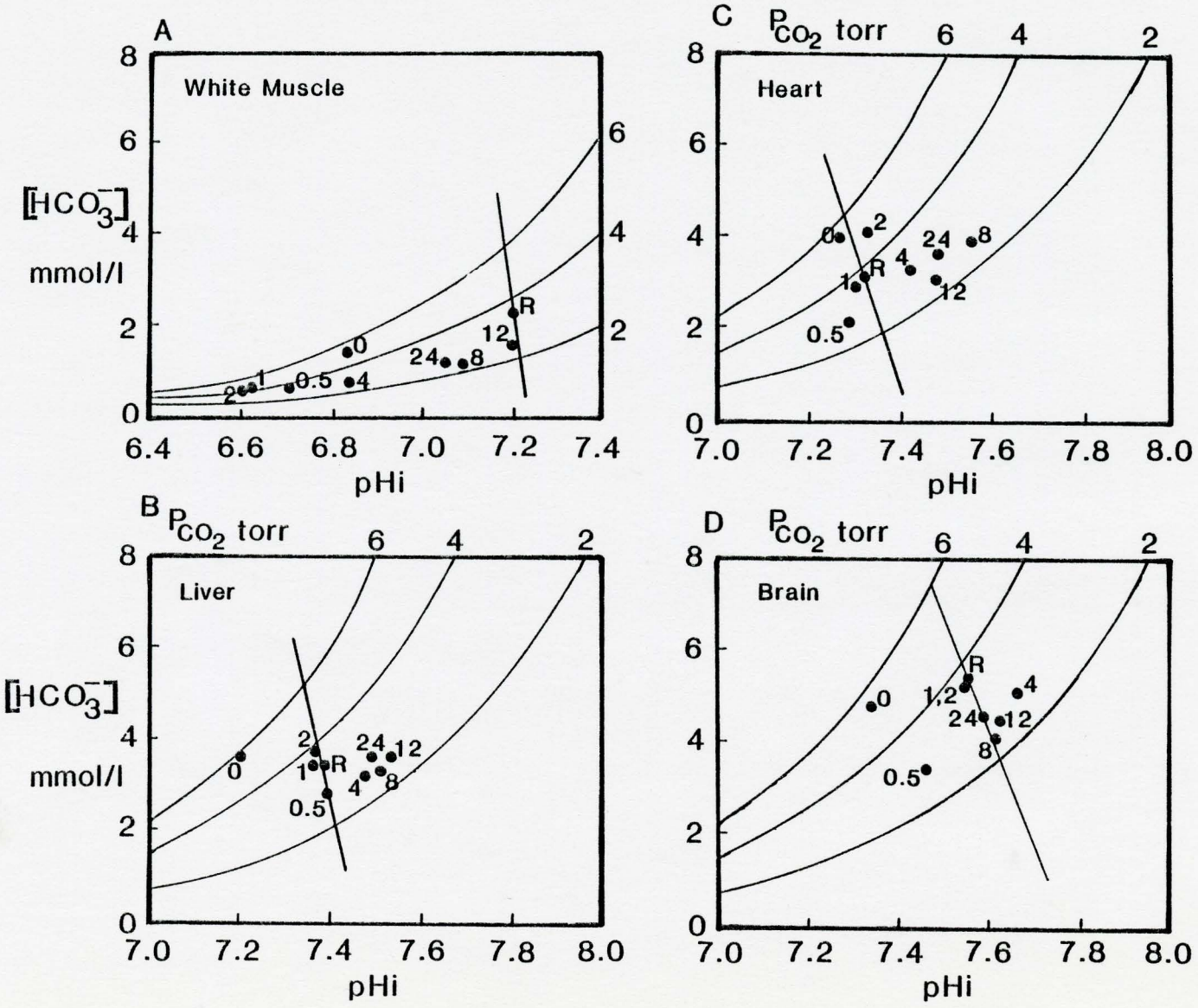
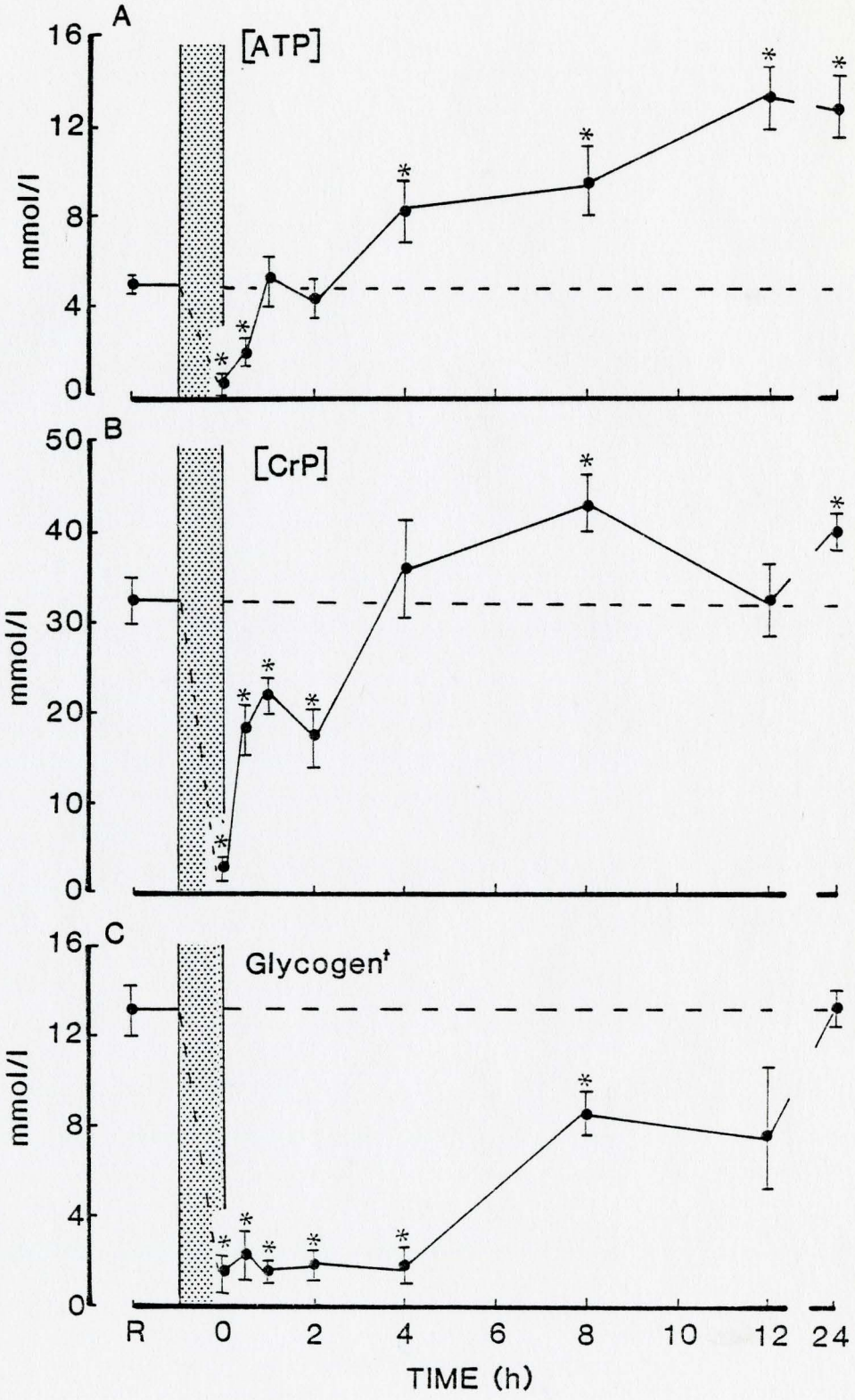

A
White Muscle
[HCO3-] mmol/l
pHi
B
Liver
PCO2 torr
C
Heart
D
Brain

Figure 4.6. Muscle concentrations of (A) ATP, (B) creatine phosphate (CrP), and (C) glycogen prior to and following exhaustive exercise. + - glycogen is expressed as glucose units, mmol/l. Means $\pm$ 1 SEM. N= 9 at rest; 8 at 0h; 6 at 0.5h; 7 at 1h; 6 at 2h; 6 at 4h; 6 at 8h; 7 at 12h and 7 at 24h. Shaded bar indicates 6 minutes of exercise, R indicates rest, and 0 indicates immediately after exercise. Dashed line indicates the mean of the rest value. * indicates a significant ($p<0.05$) difference from corresponding rest value.


A
[ATP]
mmol/l
16
12
8
4
0
B
[CrP]
mmol/l
50
40
30
20
10
0
C
Glycogen†
mmol/l
16
12
8
4
0
R
0
2
4
6
8
10
12
24
TIME (h)

occur while pHi was maximally depressed (0-2h), but began to show signs of recovery once pHi began to increase. In contrast, ATP resynthesis was quite rapid and complete (at 1h) well before pHi began to recover (Fig. 4.6A). In fact, [ATP] continued to accumulate, reaching levels at 24h, 3-4 times those at rest. [Creatine phosphate] restoration followed a different pattern still, with about 50% restoration by 2h, during the period of maximal acidosis, with full restoration complete by 4h (Fig. 4.6B). There was some evidence of overshoot at 8 and 24h.

Liver and heart pHi at rest were 0.15-0.2 pH units higher than that of muscle (Fig. 4.4B,C, respectively). The response of both tissues to exercise was nearly identical. Although pHi tended to fall slightly, neither tissue showed a significant acidosis during the period of the maximal extracellular acid-base disturbance. Similarly, both tissues showed a significant alkalosis during the latter period (8-24h) of recovery. The origin of this alkalosis was metabolic, as shown in Fig. 4.5B,C. Resting [lactate] in both tissues was similar (3-5 mmol/l) and increased 5-6 fold after exercise (Tables 4.3 and 4.4). Liver and heart [lactate] appeared to be more or less in equilibrium with whole blood [lactate] after exercise; from 0.5 through 8h, there was no significant difference between the three compartments (Tables 4.3, 4.4., Fig. 4.3A) Peak intracellular lactate levels were attained 2h into recovery, though this was not associated with an accumulation of metabolic acid (Fig. 4.5B,C). Liver pyruvate concentrations increased and then recovered over a faster time course than lactate and did not equilibrate with blood levels. Heart

Table 4.3. Liver levels of lactate, pyruvate and glycogen prior to and following exhaustive exercise in the rainbow trout.

	Time after exercise (hours)							
REST	0	0.5	1	2	4	8	12	24
Lactate								
4.33 ± 1.07 (9)	8.98 ± 1.48 (8) *	15.53 ± 2.21 (6) *	19.24 ± 2.03 (7) *	21.94 ± 3.06 (5) *	15.21 ± 1.36 (6) *	6.06 ± 0.99 (7)	5.61 ± 0.88 (7)	3.82 ± 0.33 (7)
Pyruvate								
0.07 ± 0.01 (8)	0.18 ± 0.04 (6) *	0.42 ± 0.12 (6) *	0.19 ± 0.06 (6)	0.19 ± 0.06 (5)	0.14 ± 0.08 (4)	0.06 ± 0.01 (7)	0.22 ± 0.12 (6)	0.03 ± 0.01 (6)
Glycogen+								
126.68 ± 22.61 (7)	166.94 ± 105.50 (6)	240.98 ± 139.54 (4)	247.63 ± 65.83 (7)	87.21 ± 40.34 (6)	126.26 ± 52.66 (6)	109.49 ± 14.74 (8)	109.71 ± 33.12 (7)	127.45 ± 24.79 (7)

Means ± 1 SEM. (n). All values are expressed as mmol/l intracellular fluid.

*-significantly different ($p<0.05$) from corresponding rest value

+ glycogen expressed as glucose units (mmol/l).

Table 4.4. Heart levels of lactate and pyruvate prior to and following exhaustive exercise in the rainbow trout.

	Time after exercise (hours)							
REST	0	0.5	1	2	4	8	12	24
Lactate								
3.72 ± 0.34 (6)	13.00 ± 2.16 (4) *	12.76 ± 1.44 (4) *	15.75 ± 2.43 (5) *	22.69 ± 2.25 (4) *	14.61 ± 1.33 (4) *	3.90 ± 1.22 (5)	4.58 ± 1.38 (4)	3.56 ± 0.50 (6)
Pyruvate								
0.14 ± 0.04 (6)	0.23 ± 0.08 (4)	0.22 ± 0.09 (4)	0.20 ± 0.04 (5)	0.11 ± 0.06 (4)	0.14 ± 0.03 (4)	0.09 ± 0.05 (5)	0.13 ± 0.03 (4)	0.08 ± 0.04 (6)

Means ± 1 SEM. (n). All values are expressed as mmol/l intracellular fluid.

*-significantly different ($p<0.05$) from corresponding rest value

pyruvate levels did not change appreciably. Liver glycogen content was quite variable in each group, and no significant changes were observed after exercise (Table 4.3). Glycogen was not measured in the heart.

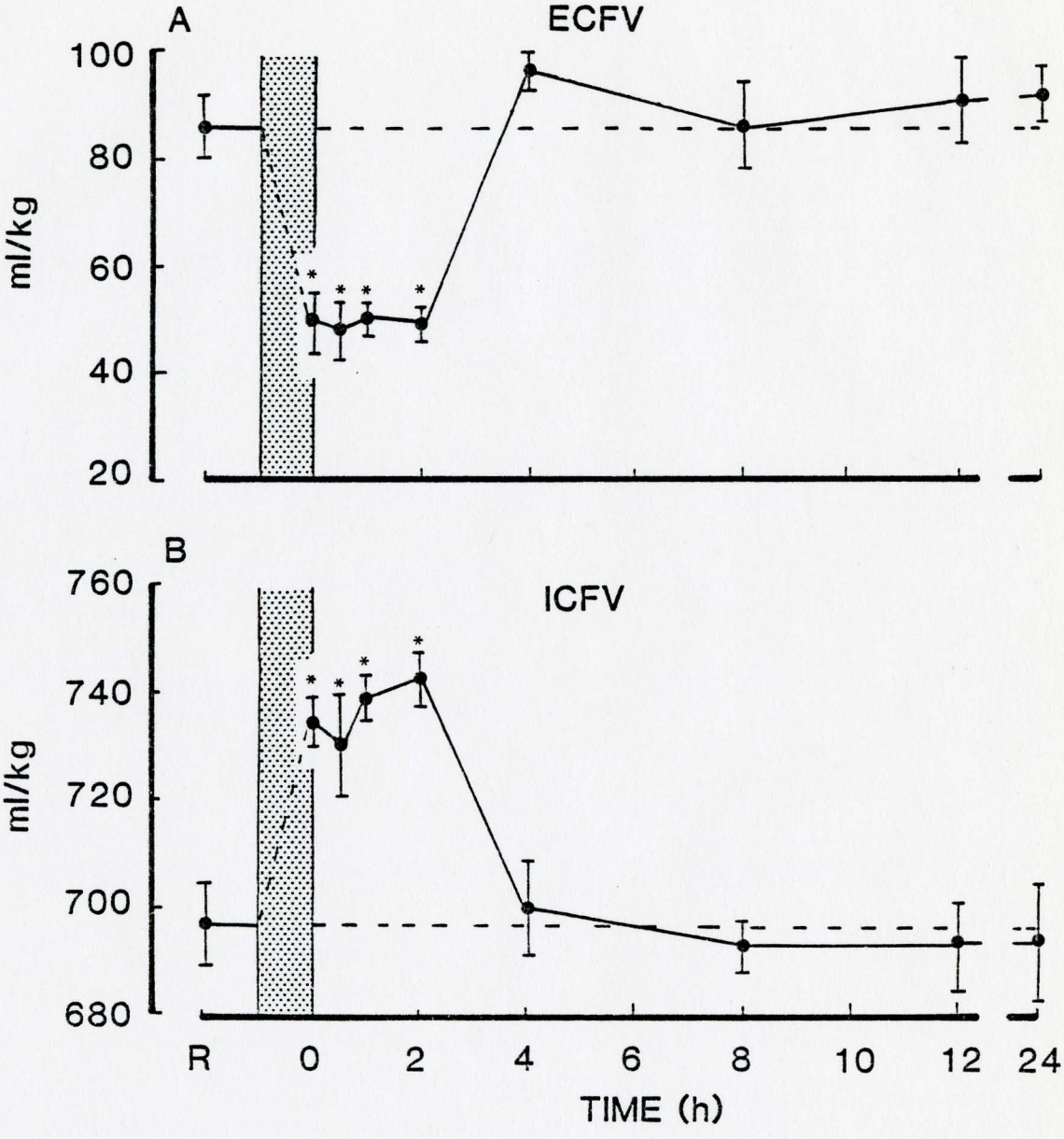
Of the tissues examined, the trout brain had the highest resting pHi, averaging 7.55 ± 0.04 (n=9, Fig. 4.4D) at rest. Unlike heart and liver, brain pHi fell immediately after exercise (Fig. 4.4D). While this acidosis appeared to be of mixed respiratory and metabolic origin (Fig. 4.5D), its significance is difficult to assess given the limitations of the methodology (see Discussion). No further perturbations in brain acid-base status were evident.

Arterial red cell pHi (measured directly) was remarkably stable after exercise (Fig. 4.4E), despite the increase in P_{CO_2} and pronounced decline in plasma pH (Fig. 4.1A,C). Only at 12 and 24 h after exercise, when pHi increased above the control value, was there any significant variation from the rest value of 7.31 ± 0.03 (n=8, Fig. 4.4E).

Fluid Volume Distribution

White muscle experienced a significant swelling after exercise, with ICFV increasing by approximately 40 ml/kg from 0-2h (Fig. 4.7). Since total tissue water did not change (788.6 ± 4.6 ml/kg wet weight, n=61), the expansion of the intracellular space was at the expense of the extracellular space. The fluid distribution of the brain was not affected by exercise, as neither ECFV (131.1 ± 4.8 ml/kg, n=61), ICFV (700.0 ± 5.6 ml/kg, n=61) nor total water content (830.1 ± 2.4 ml/kg, n=61) changed appreciably. Whilst the mannitol

Figure 4.7. (A) White muscle extracellular (ECFV) and (B) intracellular fluid volumes (ICFV) prior to and following exhaustive exercise. Means $\pm$ 1 SEM. N=9 at rest; 8 at 0 h; 6 at 0.5h; 7 at 1 h; 6 at 2 h; 6 at 4 h; 6 at 8 h; 7 at 12 h and 7 at 24 h. Shaded bar indicates 6 minutes of exercise, R indicates rest, and 0 indicates immediately after exercise. Dashed line indicates the mean of the rest value. * indicates a significant difference ($p<0.05$) from corresponding rest value.


A
ECFV
100
80
60
40
20
ml/kg
B
760
740
720
700
680
ml/kg
ICFV
R
0
2
4
6
8
10
12
24
TIME (h)

estimates of ECFV for liver and heart proved unreliable, they exhibited no significant change after exercise. Thus constant inulin-based ECFV values were used in the pHi calculations for these tissues as outlined in Materials and Methods. Neither liver (769.4 ± 2.4 ml/kg, n = 61), heart (820.8 ± 2.8 ml/kg, n = 61) nor whole body total water content (735.9 ± 4.3 ml/kg, n = 61) changed appreciably at any time as a result of exercise.

DISCUSSION

Methodology

Potential sources of error in the pHi estimates were discussed in the previous chapter where it was demonstrated that arterial *versus* venous measurement sites for pH, [^{14}C-DMO] and [^{3}H-mannitol] had negligible effects on the calculated mean whole body pHi. Comparable calculations for the individual tissues of the present study similarly indicated a negligible influence of arterial *versus* venous measurement site, and did not alter the significance of any of the differences demonstrated.

In this study mannitol proved to be an unsuitable ECFV marker for trout liver or heart, so ^{14}C-inulin derived ECFV estimates were used in the pHi calculations for these tissues. These inulin derived ECFV estimates from resting fish (C.M. Wood and S. Munger, unpublished results) were similar to those obtained from PEG-4000 (polyethylene glycol of a molecular weight of 4000 D) distribution and Cl^{-}-K^{+} space estimates in trout (Houston and Mearow, 1979). Inherent in this correction is the assumption that exercise

did not cause a fluid shift in either the liver or heart. In the calculation of pHi, a ± 50 ml/kg change in ECFV will alter pHi by only ± 0.02-0.03 pH units, which is outside the limits of detection of the DMO method.

Resting Tissue Acid-Base Status

White muscle had the greatest buffer value of the tissues examined, followed by liver, heart and brain, the latter with a buffer value only 1/3 that of muscle (Table 4.1). The high β value of white muscle is to be expected, for it has a high anaerobic potential (Castellini and Somero, 1981). While these β values were similar to those reported for heart and white muscle in dogfish (Heisler and Neumann, 1980) and channel catfish (Cameron and Kormanik, 1982), they were very different from those determined by the same methods in the sea raven (white muscle: about 39, heart: about 85 mmol/pH/l ICF; Milligan and Farrell, 1986, see Appendix I). The lower β value in the sea raven muscle most likely reflected the lower glycolytic potential of this species (Castellini and Somero, 1980).

The resting pHi values followed almost the reverse pattern to that of β values, with brain having the highest pHi, followed by liver, heart, red cell and white muscle (Fig. 4.4). Similar trends have been observed in the American eel (Walsh and Moon, 1982) and the channel catfish (Cameron and Kormanik, 1982), as well as in the rat (Roos and Boron, 1981). Thus, it is evident that there is a great deal of inter-tissue heterogeneity with respect to pHi. These differences may reflect differences in resting membrane potential and/or metabolic profiles of the tissues (Roos and Boron, 1981). Note

for example, the highest resting lactate level in the tissue with the lowest resting pHi, white muscle (Fig. 4.3A and Fig. 4.4A).

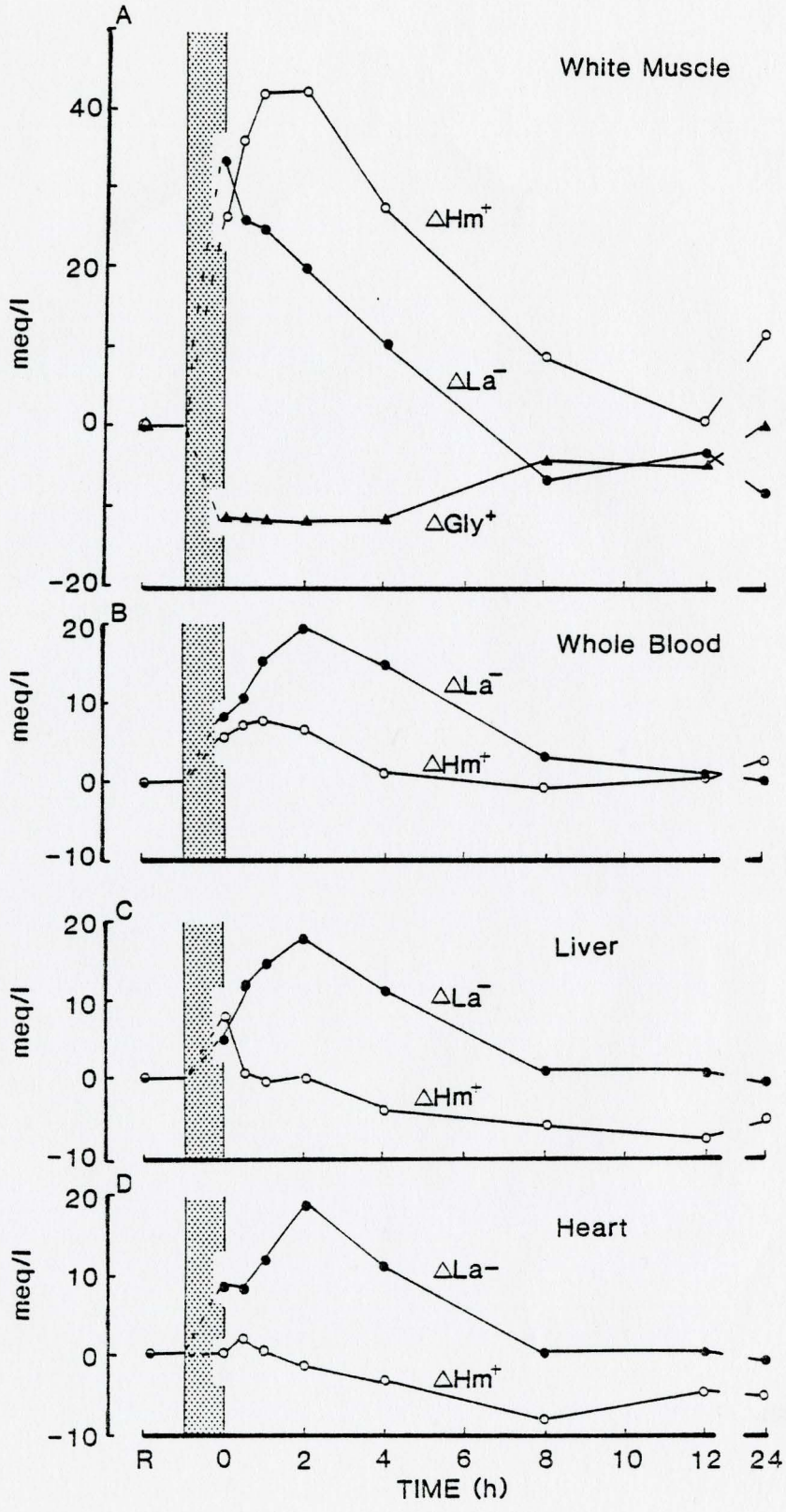
Post-exercise Changes

After exercise, white muscle experienced a severe drop in pHi, which was due almost entirely to an accumulation of metabolic acid. The brain exhibited an apparent mixed respiratory and metabolic acidosis immediately after exercise (though see cautionary note on DMO response time in Chapter III) but had recovered by 0.5h. The acid-base status of the heart and liver did not change appreciably (Fig. 4.4, 4.5). The response of red cell pHi in arterial blood in the present study was virtually identical to that observed in series II of Chapter III (Fig.3.3D). RBC pHi did not fall, despite the erythrocytes' intimate contact with the acidotic extracellular fluid. In fact, RBC pHi showed a significant alkalosis during the latter part of the recovery period (12-24 h; Fig. 4.4E). A similar trend was observed in Chapter III and by Primmett et al. (1986), though the underlying physiological reasons are not clear. Passive nonbicarbonate buffering, which is much higher than in the blood, obviously plays an important role in minimizing the pHi depressions. A post-exercise catecholamine surge is also thought to play an important role in pHi regulation in red blood cells. This question is addressed in Chapter VII. However, the role of circulating catecholamines in pHi regulation in other tissues (eg. liver, brain, heart) is less clear. In mammalian cardiac muscle in vitro , it has been suggested that circulating catecholamines may stimulate Cl^-/HCO_3^- exchange in the myocardium, thus aiding pHi regulation (Fenton,

Gonzalez and Clancy, 1978; Gonzalez and Clancy, 1984). However, recent studies with an *in situ* perfused rainbow trout heart preparation indicate that adrenaline does not appear to be important in cardiac muscle pHi regulation (Farrell and Milligan, 1986, see Appendix II).

In trout white muscle after exercise, there was an excess of protons (ΔH^+m) over lactate (ΔLa^-) of about 7 meq/l (Fig. 4.8A). This difference was equivalent to the decline in ATP levels (see Fig. 4.5A), suggesting glycolysis and ATP hydrolysis had become uncoupled (Chapter I). Accumulation of hexose-phosphates could also have contributed to the H^+ excess (Chapter I). Alternatively, other glycolytic endproducts (eg. succinate, and/or proprionate) may have been formed, and contributed to the excess proton load. However, evidence suggests these products are formed in significant amounts only during periods of extreme hypoxia, often when the fish is near death (Driedzic and Hochachka, 1975; Johnston, 1975a,b, 1977; Smith and Heath, 1980). Since terminal rather that serial samples were taken, with ΔH^+m and ΔLa^- calculated from the mean at each time, the significance of the discrepancy between ΔH^+m and ΔLa^- cannot be assessed statistically. However, a similar pattern (ΔH^+m in excess of ΔLa^-) has been observed after exercise in the white muscle of the sea raven (Milligan and Farrell, 1986, see Appendix I) and the starry flounder (Chapter VI, Fig. 6.7). Regardless of the source of these excess protons, the inherent dangers of assuming a strict 1:1 stoichiometry between lactate and proton production are clearly evident.

Figure 4.8. Metabolic acid (ΔH^+m), lactate (ΔLa^-) and glycogen (ΔGly) loads to (A) white muscle and metabolic acid (ΔH^+m) and lactate (ΔLa^-) loads to (B) whole blood, (C) liver and (D) heart after exercise in rainbow trout. + - glycogen is expressed as glucose units, mmol/l. R indicates rest, bar indicates period of exercise; 0 indicates immediately after exercise. Values plotted are calculated from means at each time. See text for details.


A
White Muscle
40
20
0
-20
meq/l
ΔHm+
ΔLa−
ΔGly+
B
Whole Blood
20
10
0
-10
meq/l
ΔLa−
ΔHm+
C
Liver
20
10
0
-10
meq/l
ΔLa−
ΔHm+
D
Heart
20
10
0
-10
meq/l
ΔLa−
ΔHm+
R
0
2
4
6
8
10
12
24
TIME (h)

After exercise, the blood lactate/pyruvate ratio (calculated from mean values) increased, from 12.1 at rest to 65.7 immediately after exercise, indicating that lactate was released from the muscle in excess of pyruvate. This contrasts sharply with that observed in starry flounder (Chapter VI) and flathead sole (Turner, Wood and Hobe, 1983) after exhaustive exercise, in which the blood lactate/pyruvate ratio decreased.

The pattern of lactate accumulation in the liver (Fig. 4.8C) and heart (Fig. 4.8D) was similar to that of the blood (Fig. 4.8B), with peak levels attained 2 hours after exercise. However in contrast to the blood, liver and heart did not show an ΔH^+m accumulation, but rather experienced a ΔH^+m deficit. The appearance of lactate in these tissues could have resulted from either glycolysis *in situ* or *via* uptake from the blood, though a number of factors suggest the latter. First, both these tissues are capable of taking up and oxidizing exogenous lactate (Bilinski and Jonas, 1972). In fact, lactate is a preferred substrate for the trout heart (Lanctin, McMorran and Driedzic, 1980). Secondly, the pattern of lactate appearance in heart and liver paralleled that of the blood, and absolute levels of lactate were equal in the three compartments from 0.5 to 8h. Had lactate appeared in these tissues as a result of exercise-induced glycolysis *in situ* , peaks at 0h, as in white muscle, rather that at 2h would have been anticipated. Finally, there was no accumulation of metabolic protons, which would have been expected had lactate been produced *in situ* .

The mechanism(s) of lactate uptake is(are) poorly understood. In some tissues, the bulk of lactate is transported in conjunction with H^+ or in exchange for OH^- or HCO_3^- (eg. human red cells, Dubinsky and Racker, 1978; Ehrlich-Ascites tumor cells, Spencer and Lehninger, 1976), whereas in others, co-transport with Na^+ predominates (eg. rat kidney, Ullrich, Rumrich and Kloss, 1982). As lactate metabolism is a net H^+ consuming process (Hochachka and Mommsen, 1983), a net acid deficit (i.e. metabolic alkalosis) would result if lactate were transported in conjunction with a cation (other than H^+) or in exchange for an anion (other than OH^- or HCO_3^-) and subsequently metabolized. In perfused rat liver, a direct relationship between intracellular alkalinization and lactate uptake was observed (Cohen, Iles, Barnett, Howell and Strunin, 1971). The alkalosis associated with lactate accumulation in the trout heart and liver (Fig. 4.8C,D) suggests that at least some of the lactate was transported without an accompanying proton and subsequently metabolized (see below).

The Fate of Lactate and H^+

During the first 4 hours of recovery, lactate disappeared from the muscle mass much more quickly than did the proton load, a difference which was reflected in the appearance of these metabolites in whole blood, where ΔLa^- was greater than ΔH^+m (Fig. 4.8A,B). What is the fate of this lactate and what are the overall contributions of the heart, liver and other lactate-utilizing 'aerobic tissues' (eg. red muscle, kidney, gills; Bilinski and Jonas, 1972) to the clearance of the muscle lactate load? In a 1 kg trout, the total

lactate load immediately after exercise averaged 17.3 mmol, assuming that the muscle mass constituted 60% of the body weight (Stevens, 1968), that whole blood lactate levels were representative of the average extracellular level, and that the whole body ECFV estimates of Chapter III (Fig. 3.4) were applicable. Of this total, 15.2 mmol were in the white muscle (Fig. 4.3A). By 4h, 11.0 mmol had disappeared from the muscle, of which only 1.6 mmol could be accounted for by additional accumulation in the extracellular fluid (Fig. 4.3B). Excretion to the water was insignificant (Table 3.5; Chapter III). The observations that neither liver nor muscle glycogen were elevated over this 0-4h period (Table 4.3, Fig. 4.6C) and that the rise in blood [glucose] was quite small (Table 4.2) suggest that the bulk of this 9.4 mmol lactate which disappeared was oxidized. For this to occur, a whole animal O_2 consumption rate of at least 7 mmol O_2/kg/h would be required assuming that for every mole of lactate metabolized, 3 moles of O_2 are consumed (Newsholme and Leech, 1983). This value is not unreasonable, for O_2 consumption can reach levels as high as 25 - 31 mmol O_2/kg/h following exhaustive exercise in salmonids (Brett, 1972) and remains significantly elevated above pre-exercise levels for up to 8 h (Brett, 1964). If the burden of oxidation were placed solely on the 'aerobic' tissues (approximately 7% of body weight, Stevens, 1968), then minimal O_2 consumptions of 100 mmol O_2/kg/h would be required of this tissue mass. An oxygen consumption of about 78 mmol O_2/kg/h has been observed for the <u>in situ</u> perfused trout heart (Farrell and Milligan, 1986; see Appendix II) and <u>in vitro</u> red muscle preparations from a

variety of fish (Gordon, 1972a), so the requirement of 100 mmol O_2/kg/h appears reasonable. However, these reported tissue O_2 consumption rates represent total aerobic metabolism, of which lactate oxidation is only a part; thus, a minimal requirement of 100 mmol O_2/kg/h is probably an underestimate.

The white muscle is also capable of lactate oxidation, albeit at rates lower than those of the 'aerobic' tissues (Bilinski and Jonas, 1972). As white muscle constitutes the bulk of the body mass, it does not seem unreasonable to suggest that lactate may be oxidized *in situ*. Indeed, this may have supplied the energy for the restoration (and overshoot) of ATP and CrP stores which occurred at this time (Fig. 4.6). The observation of Turner and Wood (1983) in rainbow trout that only about 10% of the lactate produced after exercise leaves the muscle mass supports this contention. If this were the case, then 'aerobic' tissue O_2 consumption requirement would only be 10 mmol O_2/kg/h and that of the white muscle 10.6 mmol O_2/kg/h, well within the reported *in vitro* values (Gordon, 1972a,b). Thus in trout, it would appear that in the early period of recovery lactate clearance can be attributed to a combination of export to the 'aerobic' tissues and *in situ* oxidation. Schwalme and Mackay (1985b) reached a similar conclusion regarding the fate of lactate produced during angling-induced exercise in pike (*Esox lucius* L.)

During the remainder of the recovery period, from 4 to 24 hours, lactate and proton clearance from the muscle were about equal and correlated with a restoration of glycogen stores (Fig. 4.6C) as

pHi returned to resting levels (Fig. 4.4A). In fact, by 24h, approximately 75% of the glycogen resynthesized could be accounted for by the disappearance of lactate and protons. It appears that the muscle even tapped into its lactate 'reserve' to replenish its glycogen store, for by 24h, lactate had fallen to approximately 30% of the resting level (Fig. 4.3A, 4.6C).

The classical picture of the restoration of glycogen stores after exercise is the Cori cycle (Newsholme and Leech, 1983): lactate leaves the muscle, is taken up by the liver, and is synthesized into glucose which then enters the bloodstream to be taken up by the muscle and made into glycogen. However, the results of this study and others (Hermansen and Vaage, 1977; Connett, 1979; McLane and Holloszy, 1979; Constable, Young, Higuchi and Holloszy, 1984) indicate that vertebrate white muscle is capable of *in situ* glyconeogenesis. These physiological studies, in conjunction with biochemical investigations (Dyson, Cardenas and Barsotti, 1975; Hochachka and Somero, 1984) argue against a prominent role for the Cori cycle in restoring muscle glycogen stores in vertebrate muscle after exercise. Instead, it is postulated that lactate and proton clearance from the muscle initially occurs via export to 'aerobic' tissues and *in situ* oxidation, which aids in restoring muscle pHi to a level compatible with glycogen re-synthesis. When this point is reached, lactate and protons are used as substrates for *in situ* glyconeogenesis. In support of this idea, Kashiwagura *et al.* (1984) have shown that glycogen resynthesis does exhibit a pH dependence; at low pH (less than 7.1), glyconoegenesis was reduced in mammalian tissue.

CHAPTER V

INTRACELLULAR AND EXTRACELLULAR ACID-BASE STATUS AND H^+ EXCHANGE WITH THE ENVIRONMENT AFTER EXHAUSTIVE EXERCISE IN THE STARRY FLOUNDER

INTRODUCTION

In the benthic, rather inactive starry flounder, appearance of lactate in the blood is minimal after exercise, and the H^+ load is far in excess of the lactate load (Wood et al. , 1977), whereas in active species, such as rainbow trout the reverse is seen (Fig. 3.7, Chapter III; also Turner, Wood and Clark, 1983). Little is known about the mechanism(s) leading to these different patterns of lactate and H^+ accumulation in the blood of the flounder, and nothing is known about intracellular changes. In the trout, lactate accumulation in the blood well in excess of H^+ was due mainly to differential release of lactate and H^+ from the white muscle (Chapters III, IV), though H^+ exchange with the environment was important in restoration of extracellular acid-base status later in recovery.

The present study focuses on acid-base changes in the whole body ICFV, ECFV, and environmental water after strenuous activity in the starry flounder, with particular emphasis on transcompartmental exchange of H^+ (metabolic acid). The goal (in combination with the work of Chapter VI) was to understand the genesis of a fundamentally different pattern of acid-base and lactate dynamics.

MATERIALS AND METHODS

Experimental Animals

Starry flounder (Platichthys stellatus), weighing 150-1200 g, of both sexes were collected by otter trawl from East Sound, Orcas Island and Birch Bay, Washington from October through December, 1982. Fish were held in large (6800 liter) tanks with sand-covered bottoms supplied with fresh running seawater (29^{o}/oo) at seasonal temperature (11 $\pm$ 1^{o}C) at Friday Harbor Laboratories, University of Washington. During holding, fish fed ad libitum on other small fishes and invertebrates present in the tank. Prior to experimentation, flounder were acclimated to laboratory conditions indoors in plexiglass, sand-covered tanks supplied with fresh seawater for 3-5 days, during which period they were starved.

Caudal artery catheters were surgically implanted according to the method of Watters and Smith (1973) while the fish were under MS:222 (1:10,000; Sigma) anaesthesia. To prevent infection, the wound was dusted with the antibiotic oxytetracycline hydrochloride (Syndel Labs, Vancouver) prior to closure with silk sutures. Catheters were filled with heparinized (50 i.u./ml) Cortland saline adjusted to 160 mM NaCl. Fish were then placed in 15 l plastic tubs supplied with fresh flowing seawater (1.5 l/min) at 11 $\pm$ 1^{o}C and allowed to recover at least 72 h before experimentation. These tubs were fitted with an aeration ring around the perimeter which allowed them to be operated as low volume (1 l per 100 g body weight) closed systems for flux measurements. During these periods temperature was maintained at 11 $\pm$ 1 ^{o}C by bathing the tubs in flowing seawater.

It has been shown that resting values for a number of physiological parameters are never attained if the flounder are held in bare tanks, for they lack substrate in which to bury (Wood, McMahon and McDonald, 1979a). Sand could not be used for it prevented thorough mixing of the water and supported growth of microorganisms which could influence acidic equivalent and ammonia flux measurements. In place of sand, a piece of black plastic mesh was mounted 5-10 cm from the bottom of the tub, under which the flounder could lie. This appeared to mimic the effect of sand satisfactorily, for the fish did not exhibit any undue activity and key physiological parameters, such as pHa (Fig. 5.1A), Pa_{O_2} (Fig. 6.1D, Chapter VI) were typical of those previously reported for resting, buried flounder (Wood et al. , 1979a).

Experimental Protocol

These experiments focused on post-exercise changes in arterial acid-base and lactate status, whole body pHi and ECFV, acidic equivalent exchanges with the environment, and ammonia, ^{3}H-mannitol and ^{14}C-DMO excretion. Parallel experiments were run on two groups, only one of which was subjected to exercise (n=8). The other group (n=8) served as a control for handling and sampling effects; these fish were left at rest throughout but otherwise treated identically to the experimental group.

The experimental protocol was identical to that described for rainbow trout in series I of Chapter III, except that experiments were done in seawater ($29^{o}/oo$) at $11 \pm 1^{o}C$.

To induce strenuous activity, flounder were rapidly transferred to a shallow rectangular tank (128 cm x 98 cm x 9 cm) and then vigorously chased by hand for 10 min (cf. Wood et al. , 1977). By the end of this period, fish were unable to either burst-swim or swim slowly. Fluxes of titratable acidity and ammonia and loss of markers to the water could not be measured during the exercise period. However, even if such fluxes had occurred at up to five times the immediately post-exercise rates during the 10 min exercise period, their influence on all calculated parameters would have been negligible.

Arterial blood samples (500 ul) were drawn prior to ('rest'), immediately after exercise (time 0), as well as at 0.25, 0.5, 1,2,4,8,12 and 24h post-activity. Samples were analyzed for pH, total CO_2 (in both plasma and whole blood), hematocrit, [hemoglobin], whole blood [glucose] and [lactate], plasma [protein] and plasma levels of ^{3}H and ^{14}C radioactivity. The volume of blood sampled was replaced with saline.

Analytical Techniques, Calculations and Statistical Analyses

All analyses and calculations were performed as described in Chapter II and III, though with electrodes maintained at 11 ^{o}C rather than 15 ^{o}C, and with constants chosen for this temperature.

The relationship between β, [hemoglobin] and hematocrit for whole blood and plasma in starry flounder was determined by in vitro CO_2 titrations. Blood was drawn from the arterial catheters of 5 resting flounder, heparinized at 250 i.u./ml, pooled, gently centrifuged to separate plasma from RBC's, then reconstituted to 6

hematocrits ranging from 0-35%. Tonometry, analyses and estimation of β over the physiological range of pHa (Fig. 5.1A) for each [hemoglobin] and hematocrit were carried out as described by Wood et al. (1982).

RESULTS

Blood Buffer Capacity

In vitro tonometry of whole blood and plasma over the normal range of CO_2 tensions yielded linear relationships between β, the non-bicarbonate buffer capacity, in mmol/l/pH and [hemoglobin] in g/100 ml or hematocrit as a decimal. The regression relationships for whole blood were:

$$\beta = -1.46 \text{ X } [Hb] - 2.64; \ (n=6; \ r=-0.993; \ p<0.005) \qquad (5.1)$$

$$\beta = -40.52 \text{ X } Ht - 2.79; \ (n=6; \ r=-0.993; \ p<0.005) \qquad (5.2)$$

and for true plasma

$$\beta = -1.69 \text{ X } [Hb] - 2.08; \ (n=6; \ r=-0.989; \ p<0.005) \qquad (5.3)$$

$$\beta = -46.41 \text{ X } Ht - 2.23; \ (n=6; \ r=-0.993; \ p<0.005) \qquad (5.4)$$

The buffer value for separated plasma was -3.04 mmol/l/pH.

Behaviour of ^{3}H-mannitol and ^{14}C-DMO

There was a significant loss of both markers into the environmental water (Table 5.1) at about half the rates previously measured for rainbow trout (Table 3.2; Chapter III). Loss rates were quite variable between fish though of about the same absolute magnitude for ^{14}C-DMO and ^{3}H-mannitol. Exercise did not appear to affect the rate of marker loss, as there were no differences between the control and exercise group. Total marker loss due to repetitive blood sampling amounted to only about 5% of losses due to

Table 5.1. Percent of injected marker doses excreted to the environment in exercised and non-exercised starry flounder.

Time after injection (hours)	12	14	16	20	24	36
Experimental time (hours)	0	2	4	8	12	24
Control group						
^{14}C-DMO	3.71 ± 0.34% (8)	5.68 ± 1.14% (8)	6.01 ± 1.47% (8)	8.42 ± 3.17% (8)	11.77 ± 3.01% (8)	17.42 ± 4.07% (8)
^{3}H-mannitol	3.49 ± 0.66% (8)	5.79 ± 2.34% (8)	5.98 ± 1.49% (8)	8.07 ± 2.34% (8)	10.08 ± 3.16% (8)	16.70 ± 3.73% (8)
Exercise Group						
^{14}C-DMO	4.93 ± 0.52% (8)	5.78 ± 0.75% (8)	6.97 ± 1.13% (8)	8.93 ± 1.07% (8)	10.08 ± 1.34% (8)	15.73 ± 2.01% (8)
^{3}H-mannitol	4.70 ± 0.78% (8)	5.95 ± 0.81% (8)	7.19 ± 0.76% (8)	8.60 ± 0.83% (8)	9.91 ± 0.86% (8)	14.88 ± 1.58% (8)

Means ± 1 SEM (n)

There were no significant differences at any time between the control and experimental group.

excretion. By the time rest samples were taken (12h post-infusion), approximately 95% of the infused marker doses remained and at the end of the experiment (36h post-infusion) only about 80-85% of the dose remained. Failure to take these losses into account would have resulted in overestimates of ECFV by about 13 ml/kg (at rest) to 40 ml/kg (at 24h) and in whole body pHi by about 0.04 units (at rest) and 0.10 units (at 24h).

A further complication to the ECFV estimate was lengthy marker equilibration time. At rest (12h post-infusion), the calculated whole body ECFV in both groups was about 256 ml/kg (Table 5.2) which agreed closely with the previous estimates in rainbow trout (Table 3.3, Chapter III; also Milligan and Wood, 1982; M.V.E. Attygalle, G. Shelton and P.C. Croghan, unpublished observations). The control group exhibited a gradual rise in ECFV so that by 2h the change was significant and by 24h, ECFV had increased by about 25% (Table 5.2). In the exercise group, ECFV showed a significant decline for the first 0.5h after activity followed by a gradual rise similar to that in the control group. This expansion was probably due to ^{3}H-mannitol penetration of the ICF of the liver and kidney (Hickman, 1972) after lengthy equilibration. Clearly, the true post-exercise changes in fluid volume distribution were masked. To unmask these changes, rest values (at 12h post-infusion) were assumed correct. As in Chapter III, the mean increase over rest values in the control group was subtracted from the exercise value at each respective time to yield the corrected ECFV estimates shown in Fig. 5.4A. These corrected values were then used for calculation of mean whole body pHi (Fig.

Table 5.2. Whole body extracellular fluid volumes (uncorrected) in exercised and non-exercised starry flounder.

	REST	0H	0.25H	0.5H	1H	2H	4H	8H	12H	24H
CONTROL	255.4 ± 15.2 (8)	265.1 ± 12.6 (8)	265.7 ± 13.3 (8)	261.9 ± 12.3 (8)	265.7 ± 13.5 (8) *	280.7 ± 18.0 (8) *	271.3 ± 14.2 (8) *	298.5 ± 14.7 (8) *	297.0 ± 14.8 (8) *	319.0 ± 14.8 (8)
EXERCISE	258.9 ± 10.7 (8)	231.3 ± 10.7 (8) *	237.1 ± 9.7 (8) *	248.4 ± 17.9 (8)	246.4 ± 9.6 (8)	250.2 ± 12.8 (8)	275.3 ± 13.8 (8) *	291.4 ± 14.2 (8) *	289.4 ± 14.1 (8) *	308.9 ± 15.0 (8) *

Means ± 1 SEM. All values in ml/kg body weight.

* indicates a significant difference ($p<0.05$) from corresponding rest values.

5.5). In the control group, rest values were employed throughout.

Blood Acid-Base and Metabolite Status

Ten minutes of enforced activity resulted in a pronounced arterial blood acidosis, with pHa maximally depressed at 15 min after cessation of activity (Fig. 5.1A). The immediate acidosis (at 0h) was associated with a doubling of Pa_{CO_2} (Fig. 5.1B) and the appearance of a small metabolic acid load (ΔH^+m) in the blood (Fig. 5.2). By 0.25-0.5h, ΔH^+m peaked, while Pa_{CO_2} was beginning to decline. ΔH^+m remained significantly elevated until 8h (Fig. 5.2), while Pa_{CO_2} had returned to levels not significantly different from rest by 2h (Fig. 5.1B). Analysis based upon the principles outlined by Wood et al. (1977) revealed that immediately post-activity, the acidosis was mainly of respiratory origin (greater than 90%), with little metabolic contribution. However, ΔH^+m progressively increased (Fig. 5.2) as indicated by the depression of plasma [HCO_3^-] (Fig. 5.1B) and by 1h had become the dominant component of the acidosis as Pa_{CO_2} declined (Fig. 5.1C). While this rise in ΔH^+m was slower in flounder than in trout (Fig. 3.7, Chapter III), its magnitude was only slightly smaller. By 8h into recovery, with both P_{CO_2} and [HCO_3^-] were restored to resting levels, and the acidosis was fully corrected, without any evidence of the overshoot previously seen in trout (Fig. 3.1, Chapter III). Acid-base status was unchanged in the control group and thus was not affected by sampling.

At rest, whole blood [lactate] in both the control and exercise group was quite low, about 0.25 mmol/l (Fig. 5.1D). After

Figure 5.1. Blood acid-base status in exercised (●) and non-exercised (○) starry flounder: (A) plasma pHa, (B) plasma [HCO_3^-], (C) plasma CO_2 tension, Pa_{CO_2}, and (D) whole blood [lactate] in starry flounder. Means ± 1 SEM. Exercise group, ○, n = 8; control group, n = 8, ○. R indicates rest value; vertical bar indicates 10 minute period of activity; 0h= immediately after activity. * indicates a significant difference ($p<0.05$) from corresponding rest value.

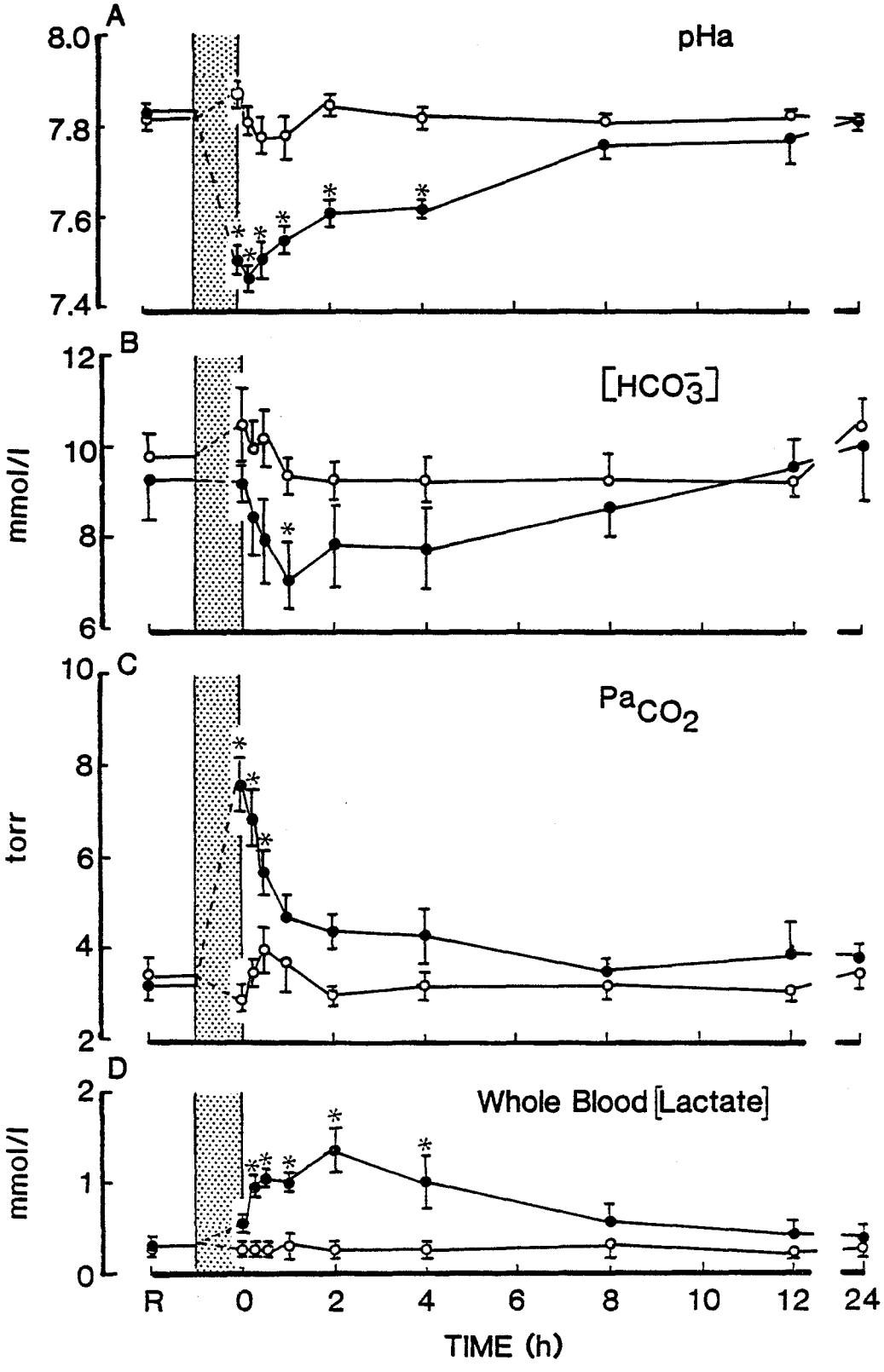
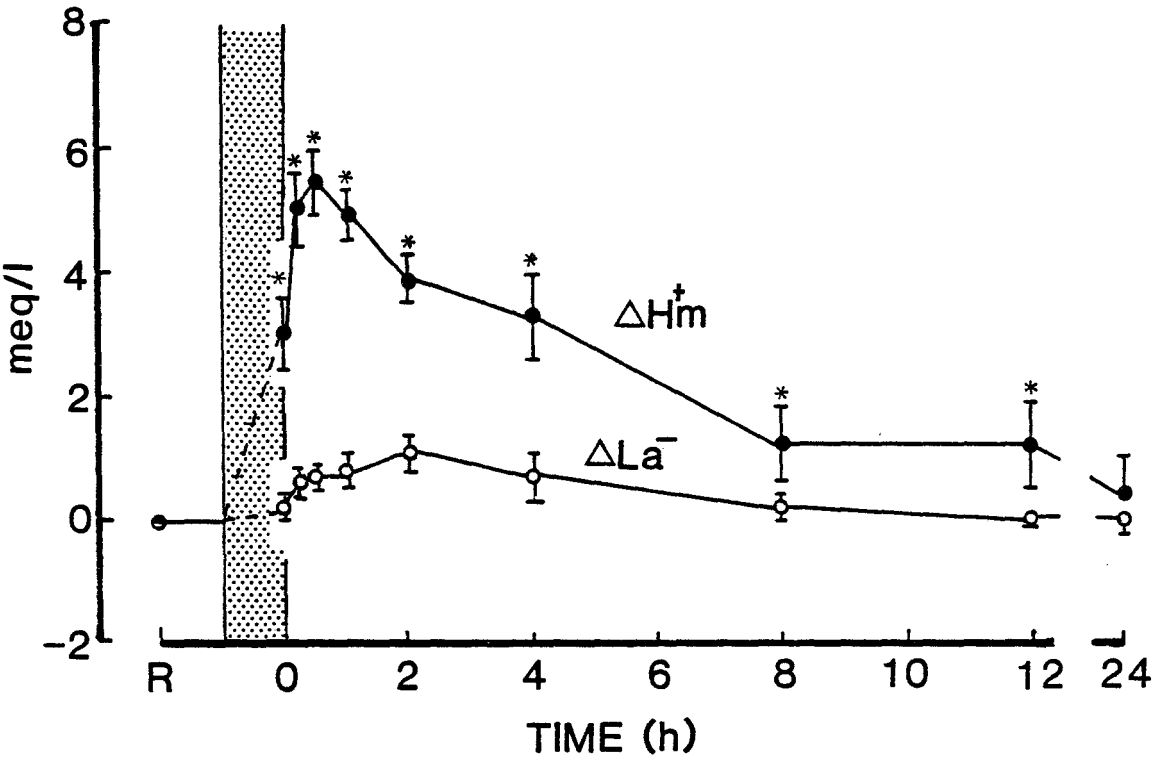

A
pHa
8.0
7.8
7.6
7.4
B
[HCO3-]
12
10
8
6
mmol/l
C
PaCO2
10
8
6
4
2
torr
D
Whole Blood [Lactate]
2
1
0
mmol/l
R
0
2
4
6
8
10
12
24
TIME (h)

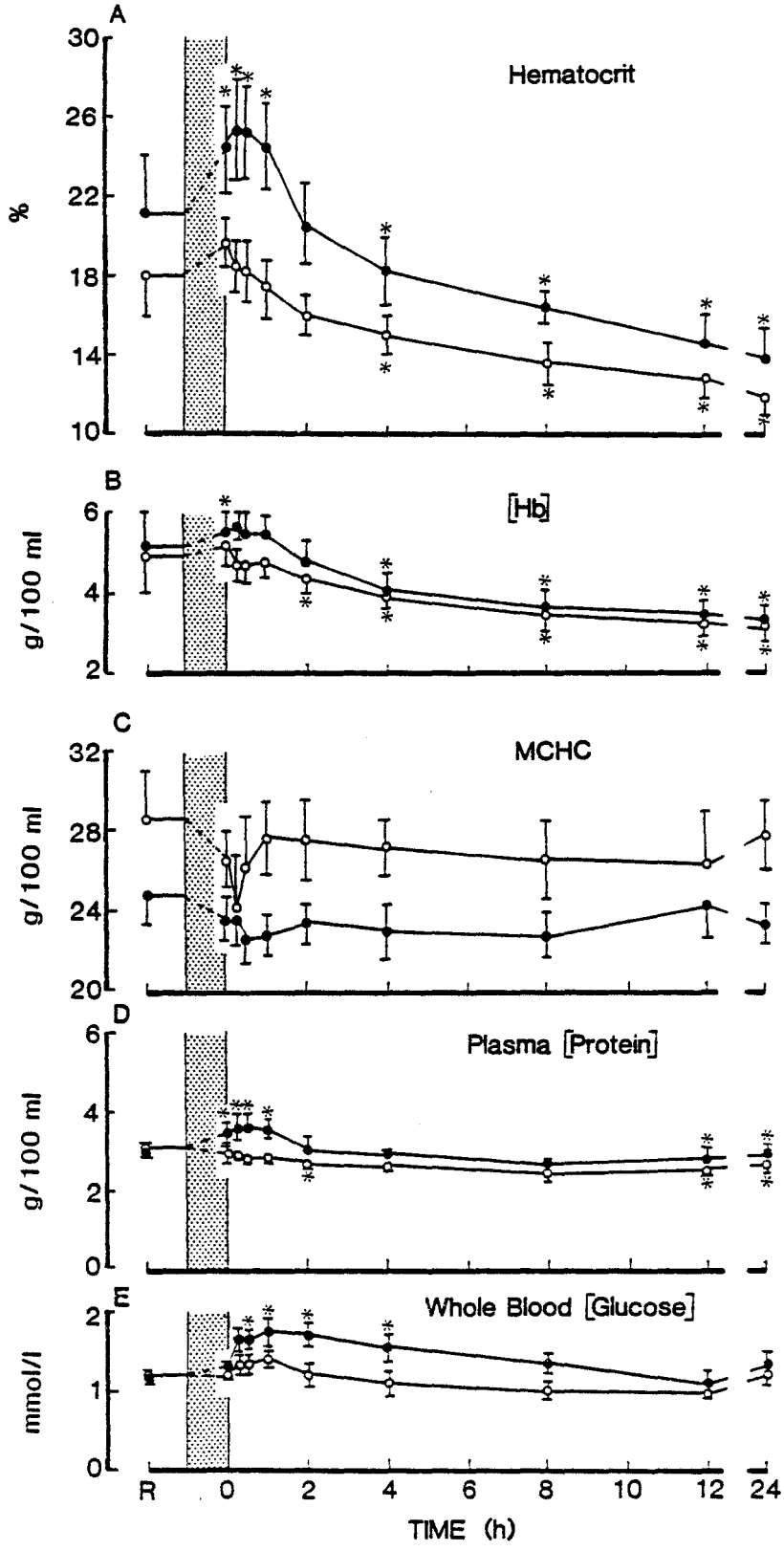
Figure 5.2. Changes in blood lactate load (ΔLa^-) and blood metabolic acid load (ΔH^+m) during recovery from exercise; see text for details of calculation. Means $\pm$ 1 SEM, n=8. R indicates rest value; vertical bar indicates 10 minute period of activity; 0h is immediately after activity. * indicates a significant difference ($p < 0.05$) between ΔLa^- and ΔH^+m at the same time.


8
6
4
2
0
−2
meq/l
ΔH⁺m
ΔLa⁻
R
0
2
4
6
8
10
12
24
TIME (h)

activity, lactate levels did increase, but the rise was minimal with peak levels (at 2-4h) averaging about 1.3 mmol/l and rarely greater than 2 mmol/l in individual fish (versus 15-20 mmol/l for trout, Fig. 3.1D, Chapter III). Thus, the blood lactate load (ΔLa^-) appeared with both a much slower time course and a much smaller absolute magnitude than the blood metabolic acid load (ΔH^+m; Fig. 5.2). Lactate levels also declined slowly, returning to rest values by 8h. [Lactate] in the control group was not affected by sampling (Fig. 5.1D).

A further consequence of enforced activity in the starry flounder was a rapid and significant elevation in hematocrit, [hemoglobin] and plasma [protein] (Fig. 5.3A,B,D). These changes most likely reflected a general hemoconcentration due to a fluid shift out of the ECFV and into the ICFV (see below). The fact that mean cell hemoglobin concentration (MCHC, Fig. 5.3C) declined only slightly and non-significantly suggests that red cell swelling was of minor importance in the hematocrit elevation. For the same reason, mobilization of reticulocytes (cells with lower hemoglobin content) from the spleen was likely not a significant component of either the increase in hematocrit or [hemoglobin]. The hemoconcentration was short-lived, with hematocrit, [hemoglobin] and plasma [protein] returning to rest levels by 2h. However, by 4h, the diluting effect due to repetitive sampling became evident, as these parameters began to decline, following a pattern similar to that of the control group (Fig. 5.3A,B,D).

Figure 5.3. Changes in (A) hematocrit, (B) [hemoglobin], (C) mean cell hemoglobin concentration (MCHC), (D)plasma [protein], and (E) whole blood [glucose] induced by 10 minutes of activity. Means $\pm$ 1 SEM. Exercise group, n = 8 , ●; control group, n = 8, O. R indicates rest value; vertical bar indicates 10 minute period of activity; 0h is immediately after activity. * indicates a significant difference ($p<0.05$) from corresponding rest value.


A
Hematocrit
%
30
26
22
18
14
10
B
[Hb]
g/100 ml
6
4
2
C
MCHC
g/100 ml
32
28
24
20
D
Plasma [Protein]
g/100 ml
6
4
2
0
E
Whole Blood [Glucose]
mmol/l
2
1
0
R
0
2
4
6
8
10
12
24
TIME (h)

Whole blood [glucose] followed a somewhat different pattern. Though the [glucose] increased by about 50%, the change was not significant until 0.5h after cessation of activity (Fig. 5.3E). Furthermore, repetitive blood sampling did not affect glucose levels in either the control or exercise group.

Whole Body Fluid Volumes and Intracellular pH

After cessation of activity, whole body ECFV fell by about 11%, or 24 ml/kg and remained depressed until 4h into recovery (Fig. 5.4A). Since total body water remained constant at various times after activity (at 784.0 $\pm$ 2.9 ml/kg, n=40; Chapter VI), the reduction in ECFV was a direct consequence of a fluid shift into the ICFV. This resulted in a reciprocal expansion of the ICFV (Fig. 5.4B) until 4h into recovery. These changes were smaller but of similar pattern to those seen in trout (Fig. 3.4, Chapter III).

At rest, whole body pHi in the control group, 7.56 $\pm$ 0.04 (n=8) was virtually identical to the exercise group at 7.58 $\pm$ 0.05 (n=8; Fig. 5.5A,B) and much higher than that of trout (7.25, Fig. 3.5A, Chapter III). Immediately after exercise, pHi fell to 7.29 $\pm$ 0.04 (n=8) and fell further to a low of 7.24 $\pm$ 0.06 (n=8) at 15 min, similar to the pattern of the pHe change. pHi remained depressed until 4h, when it showed a near complete recovery, despite the fact that pHe was still significantly depressed. By 8h post-exercise, pHi showed a significant alkalosis at a time when pHe had just returned to rest levels (Fig. 5.5A). The intracellular alkalosis had been corrected by 12h, with resting acid-base status restored. Whole body pHi remained constant in the control group (Fig. 5.5B).

Figure 5.4. Whole body (A) extracellular fluid volume (ECFV) and (B) intracellular fluid volume (ICFV) prior to and following exhaustive exercise in starry flounder. Data are corrected as described in text. Means $\pm$ 1 SEM (n=8). R indicates rest value; vertical bar indicates 10 minute period of activity; 0h is immediately after activity. Dashed line indicates mean of rest value. * indicates a significant difference ($p<0.05$) from corresponding rest value.

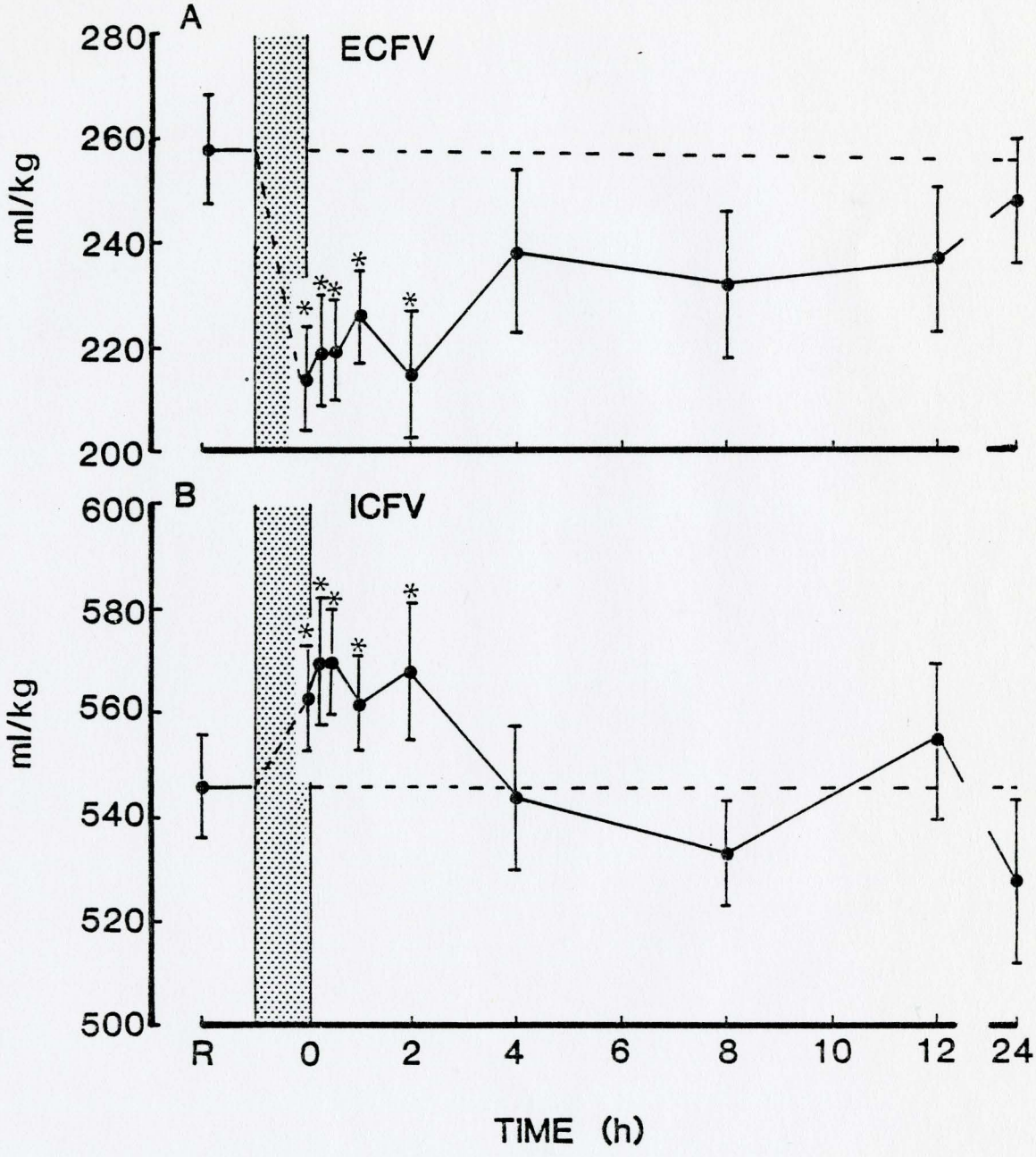
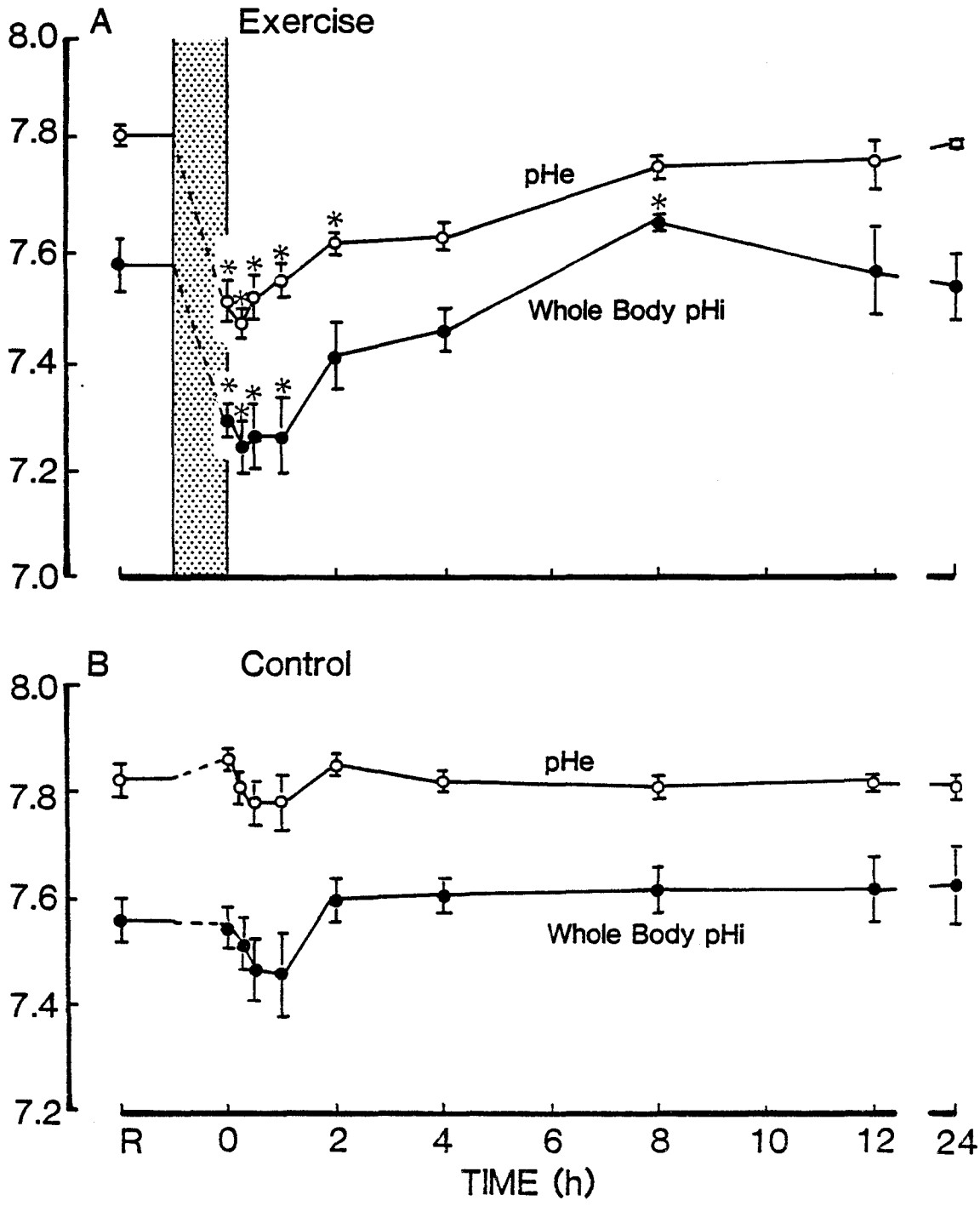

A
ECFV
280
260
240
220
200
ml/kg
B
ICFV
600
580
560
540
520
500
ml/kg
R
0
2
4
6
8
10
12
24
TIME (h)

Figure 5.5. Whole body intracellular pH (pHi-●) and extracellular pH (pHe-○) in (A) exercised and (B) non-exercised starry flounder. Means ± 1 SEM. R indicates rest value; vertical bar indicates 10 minute period of activity; 0h is immediately after activity. * indicates a significant difference ($p<0.05$) from corresponding rest value.

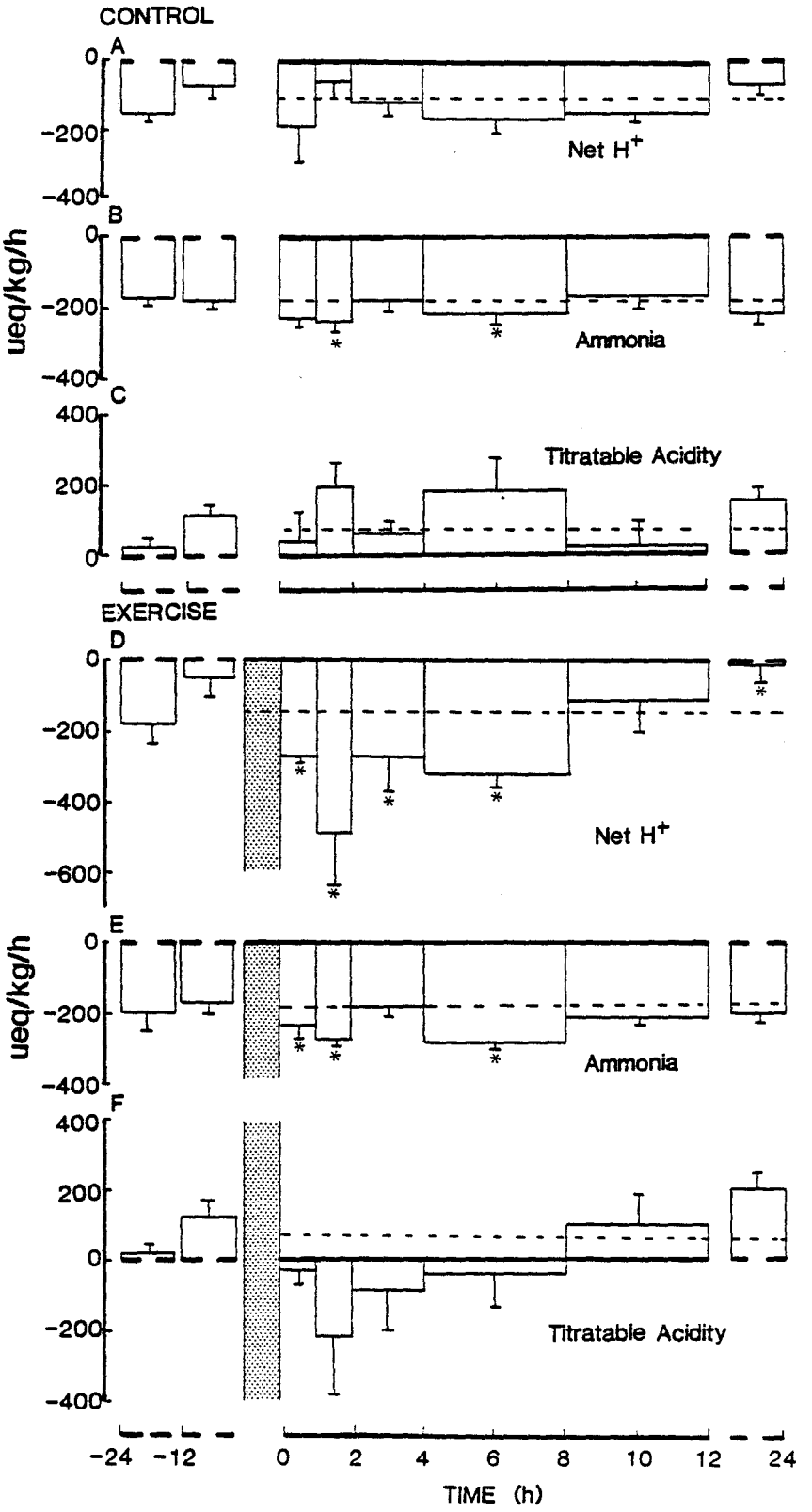

A
Exercise
8.0
7.8
7.6
7.4
7.2
7.0
pHe
Whole Body pHi
B
Control
8.0
7.8
7.6
7.4
7.2
pHe
Whole Body pHi
R
0
2
4
6
8
10
12
24
TIME (h)

H^+ Exchange with the Environment

During the two 12h periods prior to activity, net H^+ flux averaged about -160 ueq/kg/h in both the control and exercise group (Fig. 5.6). This did not change appreciably in the control group during the experimental period. However, in the exercise group, net H^+ excretion to the water increased 3-fold (Fig. 5.6D). Associated with this elevation in net H^+ excretion was a significant change in the titratable acidity uptake, from a positive (+70 ueq/kg/h) to a highly negative (-200 ueq/kg/h) value (Fig. 5.6F). Ammonia excretion also increased (Fig. 5.6E) but the change was small relative to the change in titratable acidity. The significance of the increased ammonia excretion is questionable for similar increases were also observed in the control group (Fig. 5.6B) implying the cause was other than activity-related.

Net H^+ flux returned to control values by 8-12h into recovery, coincident with a change of similar magnitude in the titratable acidity uptake. During the final 12h of recovery, net H^+ excretion declined significantly to near zero (-7.8 ± 42.6 ueq/kg/h, n=8; Fig. 5.6D), representing a period of relative H^+ uptake (or basic equivalent excretion) with respect to the pre-activity periods. A similar pattern was not evident in the control group (Fig. 5.6A), suggesting that this decline did not represent a 're-setting' of basal excretion, but rather was a true reversal of H^+ excretion. Once again this change in net H^+ excretion was associated with a change of similar magnitude in the titratable acidity flux (Fig. 5.6F).

Figure 5.6. Net H^+, ammonia and titratable acidity fluxes in control (A,B,C) and exercised (D,E,F) starry flounder, respectively. -24h, -12h indicate two 12 hour flux periods prior to the experimental period. Means $\pm$ 1 SEM, n=8 for both groups. Dashed line indicates mean of these two periods; vertical bar indicates period of activity, 0h is immediately after activity, i.e- the beginning of the experimental period. * indicates a significant difference ($p<0.05$) from the mean of the respective control periods.


CONTROL
A
0
-200
-400
Net H+
B
0
-200
-400
Ammonia
ueq/kg/h
C
400
200
0
Titratable Acidity
EXERCISE
D
0
-200
-400
-600
Net H+
E
0
-200
-400
Ammonia
ueq/kg/h
F
400
200
0
-200
-400
Titratable Acidity
-24
-12
0
2
4
6
8
10
12
24
TIME (h)

DISCUSSION

Blood Acid-Base Changes

The nature of the arterial blood acid-base disturbance (Fig. 5.1) after enforced activity in the starry flounder was qualitatively similar to those observed in other teleosts, including trout (eg. Fig. 3.1, Chapter III), with contributions from both respiratory and metabolic components (see Wood and Perry, 1985). However, in most other fish species, the peak in blood metabolic acid load (ΔH^+m) was coincident with the peak in P_{CO_2} (respiratory acid), which occurred immediately after cessation of exercise (eg. Fig. 3.7, Chapter III; also Wood and Perry, 1985). In the starry flounder however, the blood respiratory acid load was nearly cleared by the time the blood metabolic acid load had peaked (at 1h into recovery, Fig. 5.2). These patterns were very similar to those measured in venous blood of exercised starry flounder by Wood et al. (1977). Thus, there appears to be a temporal separation of respiratory and metabolic acid loading to the blood in flounder which will help attenuate the overall extent of the acidosis. The reason for this separation is not clear, though it may be related to a differential perfusion of the aerobic versus glycolytic tissue mass.

Blood Lactate and Metabolic Acid Loads

The pattern of lactate and H^+ appearance in the blood after severe exercise seems to be species dependent. In starry flounder, the rise in blood [lactate] after exhaustive exercise was very small, rarely attaining levels greater than 2 mmol/l (Fig. 5.1D). This minimal appearance of lactate in the blood appears to be

characteristic of relatively inactive, benthic and demersal species for it has also been observed in plaice (Pleuronectes platessa) (Dando, 1969; Wardle, 1978), various European flounder (Duthie, 1982), flathead sole (Turner, Wood and Hōbe, 1983), sea raven (Milligan and Farrell, 1986, see Appendix I) and Atlantic skates (C.M. Wood and M. Graham, unpublished observations cited in Wood and Perry, 1985). By way of contrast, after exercise in active pelagic species (eg. trout, Fig. 3.1D, Chapter III; dogfish, tuna; see Wood and Perry, 1985) whole blood [lactate] often attained levels as high as 15-25 mmol/l, 20-30 fold resting levels. In flounder, the post-exercise metabolic acid load appeared in excess of the lactate load (Fig. 5.3). The two did not come into equilibrium until 24h into recovery, at which time they had returned to resting levels. A similar phenomenon has been observed in the other relatively inactive species, but exactly the opposite pattern (ie. lactate load well in excess of metabolic acid load) appears to be characteristic of the active pelagic species (eg. trout, Fig. 3.7, Chapter III; dogfish, tuna, Wood and Perry, 1985).

In the starry flounder, it is possible that differential uptake of lactate in excess of H^+ from the blood could contribute to this apparent differential 'appearance'. However in the flathead sole, the rates of lactate and H^+ removal from the blood were equal after lactic acid infusion (Turner, Wood and Hōbe, 1983). The low urine flow of marine flatfish argues against the kidney as a site of significant excretion (McDonald, Walker, Wilkes and Wood, 1982). Lactate excretion at the gills was not measured in the starry flounder, but in the rainbow trout, it amounted to less than 1% of

H^+ excretion (Table 3.5, Chapter III). On balance, the most plausible hypothesis is that the phenomenon reflects differential lactate and H^+ release from the white muscle mass, though the controlling factors remain obscure. H^+ movement may be constrained by the 'equilibrium limitations' described by Holeton and Heisler (1983), including factors such as pH gradient and the extracellular buffer capacity. Lactate release may be carrier mediated (Hochachka and Mommsen, 1983). In plaice, Wardle (1978) has suggested that a lactate "non-release" mechanism is operative at the level of the muscle which may be under the influence of circulating catecholamines. However, a similar mechanism does not appear to operate in starry flounder, as Wood and Milligan (1986) were unable to demonstrate any adrenergic effect on lactate release from muscle after exercise.

Whole Body pHi

At rest, mean whole body pHi in flounder (7.5-7.6) was somewhat higher than the 7.2-7.3 observed for rainbow trout (Fig. 3.5, Chapter III; also Hobe et al. , 1984) and channel catfish (Cameron and Kormanik, 1982). This difference probably reflected the higher pHi of white muscle in the starry flounder (Fig. 6.3, Chapter VI), for this compartment constitutes the bulk of the body mass. After exercise, the drop in whole body pHi was very similar to that observed in white muscle (Fig. 6.5, Chapter VI). This intracellular acid-base disturbance was corrected much more rapidly than the extracellular disturbance, with the intracellular compartment showing an alkalosis at 8h post-exercise.

The response of flounder whole body pHi differed markedly from that of the rainbow trout. In the latter, the depression in whole body pHi after exercise was greater, probably reflecting a difference in intensity of exercise between the two species. Furthermore, in trout, whole body pHi was slower to recover, requiring up to 12h for complete recovery, and did not exhibit an alkalosis. Possible reasons for these differences are discussed in Chpater VIII.

Fluid Volume Distribution

Resting ECFV values determined with ^{3}H-mannitol in starry flounder in both the control and exercise groups were similar to values reported for other teleosts (rainbow trout, Fig. 3.4, Chapter III; also Milligan and Wood, 1982; channel catfish, Cameron, 1980). The ECFV estimates for flounder in the present study (256 ml/kg), were considerably higher than those measured with ^{3}H-inulin (164 ml/kg) in southern flounder (Paralichthys lethostigma) by Hickman (1972); however inulin is known to give lower estimates than mannitol (Cameron, 1980). Interestingly, the artifactual elevation of ECFV due to marker penetration of the tissues starting 12h post-infusion occurred at the same rate in Hickman's study as in the present experiments (Table 5.2).

Enforced activity resulted in a contraction of the ECFV and since total body water did not change, a reciprocal expansion of the ICFV occurred (Fig. 5.4). The shift of water from the whole body extracellular to intracellular compartment can be attributed almost exclusively to the fluid shift that occurred in the white muscle (Fig. 6.5, Chapter VI). As with trout, this re-distribution of muscle fluid

was no doubt a consequence of the breakdown of glycogen into lactate and increase in other osmolytes (eg. inorganic phosphate), and the consequent rise in intracellular osmotic pressure. In turn, the reduction in ECFV resulted in a pronounced hemoconcentration as indicated by the increase in hematocrit, [hemoglobin] and plasma [protein].

Mechanism of H^+ Excretion

At rest, flounder exhibited a net H^+ flux to the environment, in contrast to trout, in which net H^+ flux was virtually zero at rest. This difference between trout and flounder may reflect differences in feeding histories of wild caught flounder *versus* hatchery raised trout, as diet has been shown to influence acid excretion (Hills, 1973).

After exercise starry flounder exhibited a large increase in acidic equivalent excretion to the environmental water with little change in ammonia excretion (Fig. 5.6). The major route of acidic equivalent and ammonia excretion in the flounder is undoubtedly the gills, as in seawater the teleost kidney is relatively insignificant in acid-base regulation due to its low volume output (McDonald *et al.*, 1982).

Na^+ *versus* acidic equivalent and Cl^- *versus* basic equivalent exchanges are thought to be present in sea water fish (Evans, 1975), but their study has been hampered by the very high unidirectional fluxes of Na^+ and Cl^- *not linked* to acid-base exchange. Thus the mechanisms of acid excretion in marine fish are poorly understood. In dogfish and Conger eels (*Conger conger*)

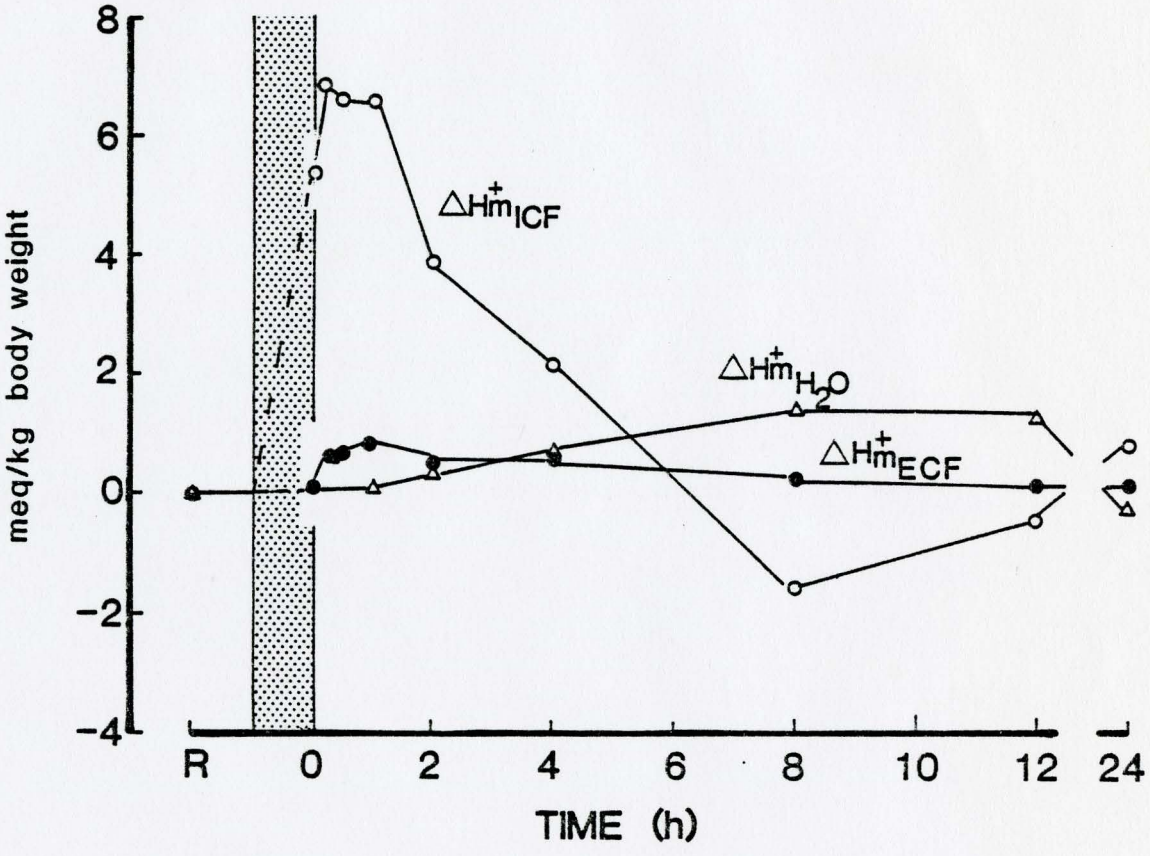
following exercise, it has been suggested that acid excretion was achieved mainly via Cl^-/HCO_3^- exchanges with Na^+/H^+, NH_4^+ exchange of lesser importance (Heisler, 1982). However, Evans (1982) has demonstrated that Na^+/H^+ exchange plays an important role in clearing a hypercapnia-induced acidosis in toadfish (Opsanus beta) and spiny dogfish. In lemon sole (Parophrys vetulus), acidic equivalent excretion in response to an infused mineral acid load was associated with an increased ammonia efflux (McDonald et al. , 1982). In the present study, the virtual lack of change in ammonia excretion after exercise would tend to argue against a prominent role for ammonia in acid excretion. By way of contrast, in the freshwater rainbow trout after exercise, acidic equivalent excretion was associated with greatly increased ammonia excretion (Fig. 3.6, Chapter III).

Distribution of H^+ Between ECF, ICF and the Environment

The data presented allow construction of a metabolic acid (ΔH^+m) budget in the 3 compartments: intracellular, extracellular and environment (Fig. 5.7). ΔH^+m to the ICF was calculated as described in Chapter III, except that the βvalue for flounder white muscle (-33.0 mmol/pH/kg = -47.8 mmol/pH/1 ICF) was used. H^+ loading to the environment was calculated assuming that pre-exercise excretion rates represented basal production.

Analysis of the ΔH^+m distribution (Fig. 5.7) revealed that up to 4h post-exercise, the bulk of the H^+ load remained intracellular. Approximately 20% of the total ΔH^+m load passed through the ECF and subsequently appeared in the environmental water.

Figure 5.7. Distribution of metabolic acid load between the intracellular compartment ($\Delta H^{+}m_{ICF}$, O), extracellular ($\Delta H^{+}m_{ECF}$, ●) compartment and environmental water ($\Delta H^{+}m_{H_2O}$, Δ) following exhaustive exercise in starry flounder. See text for details of calculation. R indicates rest value; bar indicates 10 minutes of activity; 0h is immediately after activity.


8
6
4
2
0
−2
−4
meq/kg body weight
ΔHm^+_{ICF}
$\Delta Hm^+_{H_2O}$
ΔHm^+_{ECF}
R
0
2
4
6
8
10
12
24
TIME (h)

The intracellular H^+ load was cleared rapidly, so that by 8h into recovery, the ICF experienced an alkalosis. The ΔH^+m deficit in the ICF was equivalent to the quantity of H^+ excreted into the water. The alkalosis in the whole body ICF at 8h probably reflected an alkalosis in both the white muscle mass and the liver (Fig. 6.3, Chapter VI). By 24h post-exercise, the H^+ excreted to the water was taken back, thereby aiding correction of the intracellular alkalosis. Thus it would appear that after strenuous exercise, the starry flounder temporarily stores H^+ in the environmental water to hasten restoration of intacellular acid-base status, perhaps as a means of aiding metabolic recovery.

Total metabolic acid production after exhaustive exercise in flounder (about 8 meq/kg, Fig. 5.7) was only about half that of rainbow trout (about 15 meq/kg, Fig.3.8, Chapter III). Furthermore, the distribution of H^+ between the ICF, ECF and environmental water was very different between species. In contrast to flounder, only a small portion (about 6%) of the total H^+ load in trout appeared in the water, and this transfer appeared to expedite recovery of the extracellular compartment. In trout, clearance of H^+ from the intracellular compartment occurred largely by aerobic metabolism rather than by export (Chapter IV). Possible reasons for these species differences are addressed in Chapter VIII.

CHAPTER VI

THE EFFECTS OF EXHAUSTIVE EXERCISE ON TISSUE ACID-BASE AND METABOLITE STATUS IN THE STARRY FLOUNDER

INTRODUCTION

The classical picture of the fate of muscle lactate (and presumably H^+) after exercise is the Cori cycle: lactate leaves the muscle, and is transported to the liver *via* the blood where it is converted to glucose. The glucose then enters the blood to be taken up by the muscle and utilized to replenish glycogen stores (Newsholme and Leech, 1983). The results of Chapters III and IV on the rainbow trout have already offered some challenge to this picture, with evidence presented for lactate oxidation and glyconeogenesis by the white muscle *in situ* . The extremely low appearance of lactate in the blood of the starry flounder (Chapter V) suggests that this species may represent an even more serious exception. Wardle (1978) and Batty and Wardle (1979) have earlier suggested that lactate (and presumably H^+) are not transported out of the muscle, but rather are utilized as substrates for *in situ* glyconeogenesis in the plaice.

In contrast to the rainbow trout, the flounder also showed a much more rapid recovery of whole body pHi, a relatively (though not absolutely) greater H^+ flux to the environment and an excess of H^+ over La^- appearance in the bloodstream. This suggests that the pattern of intracellular, as well as extracellular, acid-base

regulation may differ greatly between the two species.

The aim of the present study was to investigate the fates of H^+ and lactate after exercise in starry flounder through an examination of intracellular acid-base and metabolite changes in the white muscle mass, liver, and blood. As for trout, the effect of the exercise-induced extracellular acidosis on red cell pHi was also assessed.

METHODS

Experimental Animals

Adult starry flounder (Platichthys stellatus), weighing 150-560 g, of both sexes were collected by otter trawl from East Sound, Orcas Island and Birch Bay, Washington from October through December, 1982. Fish were held as described in Chapter V.

Following catheterization of the caudal artery, fish were placed in 15 l plastic tubs fitted with black plastic mesh and supplied with fresh flowing seawater at 11 $\pm$ 1 ^{o}C and allowed to recover at least 72 h prior to experimentation.

Experimental Protocol

In these experiments, fish were sampled only once (terminally) rather than sequentially, for analysis of blood, muscle, and liver acid-base and metabolite status. The experimental protocol was similar to that employed in the trout study of Chapter IV, except that fewer sampling times were employed: rest, immediately after exercise (time 0), and 0.5, 2, 4 and 12 h post-exercise.

Arterial blood was analyzed for pH, P_{O_2}, total CO_2 (in both whole blood and plasma), hematocrit, [hemoglobin], [glucose], [lactate], and [pyruvate], and red cell pHi was measured directly. Plasma was analyzed for ^{3}H- and ^{14}C- radioactivity, [protein], and levels of Na^+, Cl^-, K^+, Ca^{2+} and ammonia. Muscle and liver were analyzed for ^{3}H- and ^{14}C- radioactivity, [lactate], [pyruvate], glycogen and total water contents.

Analytical Techniques, Calculations and Statistical Analyses

All measurements and calculations were carried out as described in Chapters II, III and IV, with the following exceptions. Arterial P_{O_2} was measured with a Radiometer O_2 electrode (type E5036) maintained at the experimental temperature. Plasma concentrations of Na^+ (20 ul plasma) and K^+ (100 ul plasma) were determined after dilution (1:500 and 1:30, respectively) on an Eel flame photometer. Chloride was measured on 20 ul plasma samples using a Radiometer CMT-10 chloride titrator. Plasma [Ca^{2+}] was determined colorimetrically on 25 ul aliquots using the cresolpthalein complexone method (Connerty and Briggs, 196). Plasma [ammonia] was measured on 100 ul plasma extracted 4:1 with ice-cold 12.5% trichloracetic acid by a micromodification of the salicylate hypochlorite method of Verdouw, van Echteld and Dekkers (1978). For analysis of tissue lactate and pyruvate, tissue samples (100-250 mg) were homogenized on ice in 1 ml of 8% ice-cold perchloric acid with a glass homogenizer for 5 min. Muscle and liver glycogen were measured on tissues of similar weights using the anthrone method described by

Hassid and Abraham (1959).

As for trout, ^{3}H-mannitol proved to be an unsuitable marker for liver ECFV in the flounder. After 12 h of equilibration liver ECFV estimates were often as high as 600 ml/kg, when total tissue water was only 725 ml/kg. Therefore, it was obvious that mannitol-derived ECFV estimates were erroneous and could not be used. In a separate experiment on 8 resting fish, liver ECFV was estimated by carboxy-^{14}C-inulin and methoxy-^{14}-inulin distribution which yielded values not significantly different from one another and much lower than mannitol values. The mean of the values, 169.5 $\pm$ 38.6 ml/kg (n=8) was used in place of the mannitol derived ECFV estimates. In this same experiment, white muscle ECFV estimates based on inulin were not appreciably different from those derived from mannitol distribution. Thus mannitol derived ECFV estimates for white muscle were assumed correct and used throughout.

RESULTS

Tissue Buffer Capacities

The buffer capacity of muscle was about 1.6 times that of liver (Table 6.1). While the liver values were comparable to those measured in trout, white muscle β was only about 65% of the trout figure (Table 4.1, Chapter IV).

Extracellular Acid-Base, Metabolite and Electrolyte Status

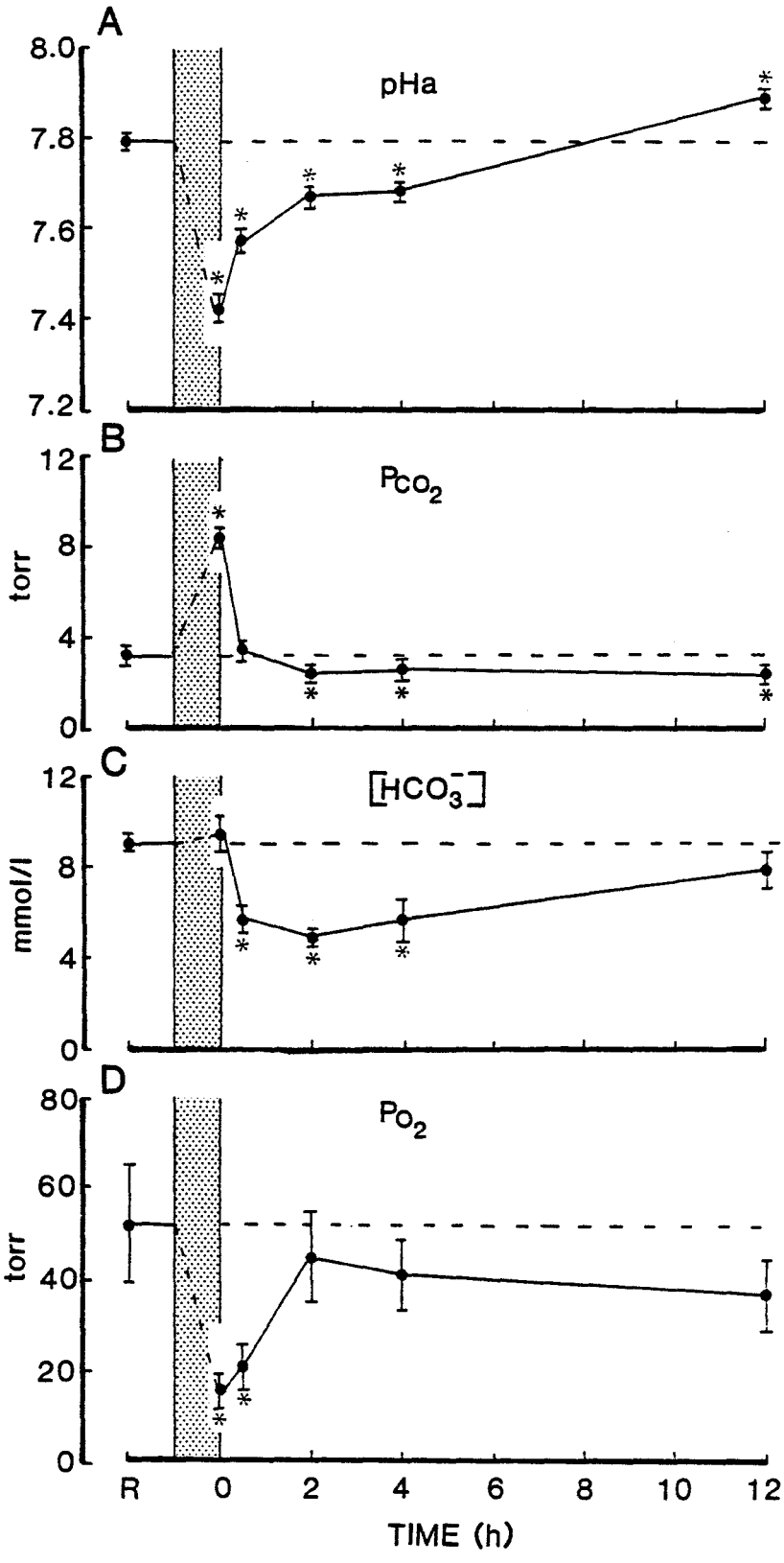
Changes in extracellular (i.e. plasma) pH, P_{CO_2} and $[HCO_3^-]$ are shown in Fig. 6.1A,B,C, and were qualitatively similar to those described for the serially sampled flounder of

Table 6. 1. Buffer capacities (β) of liver and white muscle in the starry flounder.

	β mmol/pH/kg wet weight	β mmol/pH/l ICF
White Muscle	-33.00 ± 4.56 (6)	-47.76 ± 6.83 (6)
Liver	-20.89 ± 1.68 (6)	-37.46 ± 3.16 (6)

Means ± 1 SEM (n).

Figure 6.1. Changes in (A) arterial plasma pHa, (B) plasma Pa_{CO_2} (C) plasma [HCO_3^-], and (D) whole blood oxygen tension (Pa_{O_2}) in terminally sampled flounder. Means $\pm$ 1 SEM. R, n=5; 0h, n=6; 0.5h, n=6; 2h, n=6; 4h, n=4; 12h, n=6. R indicates rest value; vertical bar indicates 10 minute period of activity; 0h is immediately after activity. Dashed line indicates mean of rest value. * indicates a significant difference ($p<0.05$) from corresponding rest value.

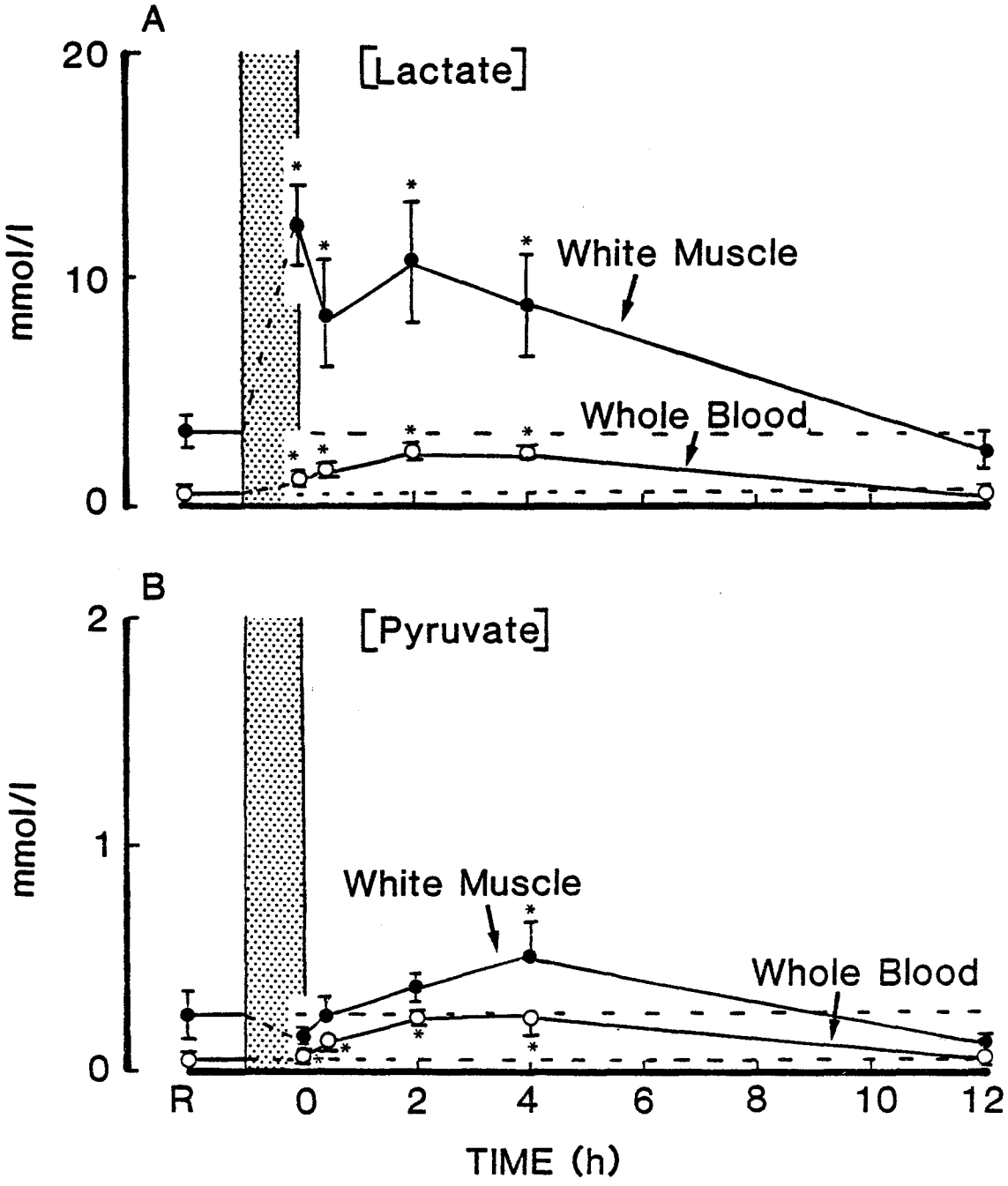

A
pHa
8.0
7.8
7.6
7.4
7.2
B
P_{CO_2}
torr
12
8
4
0
C
$[HCO_3^-]$
mmol/l
12
8
4
0
D
P_{O_2}
torr
80
60
40
20
0
R
0
2
4
6
8
10
12
TIME (h)

Chapter V (Fig. 5.1). However in the present study, the respiratory component to the post-exercise acidosis was cleared much more rapidly, so that by 0.5h, P_{CO_2} was not different from rest values. P_{CO_2} continued to decline however, so that by 2h, it was significantly lower than rest levels. This reduction in P_{CO_2} persisted resulting in a significant increase in pH at 12h, which was not observed in the previous study. The reason for this difference is not known, though it is interesting that a similar difference in post-exercise P_{CO_2} dynamics was seen between serially and terminally sampled trout (Chapters III, IV). Whole blood P_{O_2}, which was not measured in the previous study, was about 50 torr, typical of truly resting starry flounder (Wood et al. ,1979a). Pa_{O_2} fell by about 70% immediately after exercise, remained low at 0.5h, but by 2h, Pa_{O_2} had fully recovered and remained unchanged throughout the remainder of the recovery period (Fig. 6.1D).

Hematological changes were qualitatively similar to those described in the previous flounder study (Fig. 5.3, Chapter V) and are not shown.

Whole blood lactate levels were low at rest (0.1-0.2 mmol/l; Fig. 6.2A) and increased after exercise. However, the increase was quite small, with peak levels at 2-4h only 3-4 fold rest values. At all times, ΔH^+m in blood was much greater than ΔLa^- (Fig. 6.7B). This pattern is similar to that described in Chapter V (Fig. 5.2), though the peak blood lactate values attained were slightly lower in the present study (0.8 versus 1.3 mmol/l). Whole blood [pyruvate] followed a similar pattern (Fig. 6.2B) to that of

Figure 6.2. Concentrations of (A) lactate and (B) pyruvate in white muscle and whole blood prior to and following exhaustive exercise in starry flounder. Means $\pm$ 1 SEM. R, n=5; 0h, n=6; 0.5h, n=6; 2h, n=6; 4h, n=4; 12h, n=6. R indicates rest value; vertical bar indicates 10 minute period of activity; 0h is immediately after activity. Dashed line indicates mean of the rest value. * indicates a significant difference ($p<0.05$) from corresponding rest value.


A
20
10
0
mmol/l
[Lactate]
White Muscle
Whole Blood
B
2
1
0
mmol/l
[Pyruvate]
White Muscle
Whole Blood
R
0
2
4
6
8
10
12
TIME (h)

[lactate], though the absolute levels and elevations were much smaller. Whole blood [glucose] increased after exercise, reaching peak levels at 0.5-2 h, 1.6 times those at rest and returning to pre-exercise values by 4h (Table 6.2).

Plasma electrolytes were elevated immediately after exercise though by varying degrees (Table 6.3). Plasma levels of Na^+, Cl^- and Ca^{2+} rose 13-15%, similar to the observed increase in plasma [protein] (eg. Fig. 5.3D, Chapter V) suggesting these ion levels increased as a consequence of a reduction in plasma volume. The increase was short-lived though, as by 0.5h into recovery, plasma $[Ca^{2+}]$, $[Cl^-]$ and $[Na^+]$ had returned to rest levels.
Plasma $[K^+]$ responded differently (Table 6.3), increasing to a greater extent (by about 60%) and remaining elevated for a longer period (up to 4h), returning to rest levels by 12h. Plasma [ammonia], approximately 0.17 mmol/l at rest, was also elevated as a consequence of activity, peaking immediately post-exercise at about 3 times rest levels (Table 6.3).

Tissue Intracellular Acid-Base and Metabolite Status

At rest, white muscle lactate levels (3.23 $\pm$ 0.55 mmol/l, n=5; Fig. 6.2A) were only about 25% of the values seen in trout (Fig. 4.3A, Chapter IV) and pHi was correspondingly higher, by about 0.25 units, with a mean of 7.56 $\pm$ 0.05 (n=5; Fig. 6.3). This value was about 0.23 units lower than extracellular pH. Ten minutes of enforced activity drove muscle pHi to a low of 7.27 $\pm$ 0.02 (n=6, Fig. 6.3) immediately post-activity. By 4h, muscle pHi had returned to a level not significantly different from rest, and at 12h was slightly, though

Table 6.2. Whole blood levels of glucose and white muscle glycogen content prior to and following activity in the starry founder.

	Time After Exercise (hours)					
	REST	0	0.5	2	4	12
Glucose						
	1.2 ± 0.1 (5)	1.8 ± 0.4 (6)	1.8 ± 0.2 (6) *	1.9 ± 0.1 (6) *	1.3 ± 0.2 (4)	1.4 ± 0.1 (6)
Glycogen+						
	38.5 ± 4.9 (5)	19.3 ± 2.5 (6) *	22.7 ± 3.1 (6) *	35.6 ± 4.9 (6)	11.6 ± 2.9 (4) *	32.6 ± 7.5 (4)

Means ± 1 SEM (n). All values in mmol/l.

* indicates a significant difference ($p<0.05$) from corresponding rest value.

+ glycogen content expressed as glucose units in mmol/l.

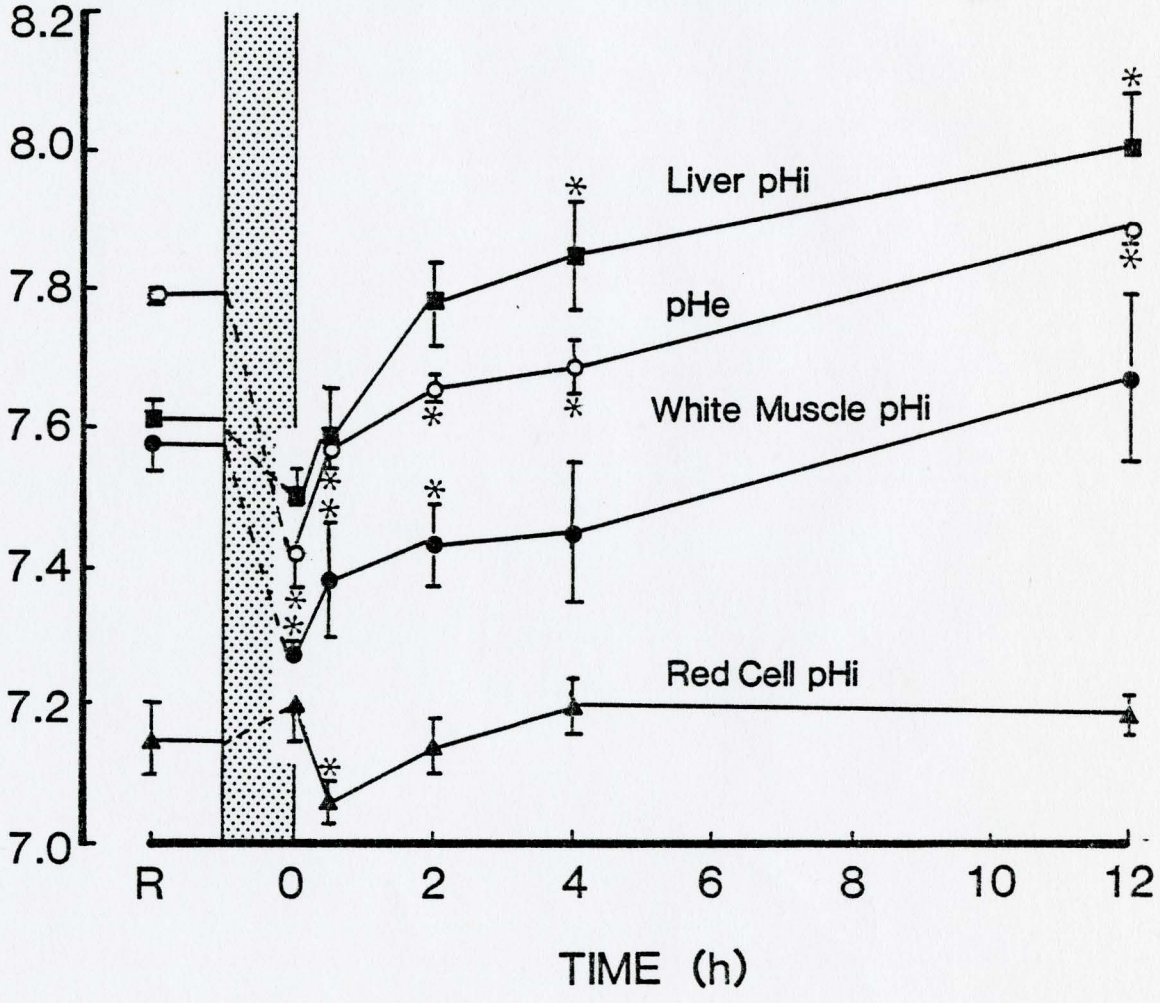
Table 6.3. The effect of exhaustive activity on plasma levels of Na^+, Cl^-, K^+, Ca^{2+} and ammonia in starry flounder.

	Time After Exercise (hours)					
	REST	0	0.5	2	4	12
Na^+ meq/l						
	167.8 ± 4.1 (5)	189.0 ± 8.9 (6) *	163.3 ± 5.4 (6)	169.4 ± 3.3 (6)	173.5 ± 8.3 (4)	160.8 ± 4.1 (6)
Cl^- meq/l						
	151.4 ± 5.1 (5)	171.3 ± 8.4 (6) *	146.2 ± 4.9 (6)	154.4 ± 4.0 (6)	158.8 ± 13.7 (4)	146.3 ± 3.7 (6)
K^+ meq/l						
	2.7 ± 0.1 (5)	4.3 ± 0.3 (6) *	2.8 ± 0.2 (6)	3.1 ± 0.1 (6) *	3.3 ± 0.3 (4) *	2.9 ± 0.1 (6)
Ca^{2+} meq/l						
	5.7 ± 0.7 (5)	6.6 ± 0.3 (6) *	5.7 ± 0.2 (6)	6.1 ± 0.4 (6)	6.4 ± 0.9 (4)	5.1 ± 0.5 (6)
Ammonia mmol/l						
	0.17 ± 0.1 (5)	0.52 ± 0.1 (6) *	0.37 ± 0.1 (6) *	0.14 ± 0.03 (5)	0.13 ± 0.05 (4)	0.09 ± 0.04 (6)

Means ± 1 SEM (n).

* indicates a significant difference ($p<0.05$) from corresponding resting value.

Figure 6.3. White muscle pHi (●), liver pHi (■), arterial red cell pHi (▲) and pHe (0) prior to and following exhaustive exercise in starry flounder. Means ± 1 SEM. R, n=5; 0h, n=6; 0.5h, n=6; 2h, n=6; 4h, n=4; 12h, n=6. R indicates rest value; vertical bar indicates 10 minute period of activity; 0h is immediately after activity. * indicates a significant difference ($p<0.05$) from corresponding rest value.

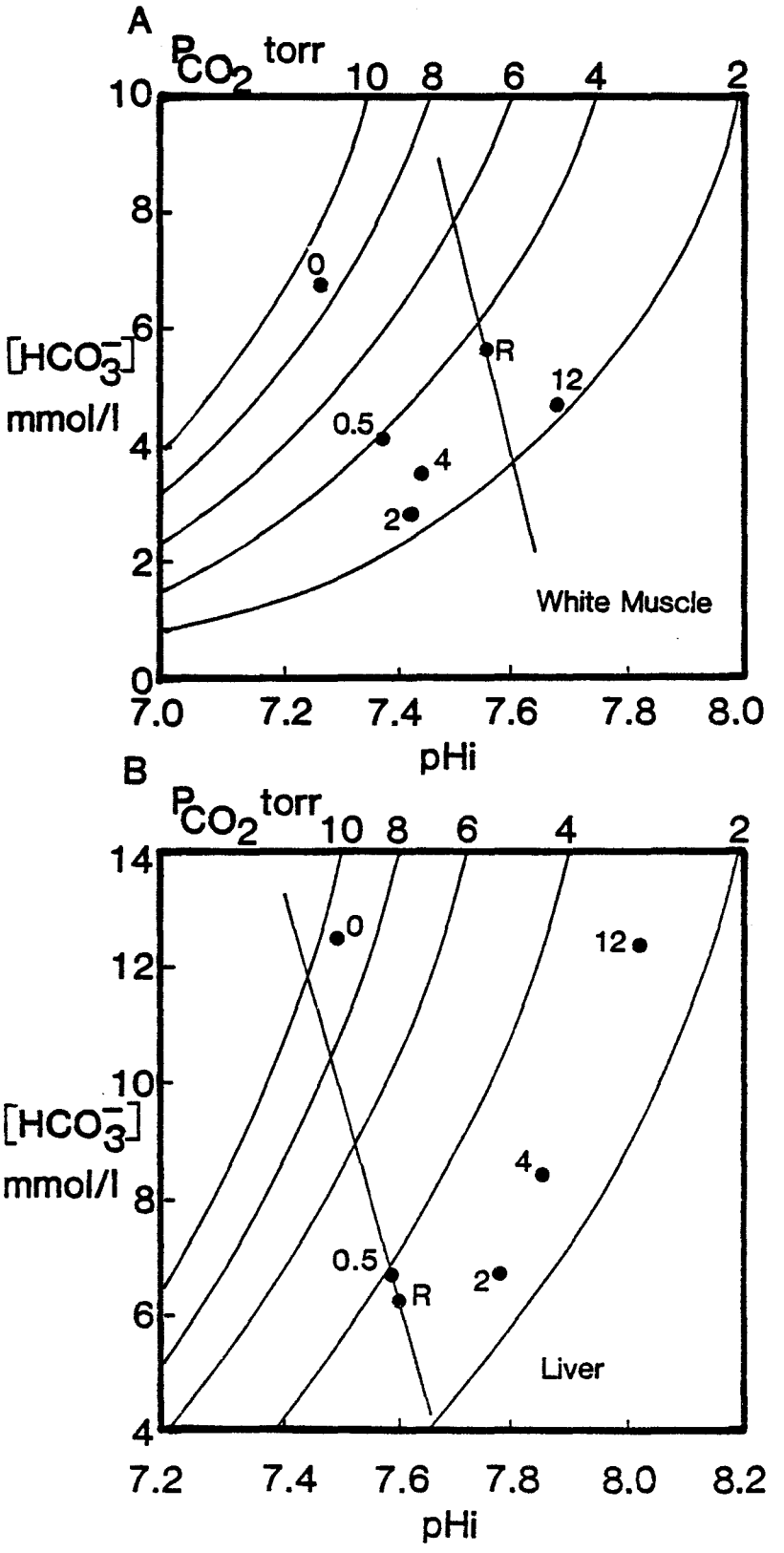

8.2
8.0
7.8
7.6
7.4
7.2
7.0
R
0
2
4
6
8
10
12
TIME (h)
Liver pHi
pHe
White Muscle pHi
Red Cell pHi

non-significantly, above the rest value. The immediate post-exercise change in pHe was greater than in pHi (0.37 versus 0.29 units) resulting in a reduction in the pHe-pHi gradient from 0.23 to about 0.15 pH units. These changes in white muscle pHi were all very similar to those seen earlier in whole body pHi for flounder (Fig. 5.5, Chapter V). A plot of the muscle data on a pH-[HCO_3^-] diagram (Fig. 6.4A) indicated that immediately after exercise the intracellular acidosis was of mixed respiratory and metabolic origin with the latter factor clearly predominating. Thereafter, the acidosis was completely metabolic.

Accompanying this metabolic acidosis was a 4-fold increase in muscle [lactate] (Fig. 6.2A) and approximately 50% reduction in glycogen content (Table 6.2). The disappearance of muscle glycogen is more than adequate to account for the increase in [lactate]. Muscle [lactate] remained elevated through 4h, returning to rest levels by 12h. Glycogen restoration tended to parallel lactate disappearance, though the data are difficult to interpret as there was a great deal of variation in muscle glycogen between fish, due no doubt to their being from a wild population. Muscle [pyruvate] followed an entirely different pattern from that of [lactate], showing a significant change only at 4h into recovery when levels doubled (Fig. 6.2B). By 12h, [pyruvate] had returned to rest levels. Peak muscle lactate and pyruvate levels in flounder were only about 25% of those seen after exercise in trout (Fig. 4.3B, Chapter IV).

Liver pHi at rest was slightly higher than that of muscle, averaging 7.60 $\pm$ 0.04 (n=5, Fig. 6.3). Immediately after cessation of

Figure 6.4. pHi-[HCO_3^-] diagrams of changes in intracellular acid-base status after 10 min of enforced activity in (A) white muscle and (B) liver in starry flounder. Straight lines plotted are the tissue in vitro non-bicarbonate buffer capacities from Table 6.1; isopleths are P_{CO_2}. Means are plotted, n as indicated in Fig. 6.3. R indicates rest value; 0h indicates immediately after activity; 0.5, 2, 4 and 12 are hours after activity.


A
P_{CO_2} torr
10 8 6 4 2
10
8
6
4
2
0
$[HCO_3^-]$
mmol/l
0
0.5
4
2
R
12
White Muscle
7.0 7.2 7.4 7.6 7.8 8.0
pHi
B
P_{CO_2} torr
10 8 6 4 2
14
12
10
8
6
4
$[HCO_3^-]$
mmol/l
0
12
4
0.5
R
2
Liver
7.2 7.4 7.6 7.8 8.0 8.2
pHi

activity, liver pHi tended to fall, though the change was not significant. A pH-[HCO_3^-] diagram analysis indicated that this tendency towards acidosis was due entirely to the increase in P_{CO_2} (Fig. 6.4B). By 2h, liver pHi began to increase, and by 4h, mean pHi, 7.86 ± 0.08 (n=4, Fig. 6.3), was significantly elevated. Metabolic base continued to accumulate (or conversely, metabolic acid was lost; Fig. 6.4B) so that by 12h, mean liver pHi was 8.02 ± 0.08 (n=6, Fig. 6.3), 0.42 units above the rest level. These changes in liver acid-base status were not accompanied by changes in metabolic status, for neither [lactate], [pyruvate], nor glycogen content changed, though again, the data were highly variable (Table 6.4).

By comparison with liver and white muscle, red cell pHi was quite low at rest, 7.16 ± 0.04 (n=5, Fig. 6.3). Despite the large drop in pHe and increase in P_{CO_2} immediately after activity, red cell pHi remained fairly constant, showing a significant decline only at 0.5h, recovering by 2h. At 12h, when pHe showed an alkalosis, pHi was unchanged from the rest value, further demonstrating the ability of the red cell to regulate pHi independently of changes in pHe.

Fluid Volume Distribution

Immediately following activity, white muscle exhibited a significant cell swelling, with ICFV increasing by 40-50 ml/kg (Fig. 6.5B). Since total tissue water did not vary at any time in the experiment (808.9 ± 2.6 ml/kg, n=40), the expansion of the intracellular space resulted in a contraction of the extracellular

Table 6.4. Liver levels of lactate, pyruvate and glycogen prior to and following activity in the starry flounder.

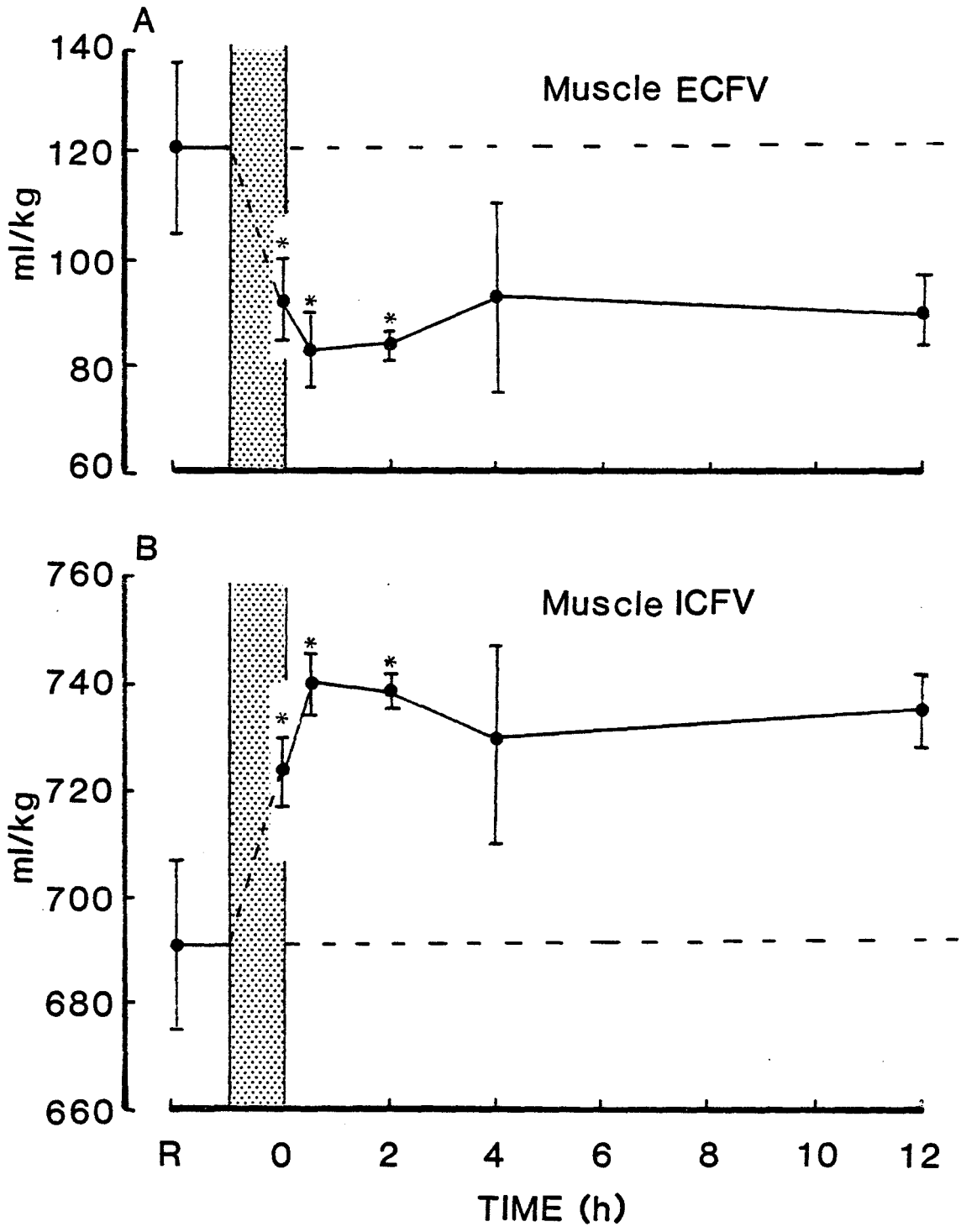
	Time After Exercise (hours)					
	REST	0	0.5	2	4	12
Lactate	1.49 ± 0.44 (5)	1.49 ± 0.23 (6)	1.71 ± 0.54 (6)	1.60 ± 0.38 (6)	1.20 ± 0.32 (4)	1.50 ± 0.28 (6)
Pyruvate	0.39 ± 0.14 (5)	0.19 ± 0.10 (6)	0.49 ± 0.34 (6)	0.18 ± 0.06 (6)	0.39 ± 0.17 (4)	0.38 ± 0.13 (6)
Glycogen+	291.3 ± 56.1 (5)	189.5 ± 60.2 (6)	128.0 ± 37.7 (6)	330.5 ± 70.3 (6)	179.6 ± 64.2 (4)	177.8 ± 18.0 (6)

Means ± 1 SEM (n). All values in mmol/l.

+ glycogen content expressed as glucose units in mmol/l.

There were no significant changes in the concentration of any metabolite after exercise.

Figure 6.5. (A) White muscle extracellular fluid volume (ECFV) and (B) intracellular fluid volume (ICFV) prior to and following 10 min of activity. Means $\pm$ 1 SEM. R, n=5; 0h, n=6; 0.5h, n=6; 2h, n=6; 4h, n=4; 12h, n=6. R indicates rest value; vertical bar indicates 10 minute period of activity; 0h is immediately after activity. Dashed line indicates mean of rest value. * indicates a significant difference ($p<0.05$) from corresponding rest value.


A
Muscle ECFV
140
120
100
80
60
ml/kg
B
Muscle ICFV
760
740
720
700
680
660
ml/kg
R
0
2
4
6
8
10
12
TIME (h)

space (Fig. 6.5A).

Neither liver (718.8 ± 8.9 ml/kg; n=40) nor total body (784.0 ± 2.9 ml/kg; n=40) water content changed as a consequence of exercise.

DISCUSSION

Extracellular Acid-Base, Metabolite and Electrolyte Status

The post-exercise changes in plasma pH, $[HCO_3^-]$ and P_{CO_2} (Fig. 6.1) were similar to those described previously (Fig. 5.1, Chapter V) and the same explanations probably apply. Whole blood P_{O_2}, which was not measured in the previous study, fell after exercise. A decline in Pa_{O_2} implies a diffusive limitation to gill O_2 transfer, perhaps as a consequence of a reduction in gill blood transit time due to an increase in cardiac output (Perry, Daxboeck and Dobson, 1985; Wood and Perry, 1985). The fact than Pa_{O_2} fell by approximately 70% and P_{CO_2} more than doubled would suggest that diffusive limitations existed.

Changes in whole blood [pyruvate] after exercise followed a pattern similar to that of blood [lactate] (Fig. 6.2A,B). The absolute changes in [pyruvate] were greater which resulted in a reduction in the blood lactate:pyruvate ratio from about 7.5 at rest to about 3.9 at 0h. A similar reduction in the blood lactate:pyruvate ratio was observed after exercise in the inactive, benthic flathead sole (Turner, Wood and Hōbe, 1983). By way of contrast, after exercise in more active fish, such as rainbow trout, there was a 4-5 fold increase in the lactate:pyruvate ratio (Chapter IV; also Turner, Wood and Clark, 1983). This species difference is not due to

differences in the pyruvate response, for the absolute increases were similar in flounder, sole and trout, but rather a difference in the lactate response. After exercise in trout peak blood lactate levels often reached 20 mmol/l (Fig. 4.3, Chapter IV), versus 0.5-2 mmol/l in flounder (Fig. 6.2) and sole. Thus it would appear that in flatfish movement of pyruvate from the muscle to blood is not subject to the same restrictions as for lactate movement.

The rather abrupt, short - lived rise in plasma [ammonia] observed in the present study (Table 6.3) is most likely a reflection of increased tissue ammoniagenesis. Similar effects have been seen in the rainbow trout and flathead sole (Turner, Wood and Clark, 1983; Turner, Wood and Hōbe, 1983). Fish white muscle is capable of ammonia production (Driedzic and Hochachka, 1976). Branchial clearance of this ammonia load probably explained the small increase in ammonia excretion to the water after exercise measured in Chapter V (Fig. 5.6) in starry flounder. Whole blood [glucose] (Table 6.2) increased presumably as a consequence of breakdown and mobilization of liver glycogen (Driedzic and Hochachka, 1975).

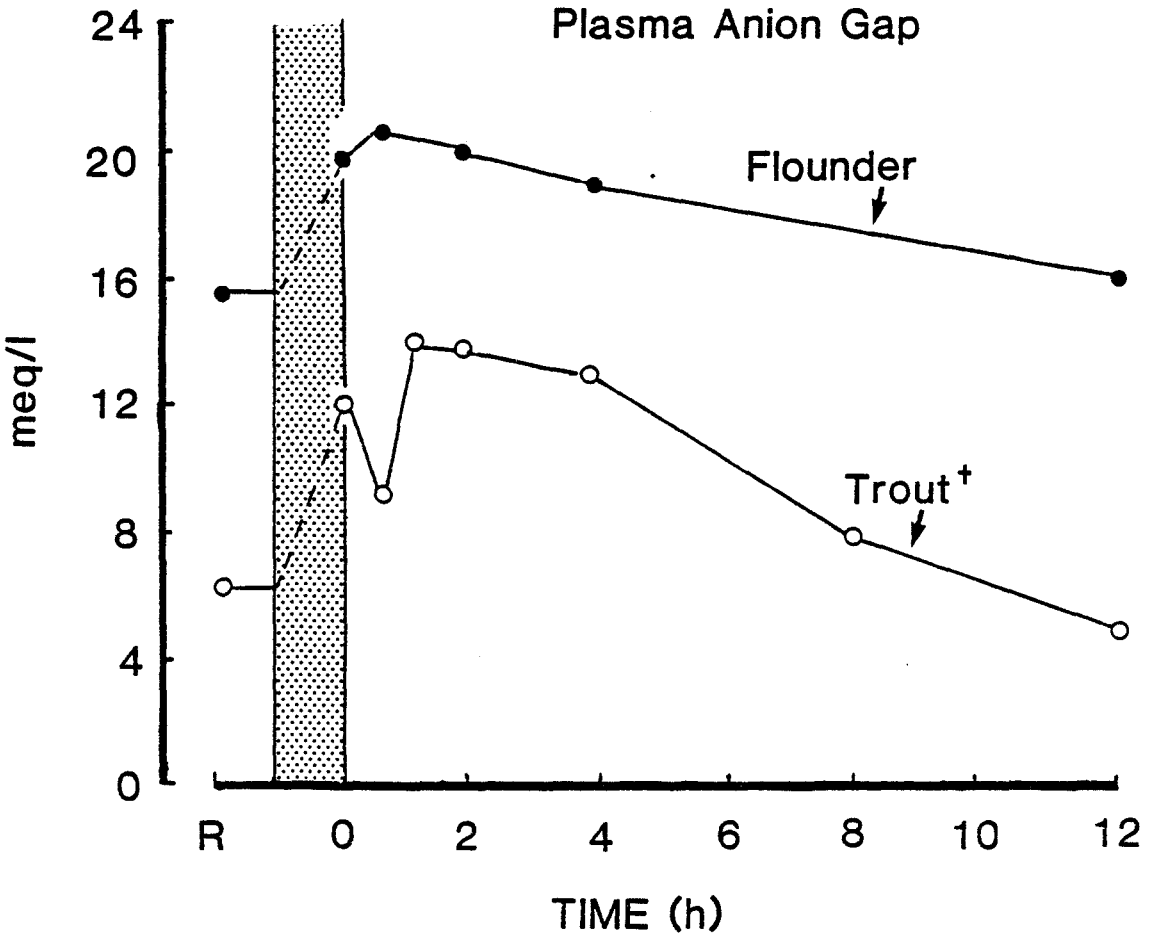
The post-exercise changes in plasma electrolytes (Table 6.3) were similar to those reported for flathead sole (Turner, Wood and Hōbe, 1983). The observation that the increases in plasma concentrations of Na^+, Cl^- and Ca^{2+} were nearly identical to the post-exercise elevation in plasma [protein] observed in the previous study (Fig. 5.3, Chapter V) indicated these ions increased as a consequence of the reduction in whole body extracellular fluid volume observed in Chapter V (Fig. 5.4). The 2-3 fold greater

increase in plasma K^+ over the other ions is commonly observed after bouts of glycolytic activity in vertebrates and has been attributed to a release of K^+ from the muscle intracellular compartment in response to an acidosis (Woodbury, 1974). In addition, release of K^+ bound to glycogen during glyconeogenesis may have contributed to the rise in plasma K^+ (Hultman and Sahlin, 1981).

Following exhaustive exercise in rainbow trout, Turner, Wood and Clark (1983) reported short-lived increases in plasma Na^+ and Cl^- levels and a persistent elevation in plasma $[K^+]$, qualitatively similar to that observed in flounder in the present study. However, plasma $[Cl^-]$ later declined below rest levels, in reciprocity to the lactate rise.

The plasma anion gap (calculated as $([Na^+] + [K^+] + [Ca^{2+}]) - ([Cl^-] + [HCO_3^-] + [\text{lactate}] + [\text{pyruvate}])$ in meq/l) in flounder at rest was about 16 meq/l and increased by about 30% after exercise (Fig. 6.6). Comparable calculations were done for trout using data from the study of Turner, Wood and Clark (1983), which followed an exercise regime identical to that of the trout studies in this thesis. The plasma anion gap in trout at rest was less than in flounder, about 6 meq/l, and increased nearly 100% after exercise (Fig. 6.6). The anion gap at rest is thought to be due to the unmeasured negative charges on plasma proteins. After exercise, H^+ entering the blood titrates these negative charges, thus tending to <u>decrease</u> the anion gap (Woodbury, 1974). However, after exercise, the anion gap <u>increased.</u> A reduction in ECFV would tend to increase the anion gap, but only by 10-15% in flounder (Chapter V)

Figure 6.6. Plasma anion gap prior to and following exercise in starry flounder and rainbow trout. R indicates rest, vertical bar indicates period of exercise (6 min for trout, 10 min for flounder), 0 is immediately after exercise. See text for details of calculation. + calculated from the data of Turner, Wood and Clark, (1983).


Plasma Anion Gap
Flounder
Trout+
meq/l
24
20
16
12
8
4
0
R
0
2
4
6
8
10
12
TIME (h)

and 25-30% in trout (Chapter III). The observed increases were much greater, suggesting unmeasured anions (perhaps keto acids) had entered the blood.

Resting Tissue Intracellular Acid-Base Status

Red cell pHi had the lowest resting pHi of the tissues examined, though was in accordance with pHi values reported for other fish, such as the rainbow trout (Fig. 4.4, Chapter IV; also Primmett et al. , 1986).

Flounder white muscle pHi (Fig. 6.3) was 0.2-0.3 units greater than values reported for white muscle from more active pelagic species, such as rainbow trout (Fig. 4.4, Chapter IV; also Hõbe et al. , 1984), dogfish (Heisler et al. , 1976) and eel (Walsh and Moon, 1982). However it was not dissimilar to values reported for the relatively inactive, demersal catfish (Cameron and Kormanik, 1982) and sea raven (Milligan and Farrell, 1986; see Appendix I). These inter-species differences in resting muscle pHi may be related to muscle lactate levels, for the active species with the lower pHi generally tended to have higher muscle lactate levels (8-15 mmol/kg versus 1-3 mmol/kg).

On the other hand, flounder liver pHi (Fig. 6.3) was very similar to values reported for eels (Walsh and Moon, 1982; 1983) and rainbow trout (Fig. 4.4, Chapter IV). In these species, liver pHi was 0.1-0.3 pH units greater than that of muscle. From these limited observations in fish and those in mammals (see Roos and Boron, 1981), it appears that liver generally has a higher resting pHi than muscle, which may reflect differences in tissue metabolic state and/or resting

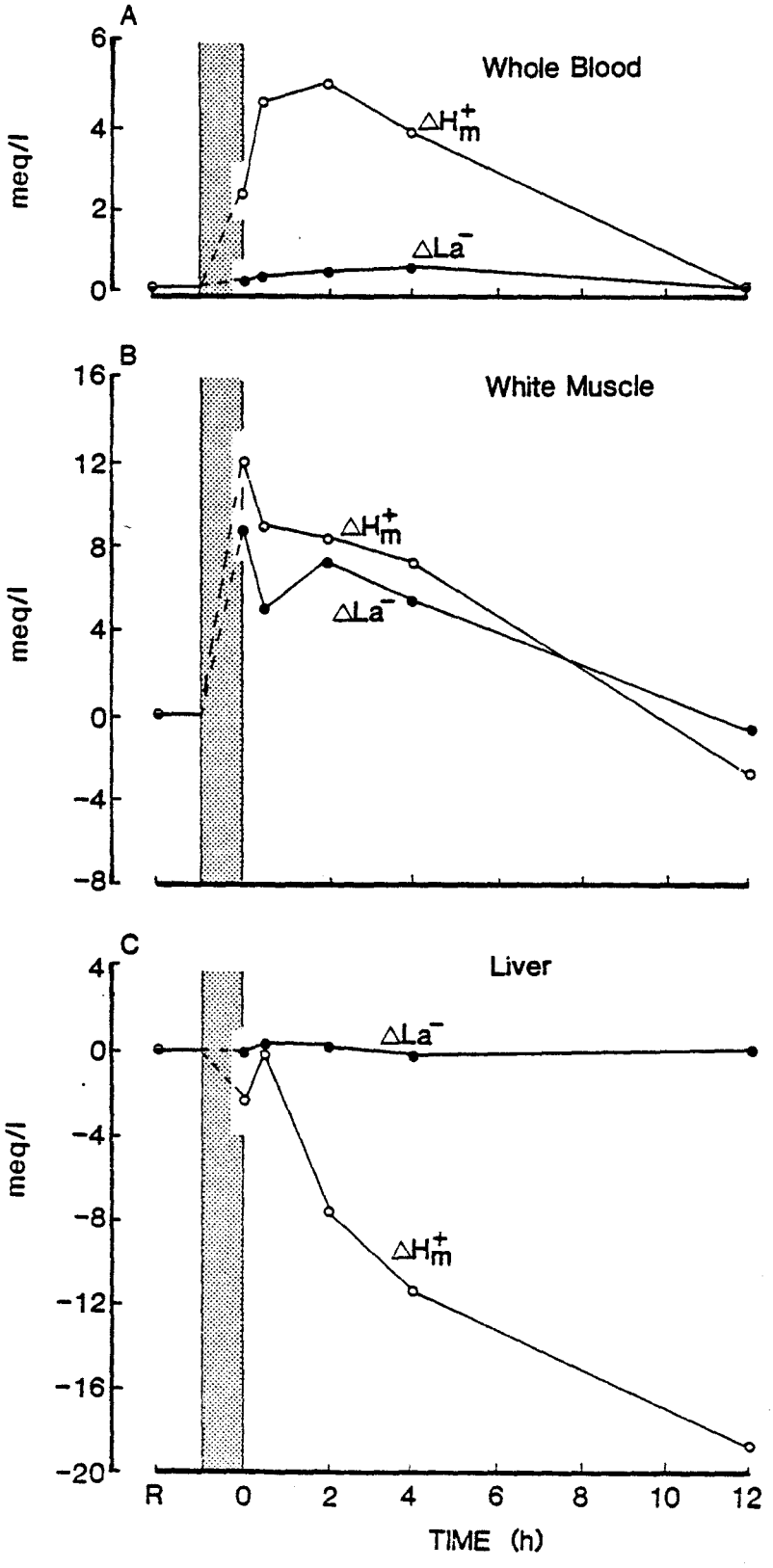
membrane potential.

The tissue buffer capacities followed a different sequence, with that of white muscle greater than that of liver (Table 6.1). Red cell buffer capacity was not measured. Similar trends were seen in trout (Table 4.1, Chapter IV), catfish, (Cameron and Kormanik, 1982), and mammals (Roos and Boron, 1981). The difference in tissue buffer capacities is thought to be related to total amount of histidine-related compounds (HRC) present; those tissues with a greater HRC content have a greater buffer capacity (Abe, Dobson, Hoeger and Parkhouse, 1985). The absolute value of flounder white muscle buffer capacity is similar to that reported for the inactive, demersal sea raven (Milligan and Farrell, 1986; see Appendix I) and catfish (Cameron and Kormanik, 1982) but much less than values reported for rainbow trout (Table 4.1, Chapter IV; also Castellini and Somero, 1981) and dogfish (Heisler and Neumann, 1980). Since white muscle buffer capacity is positively correlated with glycolytic potential (as determined by lactate dehydrogenase activity; Castellini and Somero, 1981), the relatively low buffer capacity of flounder muscle was to be expected.

Post-Exercise Changes

After 10 min of strenuous activity, flounder white muscle exhibited a severe drop in pHi which was mainly due to an accumulation of metabolic protons (Fig. 6.3, 6.4A). In flounder muscle after exercise H^+ apparently accumulated in excess of lactate (Fig. 6.7A), suggesting that ATP hydrolysis and glycolysis became uncoupled (ie. ATP consumption exceeded production) and/or there was an

Figure 6.7. Metabolic acid (ΔH^+m, O) and lactate loads (ΔLa^-, ●) in (A) white muscle, (B) whole blood, and (C) liver after 10 minutes of activity. R indicates rest value; vertical bar indicates 10 minute period of activity; 0h is immediately after activity. Values plotted are calculated from the means at each time. See text for details.


A
Whole Blood
meq/l
6
4
2
0
ΔH_m^+
ΔLa^-
B
White Muscle
16
12
8
4
0
-4
-8
R
ΔH_m^+
ΔLa^-
C
Liver
4
0
-4
-8
-12
-16
-20
ΔLa^-
ΔH_m^+
R
0
2
4
6
8
10
12
TIME (h)

accumulation of hexose-phosphates (see Chapter I). A similar pattern (ΔH^+m in excess of ΔLa^-) has been observed in rainbow trout (Fig. 4.8, Chapter IV) and sea raven (Milligan and Farrell, 1986; see Appendix I) after exhaustive exercise, which, in the former, was equivalent to the decline in ATP levels (Fig. 4.6, Chapter IV).

The liver also tended to become acidotic after exercise though this was due to an accumulation of respiratory rather than metabolic acid (Fig. 6.4B, 6.7C) which was cleared by 0.5h. Through the remainder of the recovery period, the liver exhibited a pronounced alkalosis which was of metabolic origin (Fig. 6.7C). A similar, though less severe, alkalosis has also been observed in trout liver post-exercise (Fig. 4.8, Chapter IV). However, unlike the present study (Table 6.4), the alkalosis in the trout liver was associated with an accumulation of lactate and was attributed to an uptake of lactate independent of H^+, and then subsequent metabolism. It is possible the alkalosis in flounder liver was of the same genesis, with lactate turnover so rapid that no net accumulation was evident. However, the relatively low lactate dehydrogenase activity (a necessary enzyme for lactate metabolism) generally found in the livers of inactive species (eg. plaice, sole, flounder- Dando, 1969; sea raven- Walsh, Moon and Mommsen, 1985) would tend to argue against this. Furthermore, if a tissue is metabolizing lactate, an increase in pyruvate is usually observed (Dando, 1969); this did not occur in flounder liver (Table 6.4). It is possible that the alkalosis was not due to metabolism but rather arose from transmembrane transport of acidic (or basic) equivalents. The physiological significance of the

alkalosis is unclear, though it could be important in controlling hepatic metabolism during recovery from exercise.

Unlike liver and muscle pHi, red cell pHi tended to remain constant after exercise, except for the acidosis evident at 0.5h (Fig. 6.3). This maintenance of red cell pHi post-exercise was previously seen in the rainbow trout (Fig. 3.3, Chapter III; Fig. 4.4, Chapter IV; also Primmett et al. , 1986) and the striped bass (Nikinmaa et al. , 1984) and has been attributed to a β-adrenergic influence of catecholamines released into the blood in response to the exercise stress (Nikinmaa, 1982, 1983; Nikinmaa et al. , 1984; Heming, 1984). Deoxygenation of the hemoglobin due to low Pa_{O_2} (Fig. 6.1D) may also have helped sustain pHi through the Haldane effect (Albers et al. , 1981; Jensen, 1986). The possible role of catecholamines in the regulation of red cell pHi and O_2 transport after exercise in flounder is examined in Chapter VII.

The Fate of Lactate and H^+

Strenuous activity in flounder resulted in lactate production within the muscle, though there was little accumulation of lactate in the bloodspace (Fig. 6.7B), in comparison to the more active trout (Fig. 4.8, Chapter IV). As discussed in Chapter V, this pattern of lactate 'non-accumulation' appears characteristic of relatively inactive fish species. It is possible that lactate efflux is perfusion-limited, due to a low blood flow through the white muscle mass. However, if this were the situation then metabolic acid (ΔH^+m) appearance in the blood should be affected to the same extent. However, this is not the case (see Fig. 6.7B). Furthermore

ΔH^+m declined faster than ΔLa^- in white muscle (Fig. 6.7A), and approximately 20% of the total H^+ load passed through the ECFV to be temporarily stored in the environmental water (Fig. 5.7, Chapter V). These findings indicate that in fact H^+ efflux from muscle exceeded lactate efflux, and that lactate was retained, in agreement with the work of Wardle (1978) and Batty and Wardle (1979) in plaice. However, Wood and Milligan (1986) were unable to confirm the suggestion of Wardle (1978) that catecholamines were responsible for this retention. The data of Chapter VII also tend to argue against the involvment of catecholamines.

It is suggested that most of the lactate and the greater proportion of the H^+ remained in the white muscle mass and were metabolized *in situ*, i.e. the Cori cycle was of little importance. The partial efflux of H^+ in combination with this metabolism would explain the rapid correction of muscle pHi and subsequent intracellular alkalosis (Fig. 5.5, Chapter V). Fish white muscle is capable of lactate oxidation, albeit at lower rates than 'aerobic' issues (eg. heart, red muscle; Bilinski and Jonas, 1972). For flounder muscle to clear the lactate load by *in situ* metabolism would require an average O_2 consumption of 2 mmol O_2/kg/h (assuming that 3 mol of O_2 were consumed per mol of lactate metabolized; Newsholme and Leech, 1983), well within reported O_2 consumption rates for muscle (Gordon, 1972a,b). There is now a growing body of physiological and biochemical evidence suggesting that a variety of vertebrate muscles are capable of *in situ* glyconeogenesis (eg. flatfish: Wardle, 1978; Batty and Wardle, 1979; frog: Bendall and

Taylor, 1970, Connett, 1979; rabbit: Bendall and Taylor, 1970; Dyson *et al.*, 1975; rat: McLane and Holloszy, 1979; Constable *et al.*, 1984; humans: Hermansen and Vaage, 1977). Thus it is not unreasonable to suggest that lactate and H^+ may be used as substrates for *in situ* glyconeogenesis. The observed late rise in muscle [pyruvate] at 4h (Fig. 6.2B) lends support to this argument.

In trout white muscle after exercise, it was similarly argued that lactate and H^+ were metabolized *in situ*, intially by oxidation and later, by glyconeogenesis (Chapter IV). Interestingly, recovery of white muscle pHi was more rapid in flounder (complete before pHe, at 4-8h, Fig. 6.3) than in trout (complete at 8-12h, Fig. 4.2, Chapter IV). This faster pHi recovery was associated with a more rapid restoration of muscle glycogen stores (12h in flounder, Table 6.2, *versus* 24h in trout, Fig. 4.6, Chapter IV). A similar pattern of H^+ and lactate clearance from the muscle after exercise was observed in the relatively inactive, sluggish sea raven (Milligan and Farrell, 1986; see Appendix I). Reasons for these species differences are discussed in Chapter VIII.

CHAPTER VII

REGULATION OF BLOOD OXYGEN TRANSPORT AND RED CELL pHi AFTER EXHAUSTIVE ACTIVITY IN RAINBOW TROUT AND STARRY FLOUNDER

INTRODUCTION

Exhaustive exercise resulted in a severe reduction in plasma pH by as much as 0.5 to 0.6 pH units due to metabolic and respiratory acidoses in both trout and flounder (eg. Fig. 3.1, Chapter III; Fig. 5.1, Chapter V). However, in both species, red blood cell (RBC) pHi underwent little change (eg. Fig. 3.3, Chapter III; Fig. 4.4, Chapter IV; Fig. 6.3, Chapter VI). Studies with tonometered blood *in vitro* have shown that, for a comparable reduction in plasma pH, RBC pHi falls (eg. Fig. 2.2, Chapter II) and hemoglobin-oxygen affinity is decreased via Root and Bohr effects (Eddy, 1971; Wood *et al.*, 1975; Albers *et al.*, 1981) However, *in vitro*, these effects can apparently be ameliorated by adrenaline acting on β-adrenergic receptors on the red cells (Nikinmaa, 1982, 1983; Heming, 1984). Exhaustive exercise has been reported to greatly elevate plasma catecholamine levels, at least in salmonids (see Table 7.1 for references). Nikinmaa *et al.* (1984) and Primmett *et al.* (1986) have suggested that mobilization of adrenaline into the bloodstream plays a role in maintaining RBC pHi and therefore hemoglobin-oxygen affinity *in vivo* after severe exercise in striped bass and rainbow trout, respectively. These authors concluded that catecholamines were released into the

circulation after exercise to maintain adequate blood oxygen transport during the post-exercise acidosis so that subsequent aerobic activity would not be compromised. Catecholamine mobilization has also been implicated in lactate retention in the muscle of flatfish (Wardle, 1978), though the evidence was indirect as no catecholamine measurements were made.

One goal of the present study was to measure the extent of catecholamine mobilization in rainbow trout and starry flounder. A second was to further investigate the role of circulating catecholamines, as well as possible interactive factors, such as plasma cortisol and red cell nucleoside triphosphate levels, in the regulation of RBC pHi and blood O_2 transport after exhaustive exercise.

MATERIALS AND METHODS

Experimental Animals

(1) Rainbow Trout

Adult rainbow trout (Salmo gairdneri) (736 ± 40.3 g, mean ± 1 SEM, n=15) of both sexes were obtained from Highland Springs Trout Farm, Holland Center, Ontario in November, 1985. All animals were sexually mature and in breeding condition. Fish were held as described previously. Room lights were left on continually to minimize any circadian rhythmcity in hormone levels.

Trout were surgically fitted with dorsal aorta cannulae as previously described (Chapter II) and allowed to recover at least 48 h in 20 l darkened Lucite fish boxes continually supplied with well

aerated tap water (P_{O_2} = 150 torr) at 15°C

(2) Starry Flounder

Adult starry flounder (842 ± 77.9 g, n=14) of both sexes were obtained by otter trawl from East Sound, Orcas Island and Birch Bay, Washington in November and December, 1984. All fish were sexually mature and most were in breeding condition. Fish were held as described previously (Chapter V). Room lights were left on continually.

Following catheterization of the caudal artery, fish were placed in 15 l plastic tubs fitted with black plastic mesh and supplied with fresh flowing seawater (29°/oo; P_{O_2} = 156 torr) at 9 ± 1°C and allowed to recover at least 72 h prior to experimentation.

Experimental Protocol

For each species, parallel experiments were performed on two groups, one of which was subjected to exercise (trout, n=8; flounder, n=8). The other group (trout, n=7; flounder, n=6) served as controls for handling and sampling effects. These fish were left at rest throughout but otherwise treated identically to the experimental group.

Trout were exercised by vigorously chasing them around a large circular tank (500 l) for 6 min (see Chapter III), while flounder were chased for 10 min in a shallow rectangular tank (see Chapter V). At the end of exercise, fish were returned to their boxes.

Blood samples (trout, 950 ul; flounder, 1400 ul) were drawn into gas-tight Hamilton syringes from the dorsal aorta catheter in trout or

the caudal artery catheter in flounder. Samples were taken prior to exercise ('rest'), immediately after exercise ('0 h'), then again at 0.5, 1, 2, 4, 8, 12, and 24 h after exercise. Samples were analyzed for pH, hematocrit (Ht), [hemoglobin], whole blood levels of lactate and nucleoside triphosphate (NTP), arterial oxygen tension (Pa_{O_2}), arterial oxygen content (Ca_{O_2}), and red blood cell (RBC) pHi. Plasma was analyzed for total CO_2 and levels of cortisol, adrenaline (Ad), and noradrenaline (Nad). In the flounder control group, only pH, whole blood [hemoglobin] and [NTP], RBC pHi and plasma levels of cortisol, Ad, and Nad were measured. Previous studies on flounder under control conditions have documented the effect of sampling on most of the other parameters (Chapter V). The volume of blood sampled was replaced with saline.

Analytical Techniques, Calculations and Statistical Analysis

All analyses and calculations were performed as described in the preceding chapters, except for the following. In the trout studies, plasma total CO_2 was measured with a Corning CO_2 analyzer rather than by the electrode technique of Cameron (1971) used in the flounder work. Blood oxygen content was determined with a 'Lex-O_2-Con' analyzer (Lexington Instruments) using a sample volume of 50 ul and the re-calibration procedure described by Wood et al. (1979a). To account for differences in [hemoglobin] and physically dissolved oxygen concentrations between fish, hemoglobin-bound O_2 per unit hemoglobin was calculated as:

$$\left[O_2\right]/\left[Hb\right] \text{ (mmol/g)} = \frac{Ca_{O_2} \text{ (mmol/l)} - (Pa_{O_2} \text{ (torr)} \times \alpha O_2 \text{ (mmol/l/torr)})}{\left[Hb\right] \text{ (g/l)}} \quad (7.1)$$

where αO_2 represented either the measured O_2 solubility coefficient in starry flounder blood plasma at 9 °C, 2.048 umol/l/torr, (Wood et al. , 1979a) or for trout, the tabulated value at 15 °C, 1.7745 umol/l/torr, reported by Boutilier et al. (1984). Blood NTP levels were measured by fixing either 100 ul (trout) or 200 ul (flounder) whole blood in an equal volume of ice-cold 12% trichloracetic acid and freezing the slurry in liquid N_2. No more than 48 h passed before samples were thawed and immediately analyzed. NTP levels were assayed using the technique described by Adams (1963) in which the change in [NADH] was measured spectrophotometrically (340 nm) and was directly proportional to the [NTP], as linked by the phosphoglycerate phosphokinase/glyceraldehyde phosphate dehydrogenase enzyme system. Sigma reagents were used. Since NTP is almost entirely intracellular (Wood, 1980), levels were expressed as both content per unit hemoglobin (i.e. [NTP]/[Hb]) and as cellular concentration (i.e. [NTP]/Ht).

For measurement of cortisol, Nad and Ad, 400 ul whole blood was centrifuged for 3 min at 9,000 g. Approximately 200 ul of plasma was drawn off, 10 ul preservative was added (90 mg/ml EDTA and 60 mg/ml glutathione; Sigma) and then samples were immediately frozen in liquid N_2. The remaining red cell pellet was used for measurement of red cell pHi. Plasma samples were stored at -80°C for not longer than 60 days before analysis. Cortisol was measured in duplicate on 25 ul of plasma using a commercially available ^{125}I-radioimmunoassay kit (Corning Medical). In this

radioimmunoassay, cortisol antibodies are covalently bound to glass beads. A fixed amount of ^{125}I-cortisol was added to the antibody slurry along with samples or standards. The antibody-cortisol complex was separated by centrifugation (10 min at 9,000 g) and the resultant precipitate counted on a Nuclear Chicago gamma counter. Quantification of cortisol in plasma was interpolated from a standard curve prepared with each assay. In fish, the other major plasma corticosteroids are corticosterone and cortisone. However, these constitute less than 10% of the total corticosteroids present (Butler, 1973) and according to manufacturer's specifications, exhibit less than 1% cross-reactivity with the cortisol antibody.

Plasma Ad and Nad were also measured in duplicate on 50 ul aliquots using a commercially available ^{3}H-labelled radio-enzymatic assay (Cat-A-Kit, UpJohn Diagnostics). This assay was first utilized to measure catecholamines in fish plasma by Butler, Taylor, Capra and Davison (1978). The assay utilizes the enzyme catechol-O-methyl-transferase (COMT) to catalyze the transfer of a ^{3}H-methyl group from S - adenosyl - L - methionine - [^{3}H - methyl] to adrenaline and noradrenaline. The resulting products, ^{3}H-metadrenaline and ^{3}H-normetadrenaline respectively, were isolated by thin-layer chromatography. The spots were visualized under UV (320 nm) light, scraped into glass scintillation vials, converted to ^{3}H-vanillin by periodate oxidation and then extracted. Ten ml fluor (ACS: Amersham) was added, the samples stored in the dark overnight to reduce chemiluminescence and then counted in an LKB Wallac liquid scintillation counter. A standard amount of Ad

and Nad was added to one of the plasma aliquots, and the content of Ad and Nad in the sample calculated from the ratio of the sample + standard counts to the sample counts.

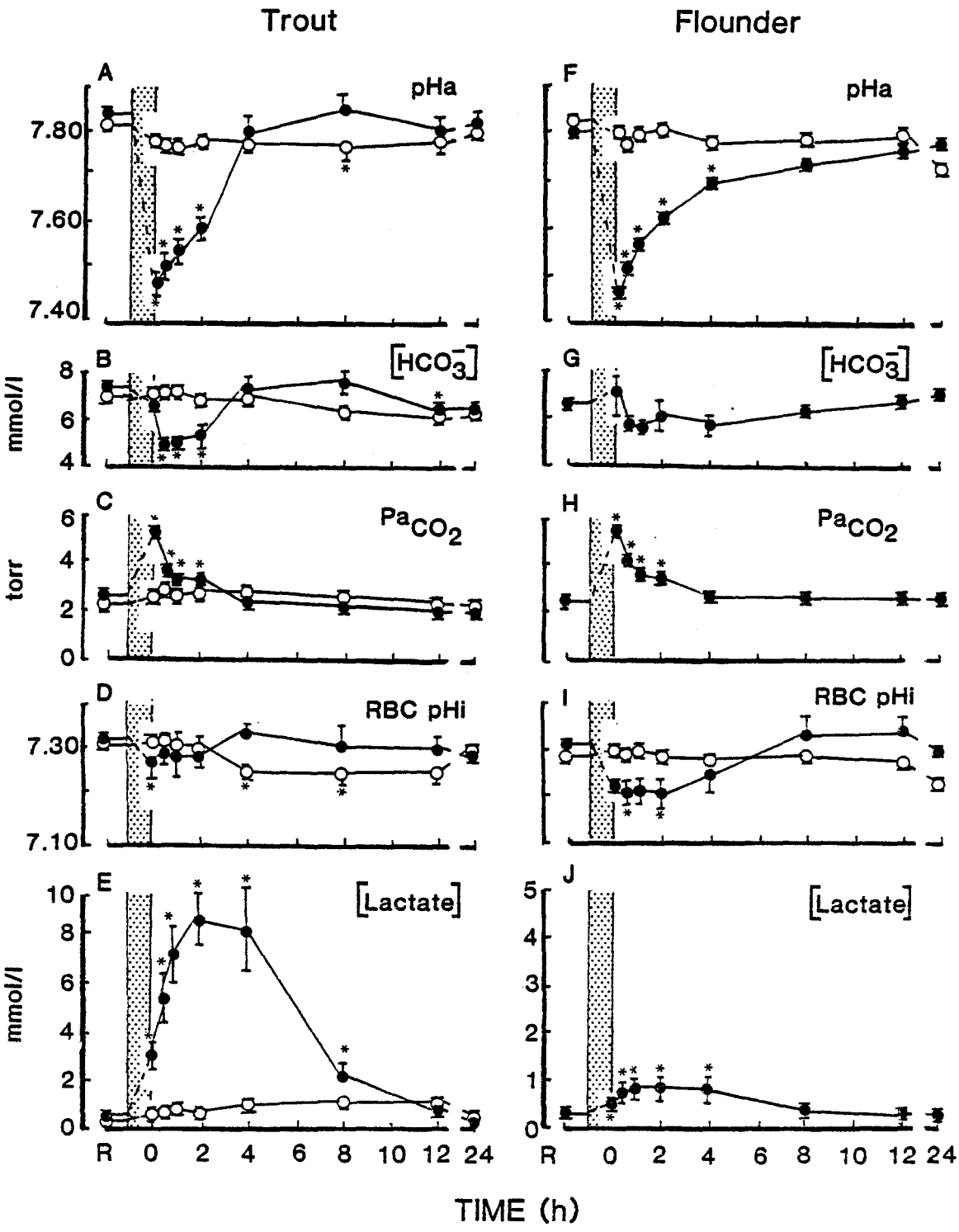
Statistical analysis was performed as described in Chapters II and III.

RESULTS

Exhaustive exercise resulted in a pronounced extracellular acidosis in both starry flounder and rainbow trout (Fig. 7.1A,F). This acid-base disturbance was qualitatively similar in both species, and was associated with an increase in Pa_{CO_2} (Fig. 7.1C,H) and reduction in plasma [HCO_3^-] (Fig. 7.1B,G). The extracellular acidosis was rapidly corrected in rainbow trout, complete at 4h, whereas in starry flounder, the correction was not complete until 8 h (Fig. 7.1A,F). There was no extracellular acid-base disturbance in either the trout or flounder control groups (Fig. 7.1A,F).

At rest, red cell pHi was virtually identical in trout and flounder, averaging 7.33 $\pm$ 0.01 (n=8) and 7.30 $\pm$ 0.03 (n=8), respectively (Fig. 7.1D,I). In both fish, RBC pHi fell significantly immediately after exercise, though to a greater extent in flounder than in trout (0.10 versus 0.07 pH units). These intracellular pH depressions were very small relative to the extracellular pH depressions (about 0.4 pH units). The RBC acidosis was short-lived in trout, with RBC pHi fully corrected by 0.5 h (Fig. 7.1D). In starry flounder, the RBC acidosis persisted through to 4 h, at which point RBC pHi returned to rest levels (Fig. 7.1I). Once RBC pHi was

Figure 7.1. Effect of exhaustive exercise on (A,F) plasma pHa, (B,G) plasma $[HCO_3^-]$, (C,H) plasma CO_2 tension (Pa_{CO_2}), (D,I) red cell pHi (RBC pHi), and (E,J) whole blood [lactate] in rainbow trout and starry flounder, respectively. Means $\pm$ 1 S.E.M. R indicates rest value, vertical bar indicates period of exercise (6 min for trout and 10 min for flounder), 0 is immediately after exercise. O - control group (trout, n=7; flounder, n=6); ● - exercise group (trout, flounder, n=8). * indicates a significant difference ($p<0.05$) from the corresponding rest value.

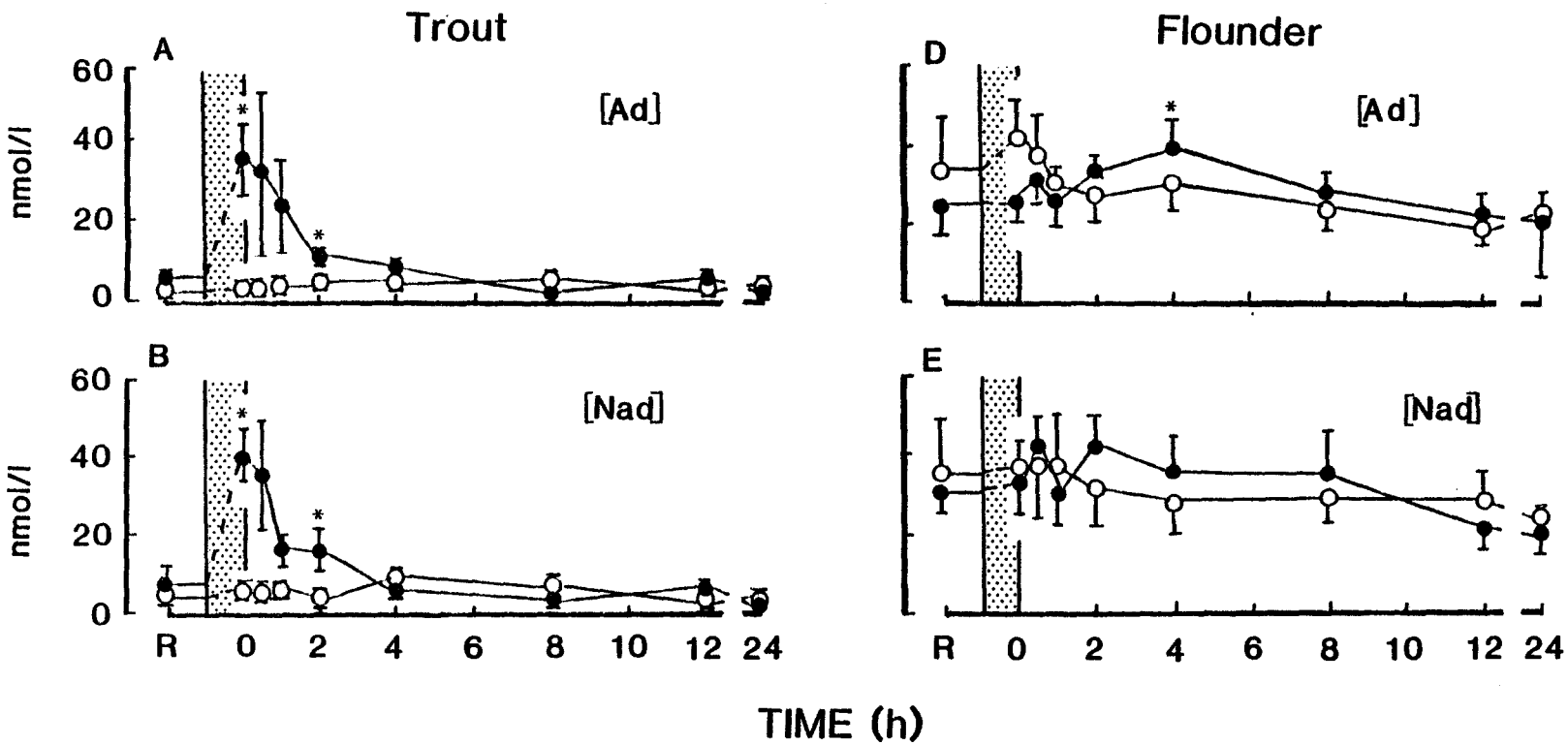

Trout
Flounder
A
pHa
7.80
7.60
7.40
F
pHa
B
[HCO3-]
mmol/l
8
6
4
G
[HCO3-]
C
PaCO2
torr
6
4
2
0
H
PaCO2
D
RBC pHi
7.30
7.10
I
RBC pHi
E
[Lactate]
mmol/l
10
8
6
4
2
0
J
[Lactate]
5
4
3
2
1
0
R 0 2 4 6 8 10 12 24
R 0 2 4 6 8 10 12 24
TIME (h)

corrected, it remained constant through 24 h. RBC pHi remained constant in the flounder control group (Fig. 7.1I), but in the trout control group, RBC pHi was significantly depressed at 4 and 8 h, but had fully recovered by 12 h and was not different at 24 h (Fig. 7.1D).

Whole blood [lactate] increased in both species after exercise, though to very different extents. Peak levels in rainbow trout were about 10-fold greater than in flounder (8.82 $\pm$ 1.64 versus 0.86 $\pm$ 0.22 mmol/l, n=8; Fig. 7.1E,J). In both fish, lactate appearance in the blood followed a similar time course, with peak levels attained 2-4 h into recovery and rest values restored by 12h.

Exhaustive exercise in trout also resulted in an 8-fold increase in plasma levels of both adrenaline (Ad; rest = 4.5 $\pm$ 3.1, 0 h = 36.7 $\pm$ 8.5 nmol/l, n=8) and noradrenaline (Nad; rest = 6.9 $\pm$ 5.4 , 0 h = 40.3 $\pm$ 6.9 nmol/l, n=8) (Fig. 7.2A,B). Levels peaked immediately after exercise (0 h), when the acidosis was most severe, and generally declined rapidly, though there was great variability at 0.5 and 1.0 h post-exercise. Rest levels were attained by 4h, in concert with the restoration of pHa. The response of starry flounder, however, was very different. Resting levels of Ad (23.6 $\pm$ 6.0 nmol/l, n=8) and Nad (30.9 $\pm$ 6.9 nmol/l, n=8) were about 6-7 times those in trout (Fig. 7.2 D,E) and did not change much after exercise, except for a small, but significant increase in Ad at 4 h (Fig. 7.2D). The previously measured reduction in ECFV after exercise (Chapter V) can account for some of the increase. Sampling in itself did not mobilize catecholamines as plasma levels of Ad and Nad remained constant in the control groups (Fig. 7.2A-E).

Figure 7.2. Plasma levels of (A,C) adrenaline (Ad), and (B,D) noradrenaline (Nad) prior to and following exhaustive exercise in rainbow trout and starry flounder, respectively. Means $\pm$ 1 S.E.M. R indicates rest value, vertical bar indicates period of exercise (6 min for trout and 10 min for flounder), 0 is immediately after exercise. 0 - control group (trout, n=7; flounder, n=6); ● - exercise group (trout, flounder, n=8). * indicates a significant difference ($p<0.05$) from the corresponding rest value.


Trout
Flounder
A
B
D
E
[Ad]
[Nad]
nmol/l
60
40
20
0
R
0
2
4
6
8
10
12
24
TIME (h)

Associated with the post-exercise acidosis and catecholamine mobilization in trout was a near 60% increase in hematocrit (Fig. 7.3A), 25% increase in [hemoglobin] (Fig. 7.3B) and a 20% reduction in mean cell [hemoglobin] (MCHC, Fig. 7.3C), the latter indicative of red cell swelling. These parameters had returned to rest levels by 2-4 h. However, MCHC continued to increase, so that by 8-12 h, it was significantly elevated over rest levels, but had returned to pre-exercise levels by 24 h. Similar relative trends, though of smaller absolute magnitude, occurred in starry flounder (Fig. 7.3F), but no secondary rise in MCHC was observed (Fig. 7.3F) MCHC did decline significantly, however, suggesting red cell swelling had occurred (Fig. 7.3G). The diluting effect of repetitive sampling became apparent at 4-8 h in both trout (Fig. 7.3A,B) and flounder (Fig. 7.3D,E), when hematocrit and [hemoglobin] declined significantly.

At rest, arterial oxygen tension (Pa_{O_2}) in trout (93.4 $\pm$ 9.4 torr, n=8; Fig. 7.4A) was about double that in flounder (44.2 $\pm$ 7.1 torr, n=8, Fig. 7.4D) and arterial blood oxygen content (Ca_{O_2}) in trout was almost triple that of flounder (5.2 $\pm$ 0.3 *versus* 1.9 $\pm$ 0.2 mmol/l, n=8; Fig. 7.4B,E). The latter was due in part to the difference in [hemoglobin] (Fig. 7.3B,E), though hemoglobin-bound O_2 content per unit hemoglobin ($[O_2]/[Hb]$) was also higher in trout than flounder (56 $\pm$ 1 *versus* 48 $\pm$ 3 umol/g, n=8; Fig. 7.4C,F). Pa_{O_2} showed small fluctuations in the trout control group, with significant increases at 2h and 8-24 h.

Figure 7.3. Hematological changes associated with exhaustive exercise in rainbow trout and starry flounder. (A,D) Hematocrit, (B,E) whole blood [hemoglobin] ([Hb]), and (C,F) mean cell [hemoglobin] (MCHC). Means $\pm$ 1 S.E.M. R indicates rest value, vertical bar indicates period of exercise (6 min for trout and 10 min for flounder), 0 is immediately after exercise. O - control group (trout, n=7; flounder, n=6); ● - exercise group (trout, flounder, n=8). * indicates a significant difference ($p<0.05$) from the corresponding rest value.

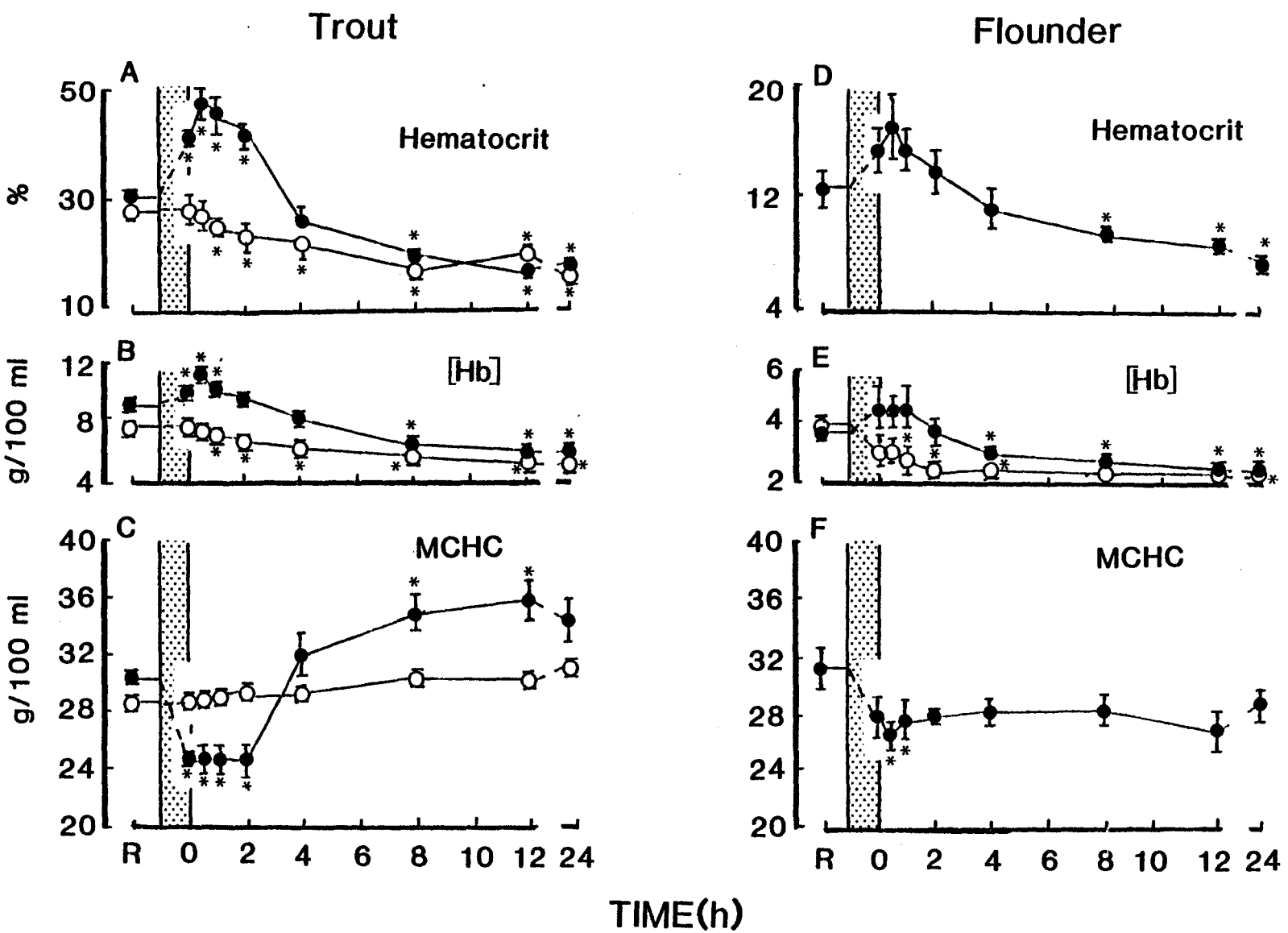
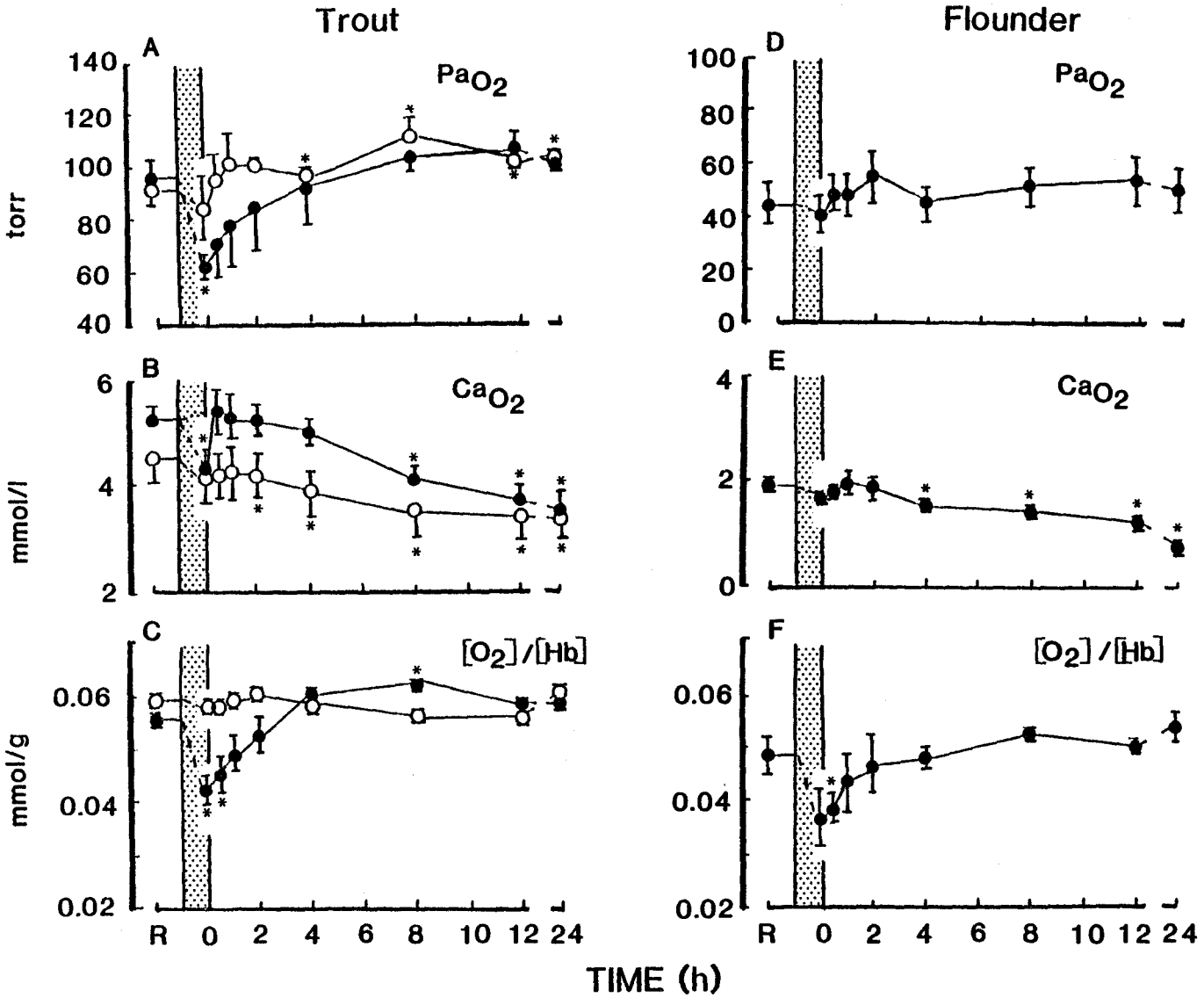

Trout
Flounder
A
Hematocrit
%
50
30
10
D
Hematocrit
20
12
4
B
[Hb]
g/100 ml
12
8
4
E
[Hb]
6
4
2
C
MCHC
g/100 ml
40
36
32
28
24
20
F
MCHC
R 0 2 4 6 8 10 12 24
TIME(h)

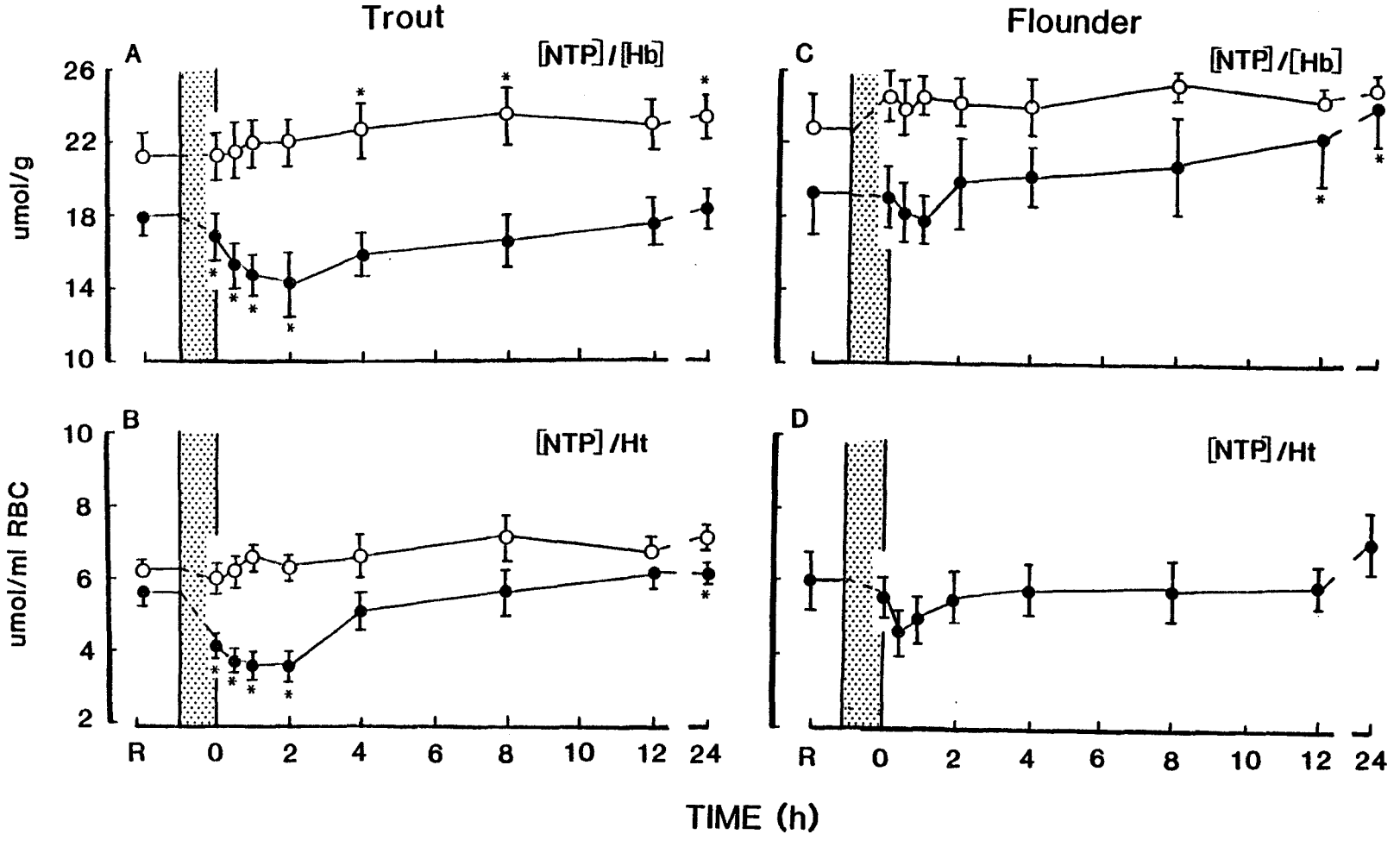
Figure 7.4. The effects of exhaustive exercise on (A,D) arterial blood oxygen tension (Pa_{O_2}), (B,E) blood oxygen content (Ca_{O_2}) and (C,F) hemoglobin-bound O_2 per unit hemoglobin ($[O_2]/[Hb]$) in rainbow trout and starry flounder, respectively. Means $\pm$ 1 S.E.M. R indicates rest value, vertical bar indicates period of exercise (6 min for trout and 10 min for flounder), 0 is immediately after exercise. O - control group (trout, n=7; flounder, n=6); ● - exercise group (trout, flounder, n=8). * indicates a significant difference ($p<0.05$) from the corresponding rest value.


Trout
Flounder
A
B
C
D
E
F
Pa_{O_2}
Ca_{O_2}
$[O_2]/[Hb]$
torr
mmol/l
mmol/g
140
120
100
80
60
40
6
4
2
0.06
0.04
0.02
100
80
60
40
20
0
R
0
2
4
6
8
10
12
24
TIME (h)

Immediately after exercise in trout, Pa_{O_2} fell significantly, by about 35%, though was restored to a level not significantly different from rest at 0.5 h (Fig. 7.4A). Ca_{O_2} in trout fell in concert with Pa_{O_2} immediately after exercise, but had returned to rest levels by 0.5h (Fig. 7.4B). Thereafter, it remained constant until 8 h, when the diluting effect of sampling on [hemoglobin] became apparent (Fig. 7.3B, 7.4B). Pa_{O_2} in flounder was not significantly affected by exercise. Similarly, Ca_{O_2} in flounder remained fairly constant after exercise until 4 h, when it fell significantly, reflecting the effect of sampling on [hemoglobin] (Fig. 7.3E, 7.4E). When post-exercise variations in [hemoglobin] were taken into account, $[O_2]/[Hb]$) fell by about 20-25% in both trout (Fig. 7.4C) and flounder (Fig. 7.4F). This decline persisted for about 1 h in both fish; thereafter $[O_2]/[Hb]$ returned to rest levels, with a slight increase evident in trout at 8 h. In the trout control group, $[O_2]/[Hb]$ remained constant throughout the experimental period. (Fig. 7.4A,C).

In trout after exercise, there was an apparent 'metabolic' degradation of NTP as indicated by the significant decline in [NTP]/[Hb] by about 20% (Fig. 7.5A). The diluting effect of red cell swelling (Fig. 7.3C) compounded this metabolic reduction, so that the actual reduction in mean cellular [NTP] was about 35% (Fig. 7.5B). Red cell NTP levels were fully restored by 4 h into recovery. In flounder, there was no apparent 'metabolic' degradation of red cell NTP, as [NTP]/[Hb] remained constant after exercise, except for a significant increase at 12 and 24 h (Fig. 7.5C). While mean cellular

Figure 7.5. Changes in (A,C) blood nucleoside triphosphate (NTP) content per unit hemoglobin ([NTP]/[Hb]) and (B,D) cellular NTP levels ([NTP]/Ht) after exhaustive exercise in rainbow trout and starry flounder, respectively. Means $\pm$ 1 S.E.M. R indicates rest value, vertical bar indicates period of exercise (6 min for trout and 10 min for flounder), 0 is immediately after exercise. O - control group (trout, n=7; flounder, n=6); ● - exercise group (trout, flounder, n=8). * indicates a significant difference ($p<0.05$) from the corresponding rest value.


Trout
Flounder
A
B
C
D
[NTP]/[Hb]
[NTP]/Ht
umol/g
umol/ml RBC
26
22
18
14
10
8
6
4
2
R
0
2
4
6
8
10
12
24
TIME (h)

[NTP] tended to decline, reflecting the red cell swelling, the changes were not significant (Fig. 7.5D). In the control groups of both trout and flounder, [NTP]/[Hb] tended to increase, though the changes were significant only in the trout group at 4, 8, and 24 h (Fig. 7.5A).

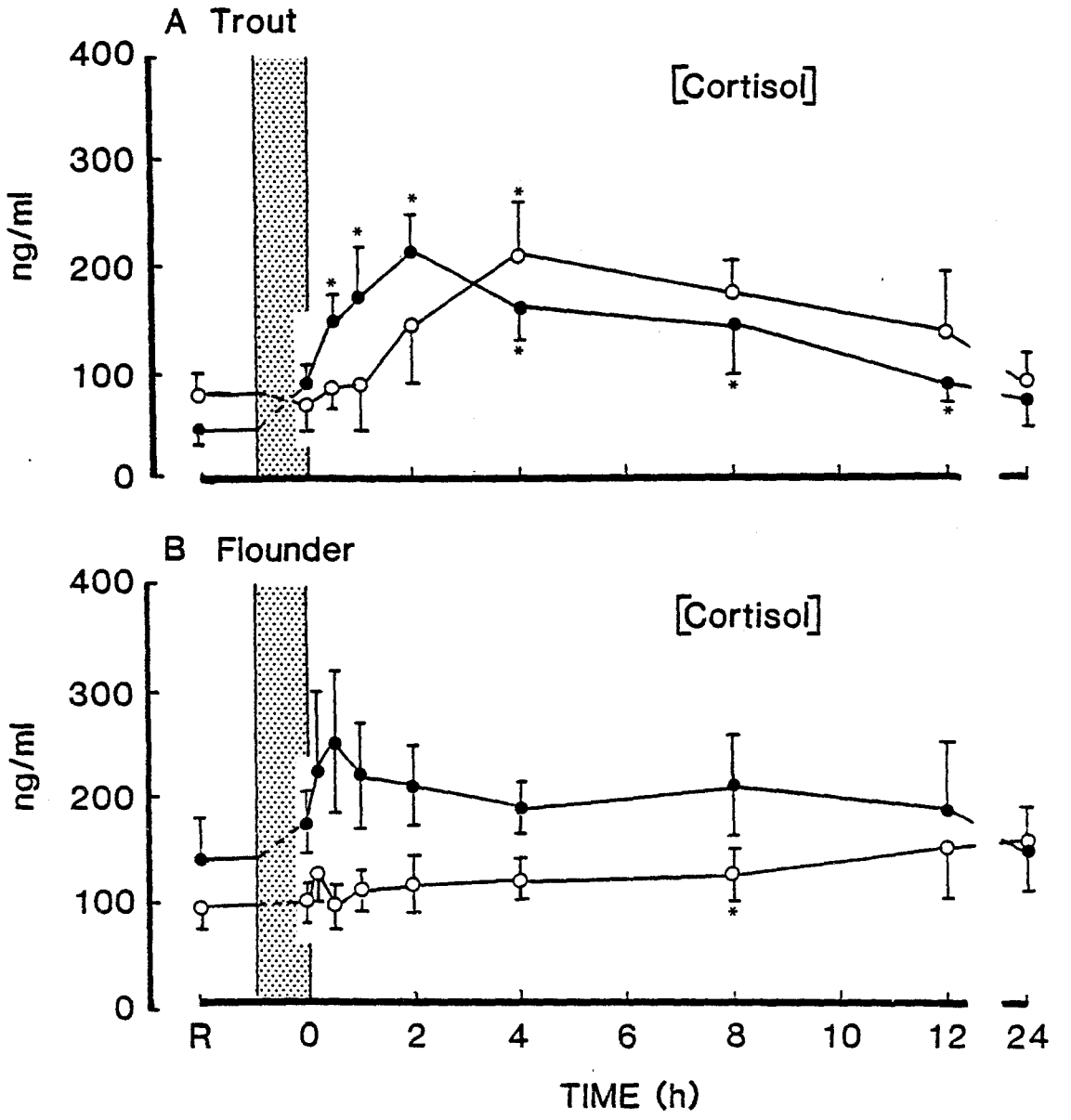
As with catechloamines (cf. Fig. 7.2), resting levels of plasma cortisol (41.8 $\pm$ 12.3 ng/ml, n=8) in trout were lower than those in flounder (133.0 $\pm$ 39.7 ng/ml, n=8, Fig. 7.6A, B). Cortisol levels tended to increase after exercise in flounder, though the changes were not significant; in the control group, cortisol remained constant (Fig. 7.6B), except for a small, but significant increase at 8 h. However in trout, plasma [cortisol] increased 3-4 fold in both the exercise and control groups (Fig. 7.6A). The changes followed similar, though not identical, patterns in both groups, making it difficult to discern any definite exercise effect. Again, the measured reduction in ECFV post-exercise (Chapter V) can account for some of the observed increases.

DISCUSSION

Control Experiments

Since a number of blood samples, each of considerable volume, were required from the fish in this study, it was anticipated that sampling alone might induce significant changes in some parameters. The control experiments showed that in general, these effects were not particularly large, apart from those directly reflecting loss of red cells from the circulation (Fig. 7.3, 7.4B), and did not confound the patterns seen in the experimental groups.

Figure 7.6. Plasma [cortisol] in (A) rainbow trout and (B) starry flounder prior to and following exhaustive exercise. Means $\pm$ 1 S.E.M. R indicates rest value, vertical bar indicates period of exercise (6 min for trout and 10 min for flounder), 0 is immediately after exercise. 0 - control group (trout, n=7; flounder, n=6); ● -exercise group (trout, flounder, n=8). * indicates a significant difference ($p<0.05$) from the corresponding rest value.


A Trout
[Cortisol]
ng/ml
400
300
200
100
0
B Flounder
[Cortisol]
ng/ml
400
300
200
100
0
R
0
2
4
6
8
10
12
24
TIME (h)

An important exception was plasma cortisol in trout, where changes in the control group were as large as in the experimental group (Fig. 7.6A). Interestingly, this was not observed in plasma catecholamines (Fig. 7.2), which are often considered a sensitive indicator of stress in fish (Mazeaud, Mazeaud and Donaldson, 1977). It is possible the control response reflected a persistent diurnal rhythm (Zelnik and Goldspink, 1981), despite the constant illumination in the present study.

In mammals, cortisol may potentiate adrenergic responses. especially those mediated by β-adrenoceptors (Kraus-Friedman, 1984). A similar synergism is thought to occur in bullfrogs in response to handling stress (Mbangkollo and deRoos, 1983). Therefore interactive effects between cortisol and catecholamines might well occur in fish after exercise (Mazeaud et al. , 1977). Unfortunately, the cortisol data from the present study were too unclear to support or refute this hypothesis. Clearly, there is a need for further studies on the possible interaction between cortisol and catecholamines in fish.

Rainbow Trout

In the rainbow trout, the post-exercise acidosis in the extracellular compartment was qualitatively similar to that previously described for the species (eg. Fig. 3.1, Chapter III) and the same explanations probably apply. Blood lactate elevations were somewhat lower than those seen previously, perhaps because the present animals were in breeding condition and did not appear to exercise quite as vigorously as those of Chapters III and IV. Despite this pronounced extracellular acidosis, red cell pHi was virtually unaffected, except

for the small, but significant, decline immediately after exercise. Given the reduction in pHe at time 0, based on the relationship between RBC pHi and pHe for trout blood determined *in vitro* (Fig. 2.5, Chapter II), RBC pHi should have fallen by about 0.23 pH units. However, *in vivo* , RBC pHi fell by only 0.07 pH units. The ability of trout red cells to better regulate pHi *in vivo* than *in vitro* is illustrated in Fig. 7.7A, which shows the relationship between pHe and RBC pHi *in vivo* and *in vitro* , with the former having a slope much less than the latter (0.20 *versus* 0.73).

This near perfect regulation of RBC pHi *in vivo* can probably be attributed to the significant elevation in circulating levels of Ad and Nad during the acidotic period (see Fig. 7.2A,B). The post-exercise levels of plasma Ad and Nad in trout in the present study are in good agreement with those recently reported for this species (Table 7.1), though the early very high values of Nakano and Tomlinson (1967) have never been confirmed. A number of *in vitro* studies have demonstrated that in the presence of Ad, trout red cells are able to better regulate pHi in the face of pHe changes (Nikinmaa, 1982, 1983; Heming, 1984). The mechanism is thought to involve β-adrenergic stimulation of Na^+/acidic equivalent exchange in excess of Cl^-/basic equivalent exchange in the cell membrane, resulting in a net efflux of acidic equivalents, net influxes of Na^+, Cl^-, and water, and thus cell swelling (Nikinmaa, 1982, 1983; Cossins and Richardson, 1985). Erythrocyte swelling also occurs passively, in response to an increase in P_{CO_2}. Cell swelling will tend to dilute the fixed negative charges in the cell (eg.

Table 7.1. Plasma levels of catecholamines prior to and following exercise in various fish species.

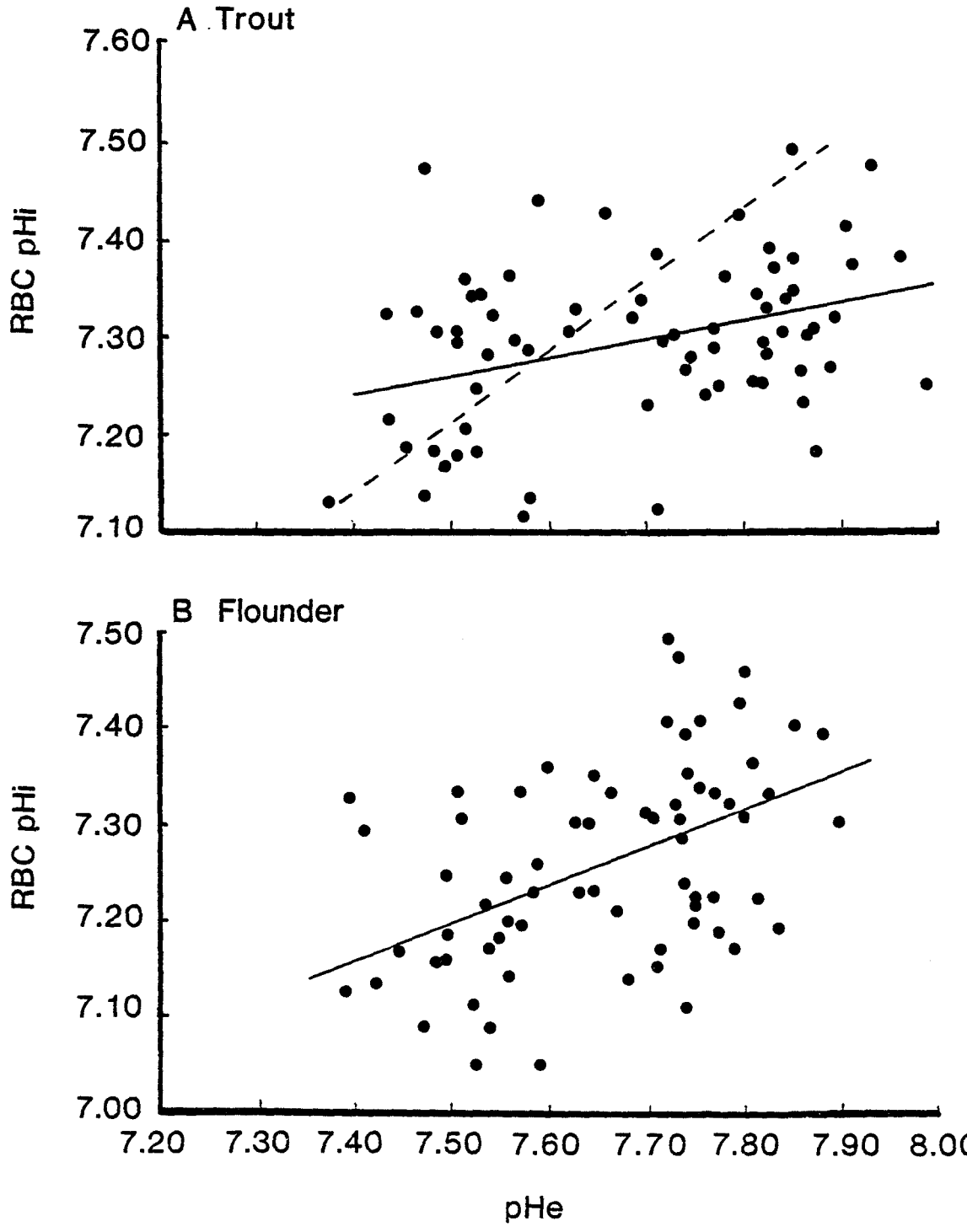
SPECIES	REST		POST-EXERCISE		SOURCE
	Ad	Nad	Ad	Nad	
Salmo gairdneri	4.5	6.9	36.8	40.3	A
	25	20	1130	310	B
(rainbow trout)	1.5	1.1	7.8	4.5	C
	0.9	0.7	38	27	D
	4	12	12	23	E_1
			190	85	E^1
Platichthys stellatus (starry flounder)	23.6	30.9	25.4	34.9	A
Scyliorhinus canicula (dogfish)	5	18	95	95	E_2^1
			20	34	E^2
Squalus acanthias (spiny dogfish)	5	8	22	38	F
Petromyzon marinus (lamprey)	21	7	12	7	G

Ad, adrenaline; Nad, noradrenaline. All values in nmol/l.

Source

A: Present study.
B: Nakano and Tomlinson (1967). Levels immediately after 15 min of violent exercise (eg. tail grasping)
C: Ristori and Laurent (1985). Levels 5 min after 3 min of 'violent' exercise (ie. tail grasping).
D: Primmett et al. (1986). Levels immediately after fish were swum to exhaustion in a swim tunnel.
E: Butler (1985): Levels immediately after fish were swum to exhaustion in a swim tunnel
E_2^1: Levels after 'violent' exercise (ie. tail grasping).
E^2: Levels in spontaneously active fish.
F: Opdyke, Carroll and Keller (1982). Levels immediately after 3 min of 'violent' exercise (ie. tail grasping).
G: Dashow, Epple and Nibbio (1982). Levels 5 min after mild agitation (10 sec prodding with glass rod).

Figure 7.7. The *in vivo* relationship (solid line) between red cell pHi and pHe in (A) rainbow trout and (B) starry flounder. The regression equations are: rainbow trout: pHi = 0.20 x pHe + 5.78 r=0.35, n=72 ($p<0.01$); starry flounder: pHi = 0.39 x pHe + 4.31, r=0.48, n=69 ($p<0.01$). The dotted line is the *in vitro* relationship between pHi and pHe for rainbow trout determined in Chapter II (Fig. 2.5), with the regression equation: pHi = 0.73 x pHe + 1.74, r=0.90, n=88 ($p<0.001$).


A Trout
7.60
7.50
7.40
7.30
7.20
7.10
RBC pHi
B Flounder
7.50
7.40
7.30
7.20
7.10
7.00
RBC pHi
7.20 7.30 7.40 7.50 7.60 7.70 7.80 7.90 8.00
pHe

hemoglobin, NTP). This will also raise RBC pHi passively by a shift in the Donnan ratio for H^+ (Nikinmaa, 1982; Heming, 1984). In the present study, red cell swelling did occur as evidenced by the significant decline in MCHC (Fig. 7.3C). Release of immature erythrocytes from the spleen in response to adrenergic stimulation may have also contributed to the decline in MCHC (Yamamoto *et al.*, 1980).

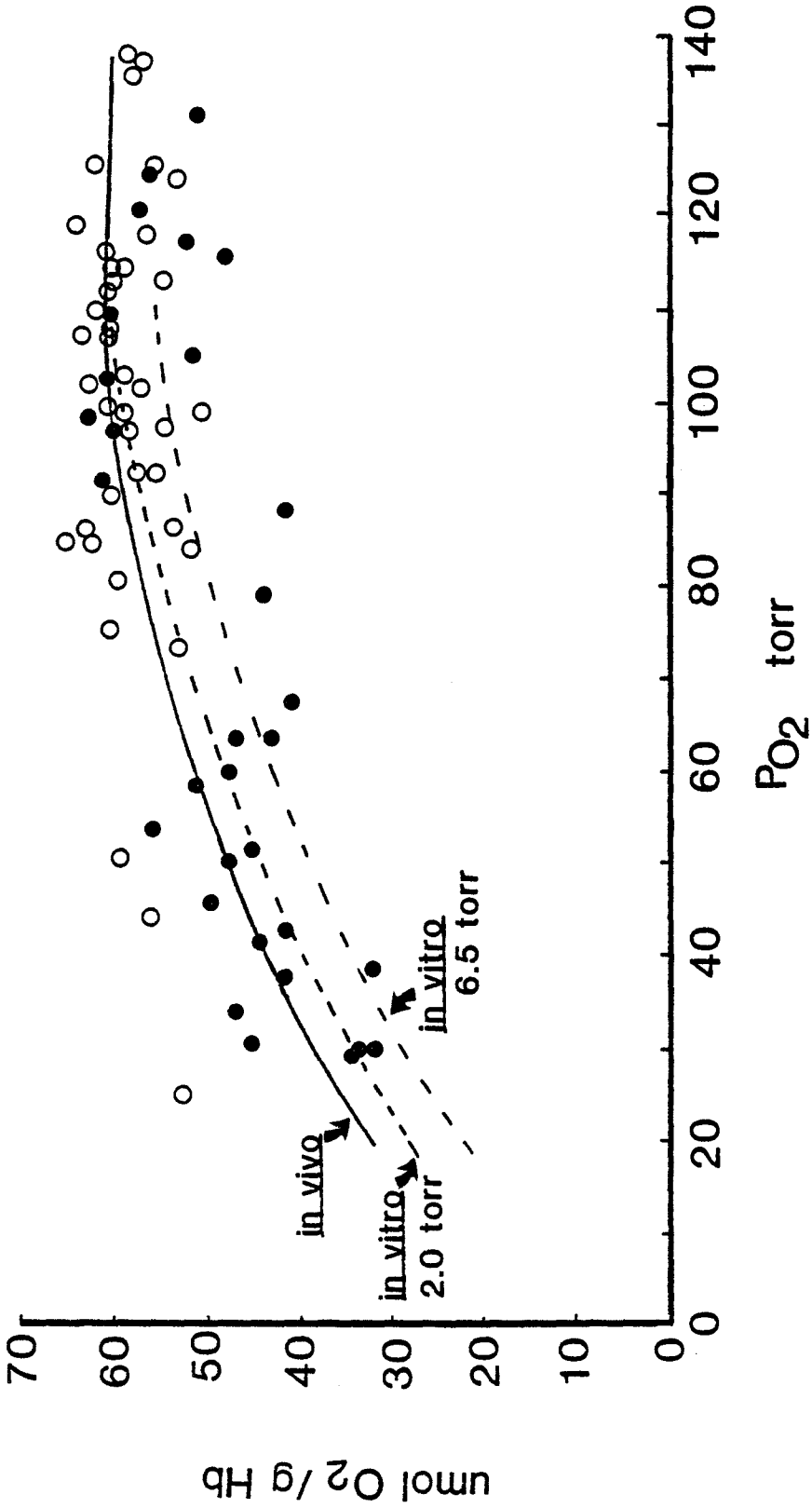
The exercise-induced catecholamine surge may also have caused the significant reduction in cellular NTP concentrations (Fig. 7.5B). In addition to the dilution of cellular NTP by cell swelling, there was evidence of a 'metabolic' degradation of red cell NTP stores because [NTP]/[[Hb] also declined (Fig. 7.5A). In trout red cells *in vitro*, Nikinmaa (1983) has demonstrated that Ad induces a 'metabolic' reduction in cellular NTP, perhaps by an inhibition of synthesis and/or a stimulation of consumption. This effect could be blocked by the β-antagonist, propranolol. Release of immature erythrocytes from the spleen may also have contributed to the reduction in [NTP]/[Hb] *in vivo*, as immature red cells possess less NTP than do their mature counterparts (Lane, Rolfe and Nelson, 1981).

Nucleoside triphosphates, which in trout and flounder are more than 90% ATP (Wood *et al.*, 1975; Bartlett, 1980) are negative allosteric modifiers of hemoglobin-oxygen affinity (Bartlett, 1980), while reductions in red cell pHi will similarly reduce hemoglobin-oxygenation via Root and Bohr shifts (Wood *et al.*, 1975; Albers *et al.*, 1981; Nikinmaa, 1983). Therefore, by minimizing the change in RBC pHi and reducing red cell NTP levels, the post-exercise surge of circulating catecholamines in trout probably exerted a

protective effect on blood oxygen transport during the period of acidosis. The catecholamine effect was not entirely successful, however, as hemoglobin-bound O_2 still fell significantly after exercise (Fig. 7.4C). Nonetheless, considering the post-exercise reduction in Pa_{O_2} (Fig. 7.4A) and rise in P_{CO_2} (Fig. 7.1C), the fall in Hb-bound oxygen *in vivo* was less than predicted from *in vitro* oxygen dissociation curves (see Fig. 7.8). These relationships were determined by Eddy (1971) in the absence of exogenous Ad, using the mixing technique of Haab, Piiper and Rahn (1960). They represent the most complete set of $Hb\text{-}O_2$ dissociation curves available for trout blood. The *in vivo* curve, based on the present data, shows that after exercise for a given P_{O_2}, the O_2 content was greater than predicted from the *in vitro* curve, thus suggesting that Ad did exert a protective effect on blood oxygen transport. Nikinmaa (1983) has demonstrated a similar effect of Ad on the hemoglobin-oxygen dissociation curve of trout red cells suspended in saline *in vitro* .

The post-exercise reduction in Pa_{O_2} (Fig. 7.4A) was similar to that reported by Primmett *et al.* (1986). These authors attributed the decline in Pa_{O_2} to a measured reduction in ventilatory frequency. However, other studies on rainbow trout have reported either constant or elevated Pa_{O_2} after exercise (Kiceniuk and Jones, 1977; Holeton *et al.* , 1983). Reasons for these differences are not immediately clear, but may include seasonality, now recognized as an increasingly important variable in fish physiology. For example, in the eel, physiological doses of Ad and

Figure 7.8. An *in vivo* blood O_2 dissociation curve (solid line) in rainbow trout constructed from all simultaneous determinations of blood hemoglobin bound O_2 ($[O_2]/[Hb]$) and P_{O_2} in the 8 fish of the exercise group. Open circles (○) are data obtained at rest and when circulating levels of Ad and Nad were not significantly elevated above rest (i.e. at 4, 8, 12, and 24 h, Fig. 7.2A,B). Darkened circles (●) are those points obtained when circulating Ad and Nad levels were significantly elevated ($p<0.05$) above resting levels (i.e. at 0, 0.5, 1, and 2 h, Fig. 7.2A,B). *In vitro* blood O_2 dissociation curves (dotted lines) for rainbow trout at 15 ^{o}C, P_{CO_2} = 2 and 6.5 torr, determined by Eddy (1971) are shown for comparison. Curves were fitted by eye.


umol O2 /g Hb
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
PO2 torr
in vivo
in vitro
2.0 torr
in vitro
6.5 torr

Nad resulted in a stimulation of heart rate and ventilation in the summer (May-July), but an inhibition in the winter (mid-December - mid-February; Peyraud-Waitzenegger, Barthelemy and Peyraud, 1980). In isolated saline-perfused rainbow trout gills, vasoconstriction was the dominant response to Ad in the winter (February-March), while at other times of the year, β-adrenergic vasodilation predominated (Pärt, Kiessling and Ring, 1982). These effects were attributed to seasonal differences in α- and β- adrenoceptors responses, with the former dominant in winter. The present experiments were performed in November and December. Therefore, the observed reduction in Pa_{O_2} post-exercise may be due to a 'winter' α-receptor mediated response to the elevated levels of circulating catecholamines.

Starry Flounder

The exercise-induced extracellular acid-base disturbance in starry flounder was virtually identical to that previously described (eg. Fig. 5.1, Chapter V), and the same explanations probably apply. In comparison to trout, pHa was depressed and P_{CO_2} elevated to the same extent, though $[HCO_3^-]$ declined and blood [lactate] rose to a lesser extent.

Circulating levels of Ad and Nad in resting starry flounder were 6-7 times greater than in trout, and generally higher than those reported for other fish species as well (Table 7.1). Circulating catecholamine levels have not been measured previously in flatfish. Perhaps these sluggish animals require higher maintenance levels than do more active species, which may be related to the observation that

hearts of pleuronectid flatfish lack adrenergic innervation (Santer, 1985), but operate with relatively high cardiac stroke volume at rest (Wood et al. , 1979b).

In contrast to the rainbow trout, circulating levels of Ad and Nad did not increase significantly after exercise in starry flounder (Fig. 7.2E,F). This probably explains why the pharmacological experiments of Wood and Milligan (1986) on flounder could not demonstrate an adrenergic involvment in lactate retention in white muscle or in the kinetics of the acid-base correction. Perhaps as a consequence of this lack of catecholamine mobilization, flounder red cell pHi was less well regulated after exercise than trout red cell pHi. The slope of the in vivo relationship between RBC pHi and pHe in flounder was about double that in trout (0.39 versus 0.20, Fig. 7.7A,B), reflecting the better regulation of trout RBC pHi in vivo . Unfortunately, in vitro data are not yet available for flounder blood. However, the in vivo slope was much lower than the in vitro slope for a number of other fish species (eg. plaice: 0.60, Wood et al. , 1975; sheatfish: 0.73, Albers et al. , 1981; carp: 0.54, Albers and Goetz, 1985; trout: 0.73, Fig. 2.5, Chapter II). This suggests that some RBC pHi regulation did occur in vivo . It is not clear why the present flounder did not regulate RBC pHi to the same extent as observed in Chapter VI, but it may be related to the use of a different batch of fish (collected in 1984 versus 1982). The lack of a post-exercise catecholamine surge may also explain why there was no evidence of metabolic degradation of red cell NTP nor significant dilution of cellular NTP stores (Fig. 7.5C,D).

Even though Pa_{O_2} remained constant in flounder, the amount of oxygen bound to hemoglobin fell by about the same amount as in trout (23%) immediately after exercise (Fig. 7.4). The fall in RBC pHi, increase in P_{CO_2}, and the lack of a compensatory reduction in cellular NTP levels were, no doubt, contributing factors. On the surface, it would appear that flounder did not attempt to regulate O_2 transport during the post-exercise acidosis, compared to trout. However, hemoglobin oxygenation was restored to rest levels 2-3 h prior to the correction of red cell pHi and reduction in Pa_{CO_2}. Furthermore, assuming the Root and Bohr shifts in starry flounder blood in vitro are similar to those reported for the closely related winter flounder (Pseudopleuronectes americanus) (Hayden, Cech and Bridges, 1975), then for the observed rise in P_{CO_2}, hemoglobin-oxygen saturation would have fallen by about 30%, rather than the observed 23%. These observations suggest that factors in addition to those considered in the present study must also influence hemoglobin-oxygen saturation in flounder.

The role of catecholamines in blood O_2 transport after exercise

In summary, this study has indicated that in rainbow trout, catecholamines released into the circulation after exhaustive exercise may play an important role in sustaining O_2 transport during the associated plasma acidosis. After a period of intense activity in this active, pelagic fish, O_2 transport to the aerobic muscles would be maintained. Therefore the capacity for continued swimming following glycolytic exhaustion of the white muscle would not be compromised. In contrast, catecholamines did not appear to play a

comparable role in starry flounder, perhaps reflecting the difference between trout and flounder in terms of their dependence on sustained swimming ability. Nonetheless, there was evidence of some protection of blood O_2 transport in flounder, suggesting other unmeasured factors were influencing hemoglobin-oxygen affinity after exhaustive exercise.

CHAPTER VIII

GENERAL SUMMARY AND CONCLUSIONS

Introduction

This thesis has examined the effects of exhaustive exercise on acid-base and metabolite status in the intracellular and extracellular compartments in two very different fish, rainbow trout and starry flounder. The results have shown that the pattern of recovery of the intracellular acid-base and metabolite disturbance in the muscle and the disposition of the lactate and metabolic acid loads differs between the two species. These different patterns are summarized in Fig. 8.1.

H^+ and Lactate Production in Muscle

Trout and flounder both experienced a severe intracellular acidosis in the white muscle mass after exhaustive exercise (1 in Fig. 8.1). In both species , this acidosis was associated with a reduction in white muscle glycogen content and an increase in lactate levels. H^+ and lactate were not produced in equimolar quantities in either trout or flounder, with H^+ produced in excess of lactate in both species. In trout, this excess of H^+ was equivalent to the net reduction in ATP levels, suggesting that glycolysis and ATP hydrolysis became uncoupled. Accumulation of hexose-phosphates may have also contributed to the H^+ excess (Chapter I). Overall, lactate and H^+ production was about 3-fold greater in trout than in flounder, reflecting the greater glycolytic capacity of the trout.

Figure 8.1. Schematic diagram of the proposed fates of H^+ and lactate after exhaustive exercise in rainbow trout (A) and starry flounder (B).

1: H^+ and lactate production

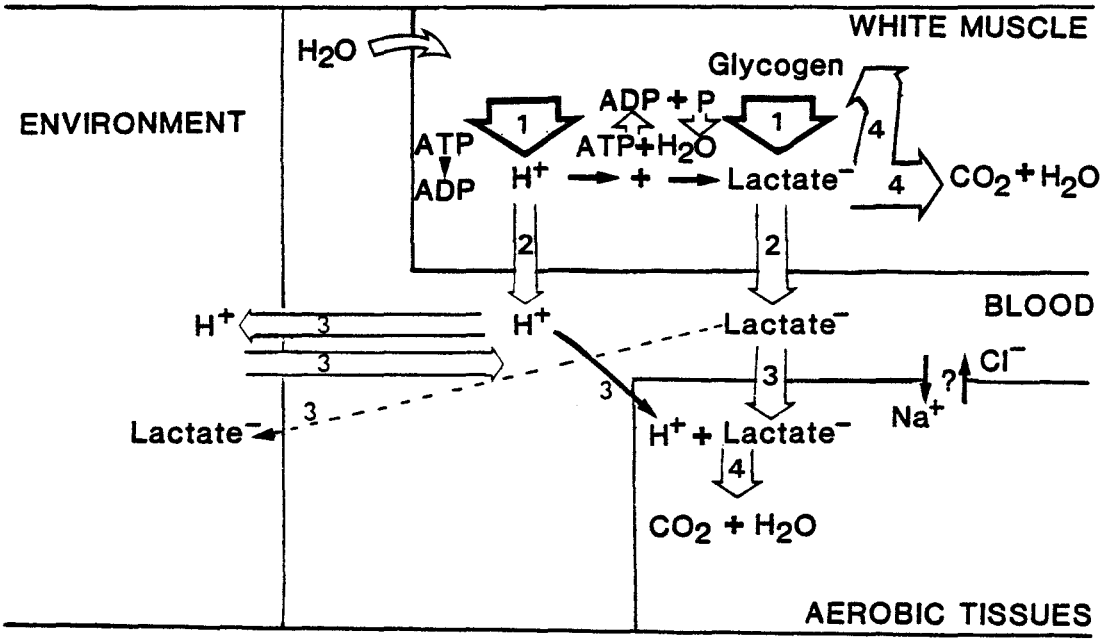
2: H^+ and lactate appearance in the blood

3: H^+ and lactate distribution between the ICF, ECF and environmental H_2O

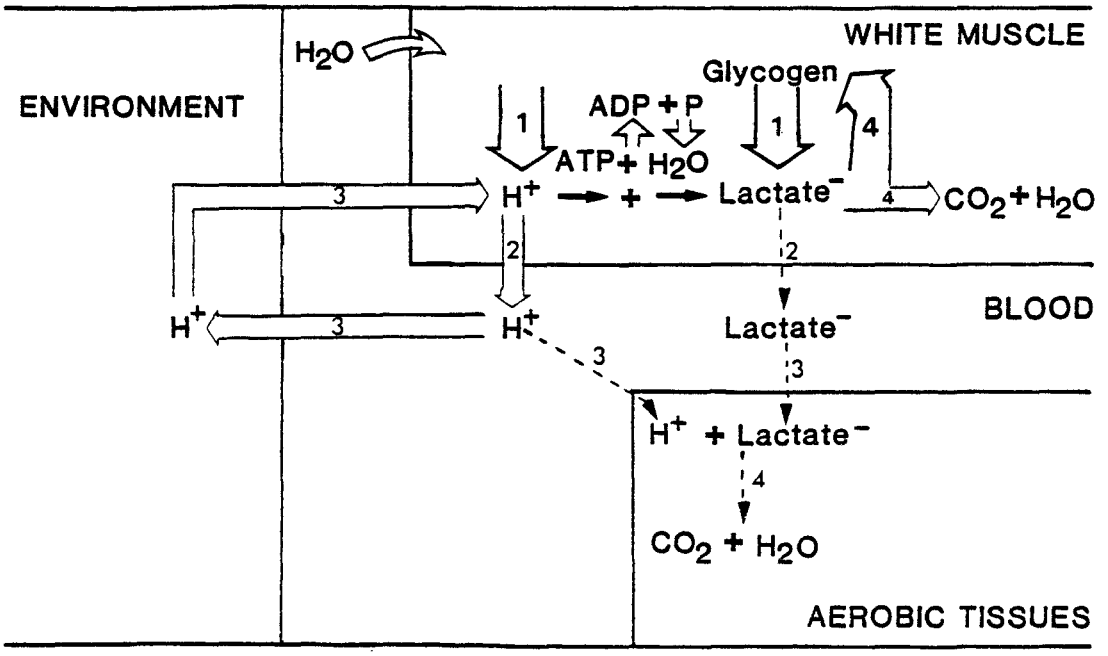
4: Fate of lactate

See text for details. Width of arrows indicate relative amounts of H^+ or lactate. Compartments are not drawn to scale.

A Rainbow Trout



B Starry Flounder



The muscle intracellular acid-base disturbance was corrected more rapidly in flounder (4-8h) than in trout (8-12). This occurred before the correction of extracellular acid-base status in flounder (8-12h), but after the correction of extracellular acid-base status in trout (4-8h). A more rapid restoration of muscle metabolite status in flounder was associated with the more rapid correction of the intracellular acid-base disturbance (by 12h). In trout, restoration of muscle metabolite status was much slower, requiring up to 24h.

In both fish, exhaustive exercise led to a contraction of the whole body extracellular fluid compartment at the expense of the intracellular compartment. The fluid shift was largely due to a shift of water from the muscle extracellular to intracellular space, by the increase in osmotically active particles, due in part to the breakdown of glycogen into lactate. The shrinkage of the extracellular compartment resulted in a general hemoconcentration, with plasma [protein], [hemoglobin] and hematocrit increasing, though red cell swelling also contributed to the increase in hematocrit.

H^+ and Lactate Appearance in Blood

The pattern of H^+ and lactate accumulation in the blood after exercise differed in trout and flounder (2 in Fig. 8.1). In the former, lactate appeared in excess of H^+, whereas in the latter, the reverse occurred. H^+ appearance was similar in the two species. The results of this thesis suggest that neither differential excretion nor uptake was responsible for the H^+ - lactate discrepancy in the blood. Instead, these results support the hypothesis that differential release of lactate from the muscle mass

was responsible for this discrepancy (Wardle, 1978; Batty and Wardle, 1979; Turner, Wood and Clark, 1983; Turner, Wood and Hōbe, 1983; Turner and Wood, 1983). However, the mechanism(s) responsible for this differential release phenomenon are not understood.

Distribution of H^+ Between the ICF, ECF, and the Environment

Both trout and flounder used the environmental water as a temporary storage site for H^+ produced during exercise. Negligible amounts of lactate were moved into the water. However, the overall contribution this played in the correction of the acid-base disturbance differed between species (3 in Fig. 8.1). In starry flounder, approximately 20% of the total metabolic acid load passed through the extracellular compartment and was transiently stored in the water. This transfer of acidic equivalents to the water resulted in an intracellular alkalosis and thus appeared to hasten the recovery of intracellular acid-base status , perhaps as a means of aiding metabolic recovery (see below).

In contrast, in rainbow trout, the bulk of the metabolic acid load (about 94%) remained in the intracellular compartment; only a small percentage (about 6% - though approximately the same absolute amount as in flounder) appeared in the extracellular compartment, then subsequently in the water. Storage of these acidic equivalents in the water resulted in an extracellular alkalosis, and thus appeared to expedite the recovery of extracellular acid-base status , perhaps as a means of protecting blood O_2 transport. The metabolic acid remaining in the intracellular compartment was cleared by aerobic metabolism (see below).

Fate of Lactate

The results of this thesis argue against a significant role for the Cori cycle in clearance of the lactate load produced during exhaustive exercise in rainbow trout and starry flounder. Instead, the bulk of the lactate is probably retained within the muscle mass and metabolized *in situ*, by oxidation to CO_2 or by glyconeogenesis (4 in Fig. 8.1). In flounder, this is almost the sole fate of lactate, as almost none appears in the blood space. What little is released is presumably taken up and metabolized by aerobic tissues (eg. liver, heart, red muscle, gills, kidney).

While most of the lactate is retained in the muscle for metabolism *in situ* in trout, a significant portion (approximately 20%) is exported to the blood space, then taken up and metabolized by the aerobic tissues. Evidence is presented in this thesis suggesting that some lactate is taken up by the aerobic tissues without accompanying H^+, ie. with an accompanying cation (eg. Na^+) or in exchange for an anion (eg. Cl^-) and subsequently metabolized, leading to an intracellular alkalosis. Mechanisms of lactate transport into and out of fish tissue are poorly understood.

Species Differences

Throughout this thesis, differences in the swimming performance of trout and flounder have been emphasized. These differences may explain the observed dissimilarities in the physiology of recovery from exhaustive exercise. The rainbow trout is an active, pelagic fish, heavily dependent upon the capacity for aerobic swimming. In this fish then, it is important to maintain adequate

O_2 delivery to the aerobic 'red' muscle. This is supported by the results of Chapter VII, which suggested that the trout attempted to maintain O_2 transport, *via* catecholamine mobilization, during the post-exercise extracellular acidosis. Trout further protect blood O_2 transport by releasing only a small percentage (6%) of the total H^+ produced into the extracellular compartment and by transferring acidic equivalents from the extracellular space to the environment. Furthermore, release of lactate from the muscle to the blood provides fuel for the aerobic red muscle at a time when glucose may be limiting. In trout therefore, physiological adjustments are made to protect aerobic swimming capacity after exhaustion of the glycolytic white muscle.

In contrast, the starry flounder is a benthic, sluggish fish, in which the burden of locomotion is placed on the anaerobic, white muscle mass (Duthie, 1982). Even severe anaemia does not hinder exercise performance in these fish (Wood *et al.* , 1979b), further illustrating the low dependence of flounder on 'aerobic' swimming. Therefore, in flounder, correction of the intracellular acidosis to a level compatible with further metabolism would be of greater importance than correction of the extracellular acidosis and maintenance of O_2 delivery. The results of Chapter VII lend support to this argument as flounder did not mobilize catecholamines to regulate O_2 transport during the post-exercise extracellular acidosis. The fishs' future survival depends more on the ability to use the muscle again glycolytically, rather than on the capacity for aerobic swimming.

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APPENDIX I: Extracellular and intracellular acid-base status following strenuous activity in the sea raven (*Hemiptripterus americanus*)

The work presented in this appendix is related to the subject of the thesis and was carried out in collaboration with Dr. A.P. Farrell at the Huntsman Marine Laboratory, St. Andrews, New Brunswick during the course of my Ph.D studies at McMaster. The paper is now in press in The Journal of Comparative Physiology, Volume B.

EXTRACELLULAR AND INTRACELLULAR ACID-BASE STATUS FOLLOWING STRENUOUS ACTIVITY IN THE SEA RAVEN (Hemiptripterus americanus)

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ABSTRACT

Exhausting activity in the sea raven resulted in a pronounced extracellular acidosis, which consisted of a large, short-lived respiratory component and a small, longer-lived metabolic component. This disturbance had been corrected by 12h. White muscle experienced a pronounced intracellular acidosis of chiefly metabolic origin, with pHi dropping from a rest value of 7.51 to a low of 7.10 immediately post-activity. The recovery of pHi was associated with a reduction in muscle lactate. Despite the large increase in P_{CO_2}, cardiac muscle pHi remained constant post-activity, actually showing an alkalosis at 30 min into recovery. Maintenance of cardiac muscle pHi was achieved by an accumulation of intracellular HCO_3^-.

INTRODUCTION

The nature of the extracellular acid-base disturbance following bouts of strenuous activity has been well defined in several fish species (eg. Piiper *et al.* 1971; Wood *et al.* 1977; Holeton *et al.* 1983; Turner, Wood and Clark, 1983; Turner, Wood and Hobe, 1983). This disturbance is characterized by a large, short-lived respiratory component (elevation of P_{CO_2}) and a more prolonged metabolic component (accumulation of fixed acid). The nature of the acid-base disturbance within the muscle, where acidic anaerobic endproducts are produced, has not been examined, nor have the effects of the extracellular disturbance on other tissues. These aspects have not been studied chiefly because it was thought that DMO (5,5-dimethyl -2,4- oxazolidinedione) (Waddell and Butler, 1959), the intracellular pH marker commonly used *in vivo*, would not be a reliable marker for pHi transients (Roos and Boron, 1978). Recently, it has been shown in rainbow trout tissues that the DMO distribution method is a reliable and sensitive method for measuring pHi transients *in vivo*, with a temporal resolution adequate to detect changes occuring after exercise (less than 15 min; Milligan and Wood, 1985).

In vitro studies have shown that an extracellular respiratory acidosis has a deleterious effect on cardiac performance in a variety of fish species (see Gesser and Poupa, 1983 for a review). This is manifested as a reduction in cardiac contractility, due presumably to increased competition between Ca^{2+} and H^{+} (resulting from CO_2 entry) for binding sites on troponin (Gesser and Poupa, 1983).

However, following strenuous exercise, when there is a pronounced respiratory acidosis (Wood *et al.* 1977; Turner, Wood and Clark, 1983) cardiac performance is probably not diminished, but rather may be enhanced, with cardiac output increasing by as much as 60% (Neumann *et al.* 1983). There may be some mechanism operating *in vivo* to offset the deleterious effects of high CO_2 observed *in vitro*. One such mechanism may be prevention of an intracellular acidosis in the first place, i.e., pHi regulation.

This possibility was investigated *in vivo* in sea raven following strenuous, enforced activity. In addition, the intracellular acid-base disturbance in white muscle was also examined. Extracellular acid-base, metabolite and ionic status was also assessed to provide a more complete picture of the physiological changes associated with strenuous activity in this normally inactive teleost.

MATERIALS AND METHODS

Experimental Animals

Adult sea raven (*Hemitripterus americanus*), weighing 700-3950 g, were collected by otter trawl in Passamaquaddy Bay, New Brunswick in mid-summer. Fish were held outdoors in large fiberglass tanks continuously supplied with fresh seawater at ambient temperature (10-11^{o}C, salinity 29 ^{o}/oo) at Huntsman Marine Laboratory for at least 48h prior to experimentation.

Fish were anaesthetized in MS:222 (1:10,000) and the hypobranchial artery cannulated to allow arterial blood sampling without disturbing the fish. An incision was made on the ventral

surface at the midline of the isthmus, the vessel exposed and the posterior end tied off with a silk suture. The anterior portion was then clamped shut, a nick made in the vessel between the two occlusions, a piece of PE50 polyethylene cannula tubing fed caudally 3mm into the vessel, the cannula anchored to the vessel with a silk suture and the clamp removed. The wound was dusted with the antibiotic oxytetracycline hydrochloride (Syndel Labs, Vancouver) and closed with silk sutures.

Fish were then placed in large aquaria (50 l) supplied with running seawater at ambient temperature and allowed to recover at least 24h prior to experimentation. Approximately 12h before fish were sampled, they were infused with a 0.25 ml/kg dose of 10 uCi/ml ^{14}C-DMO (New England Nuclear; specific activity: 50 mCi/mmol) plus 40 uCi/ml ^{3}H-mannitol (New England Nuclear; specific activity: 27.4 mCi/mmol) in saline followed by an equal volume of Cortland saline adjusted to 160 mM NaCl (Wolf, 1963).

To induce strenuous activity, fish were rapidly transferred to a large rectangular tank (500 l) then vigorously chased by hand for 10 min. By the end of this period, fish were refractory to further stimulation, but it is not known if the animals were completely exhausted, as muscle glycogen content was not measured (Wardle, 1978).

Individual fish were terminally sampled prior to (rest) and immediately following activity (0h), as well as 0.5h, 2h and 12h post-activity. At each sample time, 5-6 animals were sacrificed. Immediately after obtaining a blood sample (2000 ul) from the cannula, fish were removed from the water and muscle biopsies quickly taken

(less than 15 sec). Struggling by fish after removal from the water was minimal. Ten muscle samples (70-100 mg) were taken by punching 10 stainless steel biopsy needles (ID= 4.78 mm) through the dorsal epaxial muscle mass. Samples were frozen immediately in the needles in liquid nitrogen. Fish were then quickly killed by a cephalic blow and the heart excised. Excess blood was blotted off and the heart immediately frozen in liquid nitrogen.

Blood samples were analyzed for pH, total CO_2 in both plasma and whole blood, [hemoglobin], whole blood [lactate] and [glucose] and plasma for concentrations of Na^+, Cl^-, K^+, Ca^{2+}, ammonia, protein, and levels of ^3H and ^{14}C radioactivity. Muscle and heart were analyzed for ^3H and ^{14}C radioactivity, total tissue water, with the additional measurement of lactate in muscle.

Analytical Techniques and Calculations

Blood samples were drawn from the hypobranchial artery cannula using gastight Hamilton syringes. Whole blood pH was measured on 40 ul samples injected into a Radiometer pH microelectrode maintained at experimental temperature (10-11°C) and linked to a Radiometer PHM-71 acid-base analyzer. Total CO_2 (plasma and whole blood) was measured on 50 ul aliquots using a Corning CO_2 analyzer. Whole blood and plasma [HCO_3-] and P_{CO_2} were calculated using the Henderson-Hasselbach equation, as described by Wood *et al.* (1977).

Whole blood metabolic acid load (ΔH^+m) was calculated according to the equation:

$$\Delta H^+m \text{ (mmol/l)} = [HCO_3^-]_1 - [HCO_3^-]_2 - \beta(pH_1 - pH_2) \quad (1)$$

(McDonald *et al.* 1980) where 1 and 2 refer to initial and final measurements, $[HCO_3^-]$ is measured in whole blood and β, the non-bicarbonate buffer capacity of whole blood, was predicted from the [hemoglobin]. Since terminal, rather than serial blood sampling was employed, the analysis used mean values at each sample time, hence no estimate of variance in ΔH^+m is possible. The relationship between β and [hemoglobin] was determined for sea raven plasma and whole blood at experimental temperature by *in vitro* tonometry at a range of P_{CO_2}'s by methods previously described by Wood *et al.* (1977).

Whole blood [hemoglobin] (as cyanmethemoglobin) and plasma [protein] (by refractometry) were measured according to Milligan and Wood (1982). Whole blood lactate was measured on 100 ul blood, deproteinized with 200 ul chilled 8% perchloric acid and centrifuged at 9,000 g for 5 min. The supernatant was analyzed enzymatically for lactate using Sigma reagents. Whole blood [glucose] was analyzed on 100 ul samples deproteinized with 900 ul chilled 3% trichloracetic acid, using the O-toluidine method of Hyvarenon and Nikkita (1962) and Sigma reagents.

Plasma levels of Na^+ (20 ul), K^+ (100 ul) and Ca^{2+} (30 ul) were measured after dilution (1:1000; 1:30 and 1:50, respectively) on a Varian AA-1275 atomic absorption spectrophotometer. Plasma Cl^- (20 ul) levels were measured on a Radiometer CMT-10 chloride titrator. Plasma ammonia levels were measured on 100 ul

plasma, extracted 1:4 with chilled 12.5% trichloracetic acid using a micro-modification of the salicylate-hypochlorite method of Verdouw et al. (1978).

For determination of ^{3}H and ^{14}C radioactivity in the extracellular fluid, 100 ul of plasma was added to 10 ml fluor (ACS: Amersham). Tissue levels of ^{3}H and ^{14}C radioactivity were assayed by digesting duplicate samples of ventricle (50-100 mg) or white muscle (70-100 mg) in 1 ml tissue solubilizer (NCS; Amersham) for 2-4h at 50°C until a clear solution was obtained. The solution was neutralized with 30 ul glacial acetic acid, then 10 ml fluor (OCS; Amersham) was added. Samples were stored in the dark overnight to reduce chemilumnescence, then counted on an LKB Wallac liquid scintillation counter. Dual label quench correction was performed using the external standard ratio method as described by Kobayashi and Maudsley (1974). Muscle lactate was measured on 80-100 mg samples homogenized in 1 ml 8% perchloric acid with a glass homogenizer for 5 min, according to Turner, Wood and Clark (1983). Values obtained were corrected for trapped extracellular fluid using volumes determined on samples taken simultaneously (see below) and expressed as per kg muscle cells. Tissue water content was determined by drying tissues to a constant weight at 85°C.

Tissue extracellular fluid volume (ECFV) was calculated according to the equation:

$$\text{ECFV (ml/g)} = \frac{\text{Tissue } [^{3}\text{H-mannitol}] \text{ (dpm/g)}}{\text{Plasma } [^{3}\text{H-mannitol}] \text{ (dpm/ml)/Plasma } H_2O \text{ (ml/ml)}} \quad (2)$$

assuming a plasma water content of 96% (Milligan and Wood, 1985).

Tissue intracellular fluid volume (ICFV) was calculated as the difference between total water content and ECFV. Tissue pHi was calculated according to the equation:

$$pHi = pK_{DMO} + \log\{\frac{[DMO]i}{[DMO]e} \times (10^{(pHe - pK_{DMO})} + 1) - 1\} \quad (3)$$

where pHe is blood pH, pK_{DMO} is taken from Malan *et al.* (1976),

$$[DMO]e \text{ (dmp/ml)} = \frac{\text{Plasma } [^{14}\text{C-DMO}] \text{ (dpm/ml)}}{\text{Plasma } H_2O \text{ (ml/ml)}} \quad (4)$$

and

$$[DMO]i \text{ (dpm/ml)} = \frac{\text{Tissue } [^{14}\text{C-DMO}] \text{ (dpm/ml)} - (\text{ECFV (ml/g)} \times [DMO]e \text{ (dpm/ml)})}{\text{Tissue ICFV (ml/g)}} \quad (5)$$

Estimates of ventricular and white muscle ΔH^+m were calculated by equation (1) where pH refers to pHi and $[HCO_3]^-$ refers to intracellular HCO_3^- and β is the non-bicarbonate buffer capacity. Intracellular $[HCO_3^-]$ was calculated from the Henderson-Hasselbach equation, assuming P_{CO_2} was in equilibrium between the ICF and ECF. β was determined by acid titration of tissue homogenates under a nitrogen atmosphere (Cameron and Kormanik, 1982). Tissue (1-2g) was frozen and pulverized under liquid nitrogen with a mortar and pestle, then suspended in 5 ml 0.9% NaCl. The tissue homogenate was titrated to pH 8.0 with 1N NaOH, allowed to stabilize, then back titrated to pH 6.5 with 0.02N HCl, under a nitrogen atmosphere, at experimental temperature. A Radiometer G-202 pH electrode was employed. The slope of the pH *versus* mmol HCl relationship over the pHi range observed *in vivo*

(7.6-6.7) was taken as the buffer capacity of the tissue, in mmol/pH/l ICF.

Statistical Analyses

Means ± 1 SEM are reported throughout, unless otherwise stated. Significant differences ($P<0.05$) between groups were tested with Student's two-tailed t-test, unpaired design.

RESULTS

Blood and Tissue Buffer Capacities

In vitro tonometry of whole blood demonstrated a linear relationship between B, the non-bicarbonate buffer capacity, and [hemoglobin], according to equation (6), where B is in mmol HCO_3^-/pH/l and [Hb] is in g/100 ml;

$$B = -1.55\ [Hb] - 2.38,\ r=0.97,\ n=6 \qquad (6)$$

where the intercept, -2.38 represents the B value of plasma.

Acid titrations of white (n= 6 determinations from 4 fish) and ventricle (n = 4 determinations from 4 fish) muscle homogenates yielded mean values of -27.9 ± 1.7 and -30.9 ± 2.9 mmol/pH/kg wet tissue, respectively over the pH range of 6.7-7.6. On this basis, the buffer values of the two tissues are quite similar. When expressed on the basis of ICFV, heart has a greater buffer capacity (-84.6 mmol/pH/l ICF) than white muscle (-39.0 mmol/pH/l ICF) because of the much larger ECFV (344.7 ± 24.6 ml/kg, n=6) relative to ICFV (486.2 ± 24.2 ml/kg, n=6) of the heart compared to the white muscle (113.4 ± 8.1 and 710.2 ± 24.2 ml/kg, n=6, respectively). It is recognized that these values represent total physicochemical buffer capacity of the

tissue (i.e. non-bicarbonate + bicarbonate). However, intracellular bicarbonate levels (over the pH range examined) would be quite low, at most 1 mmol/l, as P_{CO_2} was kept low, and hence, would not contribute significantly to the measured buffer capacity.

Extracellular Acid-Base, Ion and Metabolite Status Following Activity

Ten minutes of enforced activity resulted in a pronounced extracellular acidosis. Whole blood pHa declined immediately post-exercise, recovering slowly by 12h (Fig. 1A). This acidosis was largely of respiratory origin, with P_{CO_2} increasing 3-fold, while plasma [HCO_3^-] did not change significantly (Fig. 1B,C). Analysis based upon the principles outlined in Wood *et al.* (1977), revealed that immediately post-activity and up to 0.5h, the acidosis was 80-90% of respiratory origin.

Resting whole blood lactate levels were quite low (0.25 $\pm$ 0.05 meq/l, n=6) and rose slightly, but significantly, following activity to peak levels (0.63 $\pm$ 0.10 meq/l, n=6) at 0.5h, returning to rest levels by 12h (0.18 $\pm$ 0.02 meq/l, n=5). The blood metabolic acid load (H^+m) was much higher than the lactate load (La-) (Fig. 2B), peaking immediately post-activity, then slowly declining, showing a deficit by 12h.

Both whole blood [glucose] and plasma [protein] rose significantly at 0.5h post-activity (3.2 $\pm$ 0.5 mmol/l and 4.8 $\pm$ 0.6 g/100 ml, n=6, respectively), returning to rest levels by 2h (1.8 $\pm$ 0.1 mmol/l and 3.03 $\pm$ 0.3 g/100 ml, n=5, respectively). There were no significant changes in either [hemoglobin] or plasma levels of Na^+, Cl^-, K^+ or Ca^{2+} post-activity. Plasma ammonia

Figure 1. Blood acid-base status in sea raven prior to and following activity. A) arterial pH; B) arterial plasma [HCO_3^-]; C) arterial P_{CO_2}. R=rest; bar = 10 min of enforced activity; 0h= immediately post-activity; * = a significant difference ($P<0.05$) from rest values. Means $\pm$ 1 SEM. R, 0.5h and 2h, n=6; 0h and 12h, n=5.

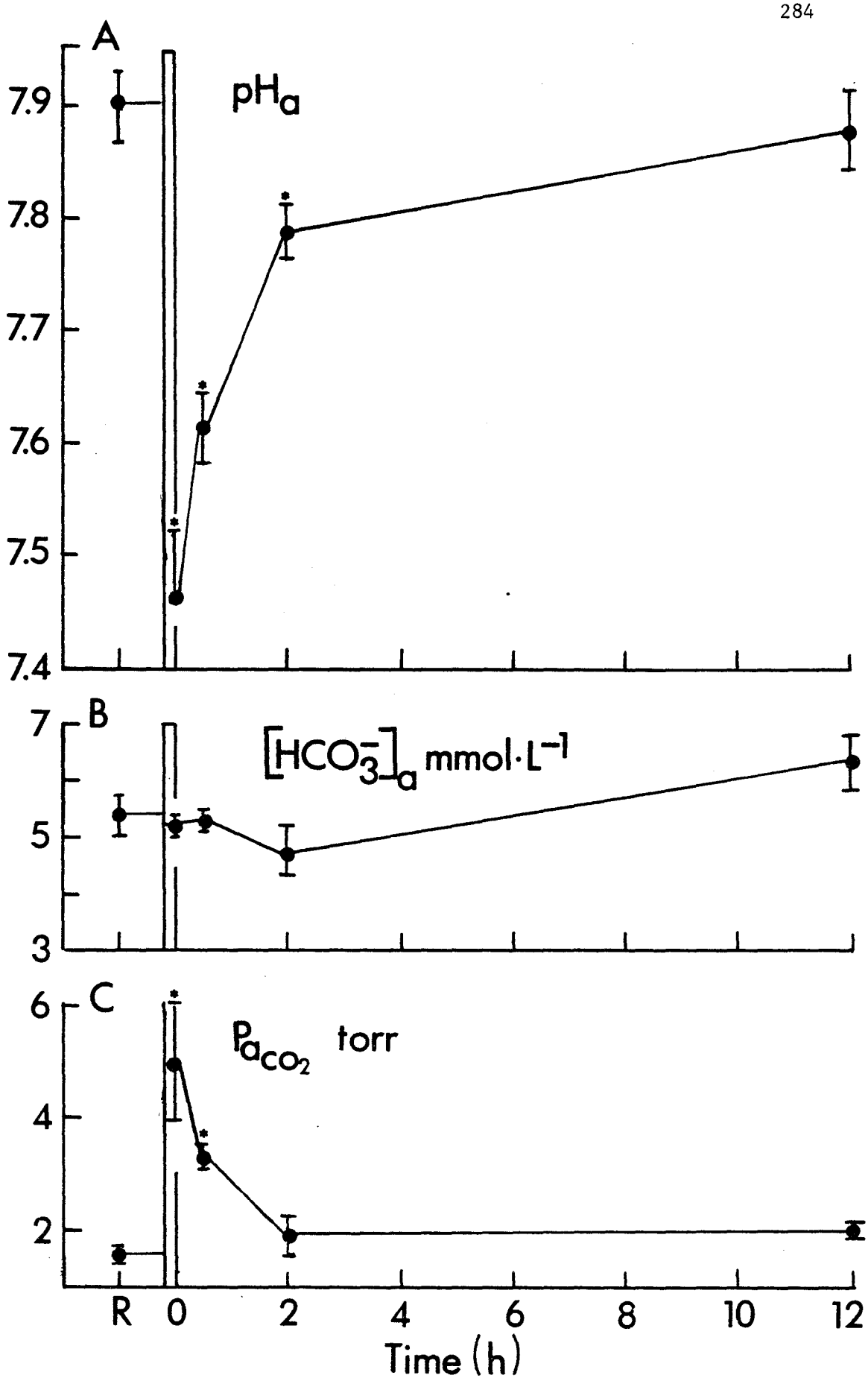
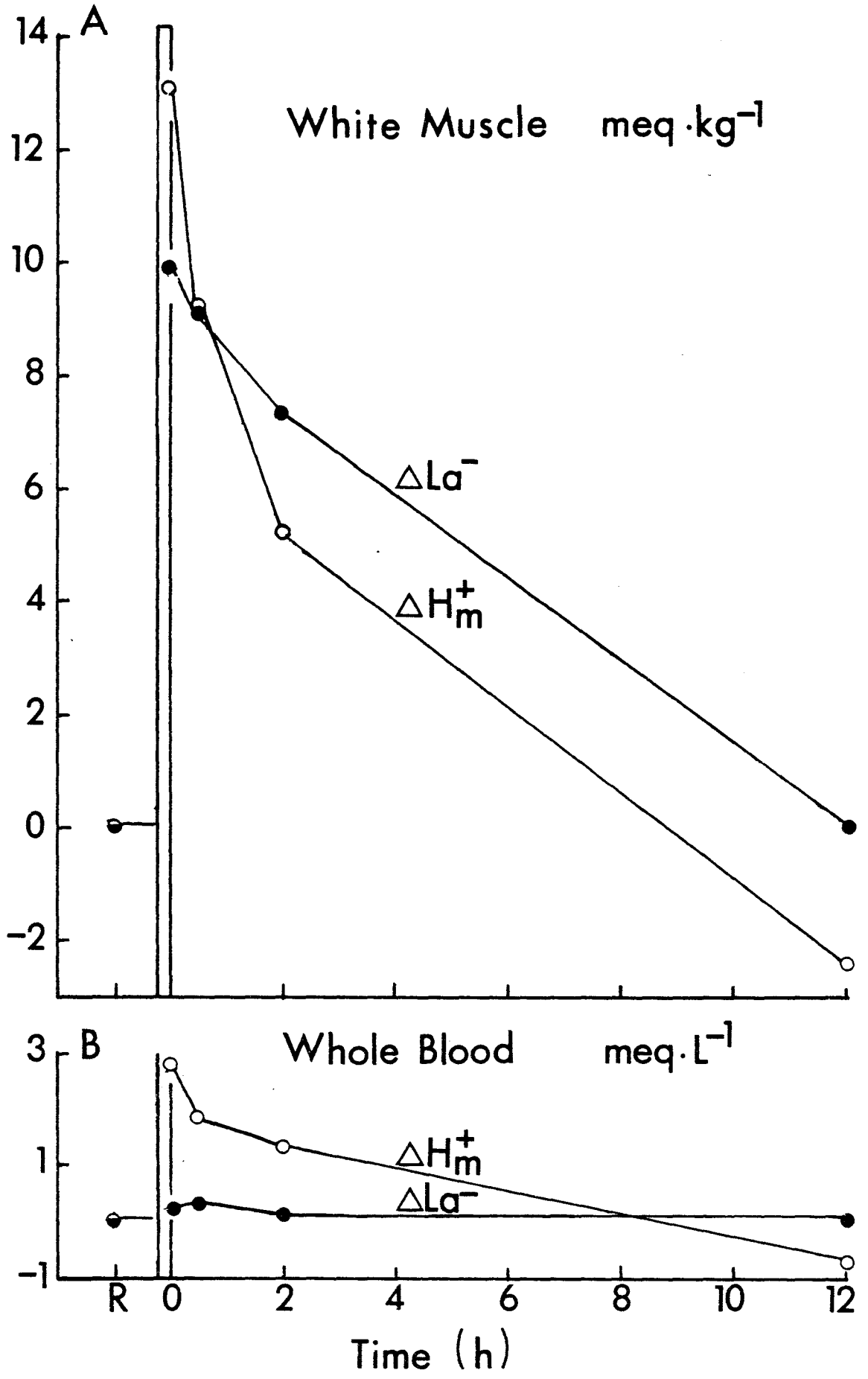

A
pHa
7.9
7.8
7.7
7.6
7.5
7.4
B
[HCO3-]a mmol·L-1
7
5
3
C
Paco2 torr
6
4
2
R
0
2
4
6
8
10
12
Time (h)

Figure 2. White muscle (A) and whole blood (B) metabolic acid ($\triangle H^{+}m$, O) and lactate ($\triangle La^{-}$, ●) loads following activity. R= rest, bar = 10 min of enforced activity; 0h= immediately post-activity.


A
White Muscle meq·kg⁻¹
14
12
10
8
6
4
2
0
-2
ΔLa⁻
ΔH⁺m
B
Whole Blood meq·L⁻¹
3
1
-1
ΔH⁺m
ΔLa⁻
R
0
2
4
6
8
10
12
Time (h)

levels, however, were significantly lower at 0.5h (350.2 ± 8.2 ueq/l, n=6), 2h (332.5 ± 27.2 ueq/l, n=6) and 12h (280.2 ± 6.5 ueq/l, n=5) post-activity than at rest (386.4 ± 58 ueq/l, n=6). The effect of this change on blood acid-base status is likely to be negligible, for blood pH would change by, at most, 0.013 units, which would not be readily detectable (Cameron and Heisler, 1983).

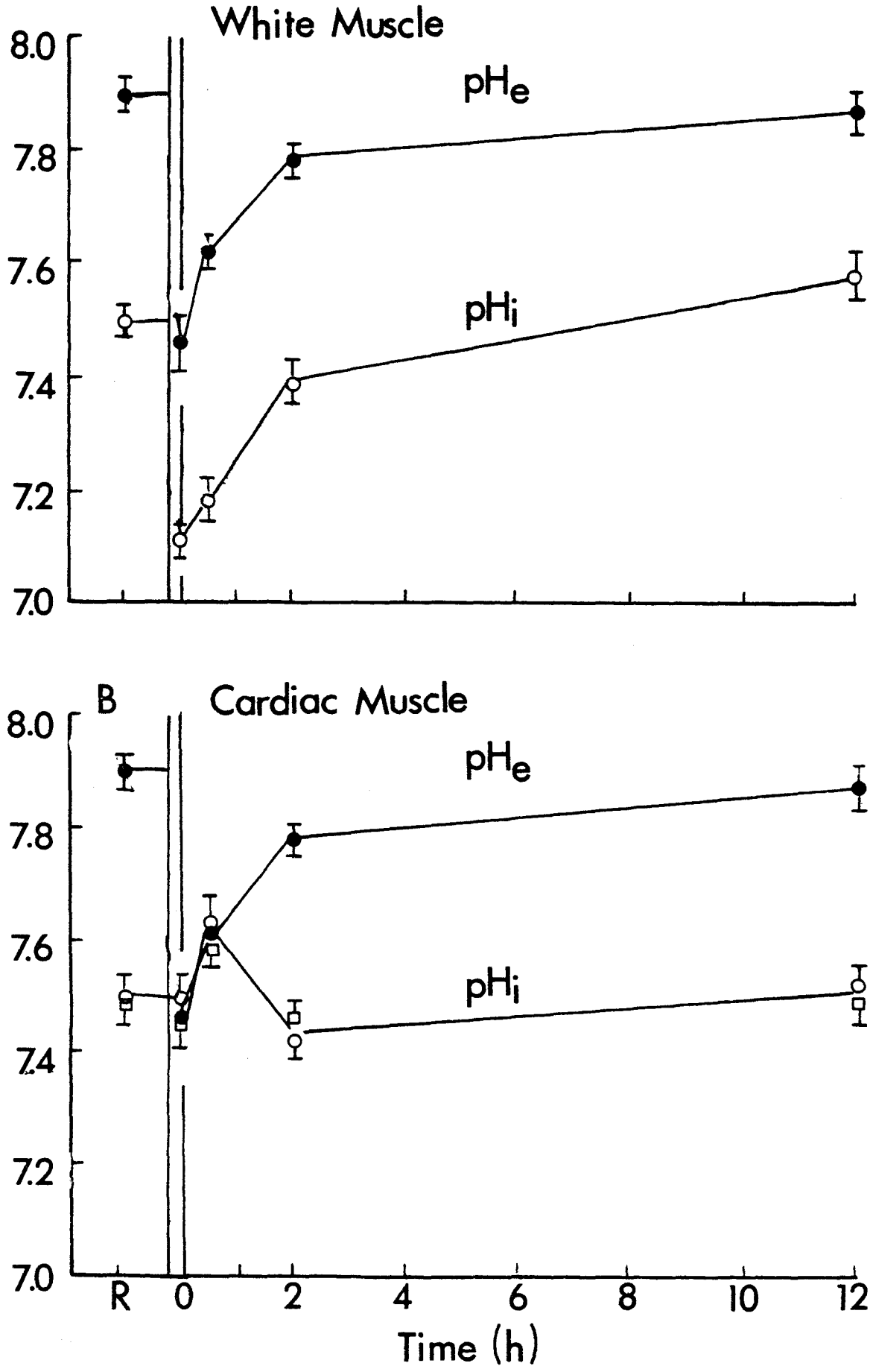
Intracellular Acid-Base and Metabolite Status Following Activity

At rest, white muscle pHi averaged 7.51 ± 0.04 (n=6), with pHe = 7.897 ± 0.036 (n=6). Immediately following activity, pHi fell precipitously to 7.10 ± 0.06 (n=6; Fig. 3A), then slowly recovered, reaching values not different from rest values by 12h.

Resting lactate levels in white muscle averaged 1.54 ± 0.58 mmol/kg (n=6) and rose significantly immediately post-activity to 11.37 ± 3.26 (n=6), remaining elevated through to 2h, returning to rest levels by 12h (1.56 ± 0.22; n=5). Analysis of H^+m and La- in white muscle (Fig. 2A) revealed that immediately post-activity, H^+m was in excess of La- by approximately 3 meq/kg. This discrepancy disappeared by 0.5h, when both were equal, then from 2h onward, La- appeared in excess of H^+m by approximately 2 meq/kg.

Heart ventricle pHi was very similar to white muscle pHi at rest (7.50 ± 0.05; n=6). However, unlike white muscle, the ventricle showed no change in pHi immediately post-activity (Fig. 3B), despite the large extracellular respiratory acidosis. Indeed, the ventricle actually became alkalotic at 0.5h into recovery, which was corrected by 2h and remained unchanged through to 12h.

Figure 3. Extracellular pH (pHe, ●) and intracellular pH (pHi, ○) of A) white muscle and B) cardiac muscle prior to and following activity. □ indicates cardiac muscle pHi calculated from venous data, see text for details. R= rest; bar= 10 min of activity; 0h= immediately post-activity; *= a significant difference ($P<0.05$) from rest values. Means ± 1 SEM; R, 0.5h and 2h, n=6; 0h and 12h, n=5.


White Muscle
8.0
7.8
7.6
7.4
7.2
7.0
pHe
pHi
B
Cardiac Muscle
8.0
7.8
7.6
7.4
7.2
7.0
pHe
pHi
R
0
2
4
6
8
10
12
Time (h)

There was no disturbance in fluid distribution in either heart ventricle or white muscle following exercise, for neither ECFV (344.7 ± 24.6 ml/kg, n=6; 113 ± 8.1 ml/kg n=6) nor total water content (831.6 ± 3.9 ml/kg, n=6; 823.7 ± 7 ml/kg, n=6) changed .

DISCUSSION

Response of the Extracellular Compartment

The blood acid-base disturbances following activity observed in sea raven are qualitatively similar to those reported for flounder (Wood *et al.* 1977) and flathead sole (Turner, Wood and Hōbe, 1983), being chiefly of respiratory origin (greater than 80%) in the early stages of recovery, followed by a smaller metabolic component. These authors have analyzed the acid-base disturbance in detail, so it will not be discussed in depth here.

Like flounder and flathead sole, resting blood lactate levels were quite low in the sea raven, approximately 0.2 meq/l, and did not increase much following activity, never attaining levels greater than 1 meq/l, unlike salmonids where blood lactate can reach 20 meq/l (Turner, Wood and Clark, 1983). This minimal appearance of lactate in the blood following activity could be characteristic of fish with a sedentary life-style, as it has also been observed in such animals as the muskellunge (*Esox masquinongy*) (Beggs *et al.* 1980) and plaice (*Pleuronectes platessa*) (Wardle, 1978). These low levels of blood lactate have been attributed to a non-release phenomenon at the level of the muscle, which may be mediated by circulating catecholamines

(Wardle, 1978).

The elevation in blood glucose concentration probably reflects the breakdown and mobilization of liver glycogen (Driedzic and Hochachka, 1978).

Reliability of pHi estimate

A potential source of error in the pHi estimate is the value chosen for pHe. In the present study, arterial pH was taken to represent extracellular pH. This does not represent a serious error in the white muscle pHi estimate, as the muscle mass 'sees' both arterial and venous blood, with the true pHe intermediate to pHa and pHv. The white muscle pHi estimate, then, is a conservative estimate. However, this problem is more serious for cardiac muscle, as the raven heart does not have coronary arteries, but rather is supplied solely by venous blood (Farrell, 1984). At rest, this will not cause a serious error as the arterial-venous pH difference is insignificant (Wood et al. 1977; Milligan, unpublished). After exercise, however; pHv can be as much as 0.06 pH units lower than arterial pH with a concomitant difference in [DMO]. Data on arterial-venous pH differences post-exercise are not available for the sea raven, however, data obtained from rainbow trout after exercise show that only immediately after exercise (time 0) is there a significant difference in both pH and [DMO] between arterial and venous blood and plasma, respectively (Milligan, unpublished). Using the mean arterial-venous pH and [DMO] differences, sea raven cardiac muscle pHi was recalculated. As shown in Fig. 3B, while the absolute values of pHi are altered, at no time is the difference significant from that

estimated using arterial data. These venous estimates represent the extreme values of heart pHi, as the arterial-venous pH difference in the sea raven is likely less than that in trout due to the less strenuous exercise performed by the sea raven. Clearly then, the maintenance of cardiac muscle pHi after exercise is real and not an artifact of the pHi calculation.

Response of the Intracellular Compartment

At rest, both sea raven white (7.51) and cardiac muscle (7.50) pHi were well within the range of values reported for these tissues in a variety of fish species at 10°C; catfish, 7.44 and 7.66 (Cameron and Kormanik, 1982); dogfish, 7.45 and 7.40 (Heisler *et al.* 1980); and eels, 7.37 and 7.36 (Walsh and Moon, 1982), respectively.

(i) White Muscle

Immediately post-activity, the metabolic acid load in the white muscle exceeded the lactate load, a phenomenon not previously reported in any vertebrate muscle. Due to the nature of the experiments, i.e. one sample time per fish, this difference (3 meq/kg) could not be tested for statistical significance. However, it is encouraging that a similar excess of ΔH^+m over Δ La- immediately post-exercise has also been observed in starry flounder and rainbow trout white muscle (Milligan and Wood, unpublished).

The source of protons produced during anaerobiosis is a complex issue, recently reviewed by Hultman and Sahlin (1980) and Hochachka and Mommsen (1983), so it will not be described in detail here. Suffice it to say that most of the proton load is produced by hydrolysis of ATP, which, when coupled with anaerobic glycolysis,

results in the equimolar production of protons and lactate. However, if these reactions should become uncoupled, i.e. the rate of ATP consumption exceeds production, then protons can be produced in excess of lactate and ATP levels will fall. While muscle ATP levels were not measured in the present study, data from other studies on fish have demonstrated a decline in muscle ATP levels following activity (Dreidzic and Hochachka, 1976; Milligan and Wood, unpublished). Alternatively, other anaerobic endproducts (eg. succinate and/or propionate) may be formed, and could contribute to the excess proton load. However, evidence suggests these products are formed in significant amounts only during periods of extreme hypoxia, often when the fish is near death (Dreidzic and Hochachka, 1975; Johnston, 1975a,b, 1977; Smith and Heath, 1980). Regardless of the source of excess protons, the inherent dangers of assuming a strict 1:1 stoichiometry between lactate and proton production is clearly evident.

Initially, the protons are cleared much more quickly from the muscle than lactate, suggesting either a net transport of protons out of the muscle or metabolism of protons independently of lactate. The metabolic fate of protons and lactate is uncertain, though there is indirect evidence to suggest lactate can be metabolized <u>in situ</u> (Wardle, 1978; Turner, Wood and Clark, 1983). Metabolism of lactate, whether to H_2O and CO_2 or to glycogen, would consume an equivalent amount of protons, which would aid in restoring pHi.

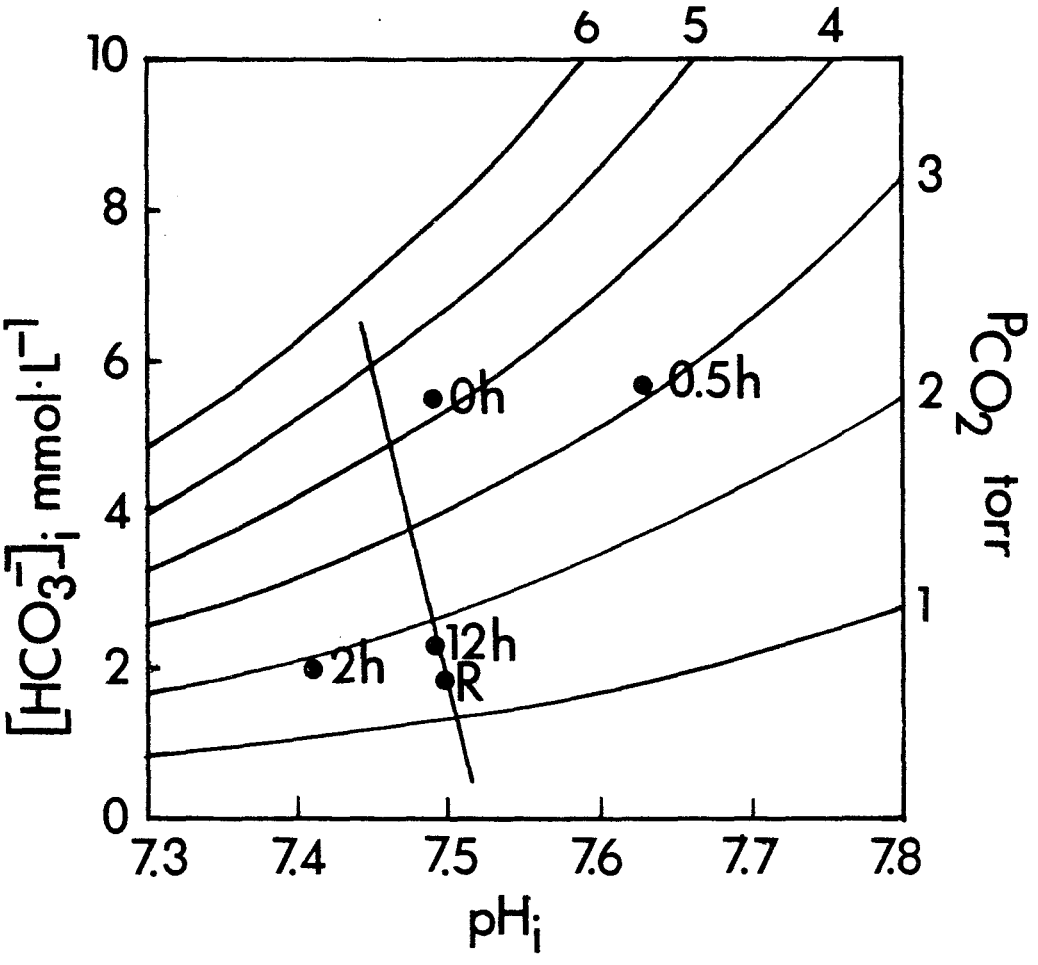
(ii) Cardiac Muscle

Despite the large increase in extracellular P_{CO_2}

immediately post-activity, cardiac muscle pHi was not affected. It is possible that CO_2 did not equilibrate across the heart cell intracellular/extracellular interface, though this is unlikely, given that CO_2 is readily permeable to the cell (Roos and Boron, 1981). From an analysis based upon the principles outlined by Davenport (1974), it is clear that the maintenance of cardiac muscle pHi immediately post activity is due, almost entirely, to passive non-bicarbonate buffering (Fig. 4). While pHi at 0h does not fall directly on the *in vitro* buffer line, given the variablilty of the estimate (7.49 ± 0.04), it is not different from the predicted value (7.46). The non-bicarbonate buffer value of the sea raven heart is high, compared to skeletal muscle (-84.6 vs. -39.0 mmol/pH/l ICF); however it is well within the range of values reported for cardiac muscle, using a similar methodology, from a variety of other vertebrates (see Burton, 1978).

Given the fall in P_{CO_2} at 0.5h post-activity, intracellular HCO_3^- levels would be expected to fall, according to the buffer line. However, HCO_3^- levels remained elevated (fig. 4). This 'active' accumulation could be achieved by a net inward flux of HCO_3^- (or outward flux of H^+). A transmembrane flux of HCO_3^- has been postulated to account for the maintenance of heart muscle pHi in *Synbranchus marmoratus*, a freshwater teleost, following transition from water to air breathing (Heisler, 1982). The presence of an active HCO_3^- (or H^+) pump has been demonstrated *in vitro* in a variety of tissues from a range of animals (see Roos and Boron, 1981, for a review).

Figure 4. Intracellular pH and [HCO_3-] in cardiac muscle plotted on a pH *versus* HCO_3- diagram (Davenport, 1974). R= rest; 0h= immediately post-activity; 0.5h, 2h and 12h are times post-activity. Values plotted are means for each time, standard errors are not shown for the sake of clarity. The *in vitro* buffer line (-84.6 mmol/pH/l) is shown.


6
5
4
10
8
6
4
2
0
3
2
1
0h
0.5h
2h
12h
R
7.3
7.4
7.5
7.6
7.7
7.8
$[HCO_3^-]_i$ mmol·L^{-1}
PCO_2 torr
pH_i

The stimulus for active HCO_3^- accumulation in cardiac muscle is unclear, though there is evidence from studies on mammals that circulating catecholamines are involved (Clancy *et al.* 1976). Certainly following stressful activity in fish plasma catecholamine levels increase (Nakano and Tomlinson, 1968; Mazeaud and Mazeaud, 1982). Catecholamines have been implicated in enhancing cardiac performance during a respiratory acidosis in the sea raven heart *in situ* (Farrell, 1984). Thus, following stressful exercise, regulation of cardiac muscle pHi via adrenergic stimulation may permit cardiac output to increase, aiding the recovery process.

Alternatively, cardiac muscle pHi regulation may have resulted from the utilization of circulating lactate as a fuel for oxidation. It is well documented that fish hearts are capable of using lactate as an aerobic fuel, which, when oxidized, could result in the net accumulation of HCO_3^- (Bilinski and Jonas, 1972; Lanctin *et al.* 1980). Only if a significant proportion of the lactate is entering the cell as the dissociated ion (i.e. in the absence of H^+ eg. in exchange for Cl^- or in conjunction with Na^+) would oxidation result in a net HCO_3^- accumulation. Documented rates of lactate oxidation for fish hearts are more than adequate to account for the observed rate of HCO_3^- accumulation (Lanctin *et al.* 1980). At present, there are no available data on the effect of circulating catecholamines on lactate utilization by fish hearts.

Clearly, either transmembrane HCO_3^- transport or lactate oxidation could account for the observed intracellular accumulation of HCO_3^- in the sea raven heart. these events need not be mutually

exclusive; they may operate in concert to minimize changes in cardiac pHi, thus offsetting the detrimental effects of acidosis on cardiac performance.

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APPENDIX II: Myocardial intracellular pH in a perfused trout heart during extracellular acidosis in the presence and absence of adrenalin.

The work presented in this appendix was carried out in collaboration with Dr. A.P. Farrell at Simon Fraser University during the course of my Ph.D studies at McMaster. The paper is now in press in The Journal of Experimental Biology.

MYOCARDIAL INTRACELLULAR pH IN A PERFUSED TROUT HEART DURING EXTRACELLULAR ACIDOSIS IN THE PRESENCE AND ABSENCE OF ADRENALIN.

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ABSTRACT

Myocardial intracellular pH was measured in a perfused rainbow trout, Salmo gairdneri , heart with DMO (5,5 - dimethyl - 2,4 - oxazolidinedione) to test the hypothesis that catecholamines promote active regulation of myocardial pH to protect contractility during a respiratory acidosis comparable to that observed post-exercise. Under control conditions (extracellular pH = 8.05, P_{CO_2} = 1.67 torr), myocardial pHi was 7.53 $\pm$ 0.01 (n=5). Acidosis (extracellular pH = 7.45, P_{CO_2} = 8.6 torr) reduced contractility, mechanical efficiency and intracellular pH (7.25 $\pm$ 0.04), but did not affect myocardial O_2 consumption. The addition of 0.5 uM adrenalin during extracellular acidosis prevented the loss of contractility, restored mechanical efficiency, but did not change intracellular pH significantly. Thus, adrenergically mediated recovery of cardiac contractility occurred without intracellular pH regulation, possibly by modulation of sarcolemmal calcium changes. The absence of a myocardial acidosis post-exercise in vivo is discussed with respect to possible intracellular pH regulation via lactate uptake and metabolism.

INTRODUCTION

An extracellular acidosis of both respiratory and metabolic origin, is a normal occurrence after exhaustive exercise in teleosts (Black, Chiu, Forbes and Hanslip, 1959; Wood, McMahon and McDonald, 1977; Graham, Wood and Turner, 1982; Holeton, Neumann and Heisler, 1983; Turner, Wood and Clark, 1983; Turner, Wood and Hobe, 1983; Milligan and Farrell, 1986). Typically, the respiratory component predominates during the initial phase of recovery (0-2 h), producing about a 0.5 pH unit reduction in extracellular pH (pHe) (see Wood and Perry, 1985). In vitro experiments clearly demonstrate that an extracellular acidosis of this magnitude depresses cardiac performance by reducing both cardiac contractility and the intrinsic heart rate (f_H) (see reviews by Gesser and Poupa, 1983; Farrell, 1984). Respiratory acidosis decreases myocardial intracellular pH (pHi) in isolated ventricle strips from trout (Gesser and Jorgensen, 1982) and the lower pHi probably reduces cardiac contractility, as is the case in the mammalian myocardium (Willamson, Safer, Rich, Schaffer and Kobayashi, 1976). However, in vivo , myocardial pHi does not fall in trout and sea raven despite the pronounced extracellular acidosis post-exercise; in fact, the myocardium tends to become alkalotic (Milligan and Farrell, 1986; Milligan and Wood, 1986). Since a physiological concentration of adrenalin protects the normal range of in vitro cardiac performance during extracellular acidosis (Farrell, MacLeod, Driedzic and Wood, 1983; Farrell, MacLeod and Chancey, 1986), it is possible, therefore, that the increased level of circulating

catecholamines associated with exercise stress (Primmett, Randall, Mazeaud and Boutilier, 1986) promotes some form of active regulation of myocardial pHi and hence prevents the deleterious effect of acidosis on contractility.

This hypothesis was tested using the DMO (5,5, - dimethyl - 2,4 - oxazolidinedione) method of Waddell and Butler (1959) to measure pHi in perfused hearts from rainbow trout, Salmo gairdneri , exposed to an extracellular acidosis of respiratory origin in the presence and absence of adrenalin.

MATERIALS AND METHODS

The experimental procedures and equipment for the perfused trout heart were essentially those fully described in the accompanying work (Farrell et al. , 1986). The perfusate was delivered to the sinus venosus via a stainless steel cannula placed in the hepatic vein. The ducti Cuvier were ligated. The ventricle pumped the perfusate against the output pressure head via a stainless steel cannula placed in the ventral aorta. The preload and diastolic afterload to the heart were set so that the heart generated a power output similar to that of a resting fish, during the equilibration period with non-recirculating control perfusate ($10^{o}C$). Cardiac output (V_b = 15 ml/min/kg fish weight) was set by the intrinsic f_H and manipulation of stroke volume (SV_H) via the preload. The mean output pressure was 45 cm H_2O. The major procedural difference was the recirculation of the experimental perfusate after

the usual equilibration period with non-recirculating, control perfusate. The volume of the experimental perfusate was 100 ml, to which 0.5 uCi ^{14}C-DMO (New England Nuclear; specific activity: 50 mCi/mmol) plus 2.0 uCi ^{3}H-mannitol (New England Nuclear; specific activity: 27.4 mCi/mmol) were added.

The equilibration time of DMO and mannitol was assessed using recirculation times of 10, 15, 20, and 25 min with condition I below (14 heart preparations). At the end of each test period, inflow and outflow perfusate (1000ul) was sampled and analyzed for pH, total CO_2, P_{O_2}, and levels of ^{3}H and ^{14}C radioactivity. The ventricle was removed, blotted dry, frozen in liquid N_2 and later analyzed for levels of ^{3}H and ^{14}C radioactivity and total water content.

Having established that marker equilibrations were complete within 20 min, (see Table 1), the following test conditions were examined over a 20 min perfusion period:

Condition I: Control: pHe =8.05 $\pm$ 0.04, P_{CO_2} = 1.67 $\pm$ 0.17 torr (n=5).

Condition II: Control + 0.5 uM adrenalin: pHe = 8.00 $\pm$ 0.02, P_{CO_2} = 1.86 $\pm$ 0.08 torr (n=6).

Condition III: Acidosis: pHe = 7.39 $\pm$ 0.03, P_{CO_2} = 8.7 $\pm$ 0.6 torr (n=6).

Condition IV: Acidosis + 0.5 uM adrenalin: pHe = 7.36 $\pm$ 0.01, P_{CO_2} = 8.5 $\pm$ 0.3 torr (n=8).

Thus a typical experiment consisted of a 20 min equilibration period during which a heart was perfused with non-recirculating control saline, followed by a 20 min 'test' period during which one of the

above conditions was examined using a recirculating perfusate containing ^{14}C-DMO and ^{3}H-mannitol. The preload and afterload were not changed throughout the 20 min test period. At the end of each test period, input and output samples (1000 ul) of perfusate were analyzed for the parameters listed above, with the additional measurement of lactate concentration in the output sample. The heart was treated as described above.

The analytical techniques and calculations are fully described elsewhere (Milligan and Wood, 1986; Farrell et al. , 1986). ^{3}H and ^{14}C radioactivity were measured in duplicate, on 100 ul aliquots of perfusate in 10 ml ACS fluor (Amersham) and 50 mg samples of ventricle digested in 2 ml NCS tissue solubilizer (Amersham), neutralized and counted in 10 ml OCS fluor (Amersham). All samples were counted on a Beckman LS-9000 liquid scintillation counter. P_{O_2} and pHe were determined with Radiometer microelectrodes at 10^{o}C and total CO_2 was measured using the method described by Cameron (1971). P_{CO_2} and [HCO_3-] were calculated from total CO_2 using the Henderson-Hasselbalch equation and values for aCO_2 and pK' reported by Boutilier, Heming and Iwama (1984). Myocardial pHi and extracellular fluid volume (ECFV) were calculated as described by Milligan and Farrell (1986) using the inflow pH, [^{3}H-mannitol] and [^{14}C-DMO] for estimates of pHe, [^{3}H-mannitol]e and [^{14}C-DMO]e respectively. The perfusate lactate concentration was determined enzymatically (lactate dehydrogenase method; Sigma bulletin #826) on 500 ul perfusate extracted with 500 ul ice-cold 8% perchloric acid.

The cardiovascular variables were analyzed as in previous work with the aid of analog-to-digital conversion using an APPLE microcomputer (Farrell et al. , 1986). Myocardial oxygen consumption ($\dot{M}_{O_2}$, nmol/s/g) was calculated as:

$$\dot{M}_{O_2}\ (\text{nmol/s/g}) = (\text{input } P_{O_2} - \text{output } P_{O_2})\ (\text{torr}) \times \alpha O_2 \times (\dot{V}_b/60)\ (\text{ml/s/g}) \qquad (1)$$

where αO_2 = 1.695 umol O_2/ml (Altman and Dittmer, 1974).
Mechanical efficiency of the heart was calculated as:

$$[\text{power output (mW/g)}] \times 2.22/[\dot{M}_{O_2}] \qquad (2).$$

Preparations showing abnormal cardiac performance during the initial equilibration period were not used. Estimates of the ventricular non-bicarbonate buffer capacity were made according to the methods outlined by Milligan and Farrell (1986) using an IL pH electrode. Hearts from 4 fish were pooled and the slope of the pH versus mmol HCl relationship over the pH range 6.9-7.7 was taken as the buffer value of the tissue, in mmol/pH/l intracellular fluid (ICF). The relationship was determined in duplicate.

Mean values ± 1 SEM are reported throughout unless otherwise stated. Significant differences ($P<0.05$) were determined using a Student's two-tailed t-test.

RESULTS

Preparation Stability: Cardiovascular variables and marker equilibration

After 10, 15, 20 and 25 min of recirculating perfusion, cardiovascular variables were generally unchanged compared to values

recorded after the equilibration period with open-circuit perfusion (Table 1). The slight decrease in f_H was not reflected in $\dot{V}_b$ or power output because of compensatory changes in SV_H. The difference between input P_{O_2} and output P_{O_2} (ΔP_{O_2}) was usually 30 to 50 torr. Myocardial oxygen consumption ($\dot{M}_{O_2}$) ranged from a low of 13.9 $\pm$ 5.5 (n=4) after 10 min of equilibration to a high of 24.3 $\pm$ 7.7 nmol/s/g (n=4) after 25 min of equilibration (Table 1), though these values were not significantly different. Equilibration of ^{14}C-DMO in the ventricle was quite rapid as indicated by the constancy in the ratio of [DMO]i/[DMO]e, hence the estimate of pHi, from 10 min onwards (Table 1). ^{3}H-mannitol equilibration was slightly longer, requiring up to 15 min for complete equilibration. The 'test' period of 20 min, therefore, allowed complete marker equilibration and adequate time for the heart to come into a new steady-state with respect to performance parameters monitored in response to the acidosis and/or adrenalin challenges.

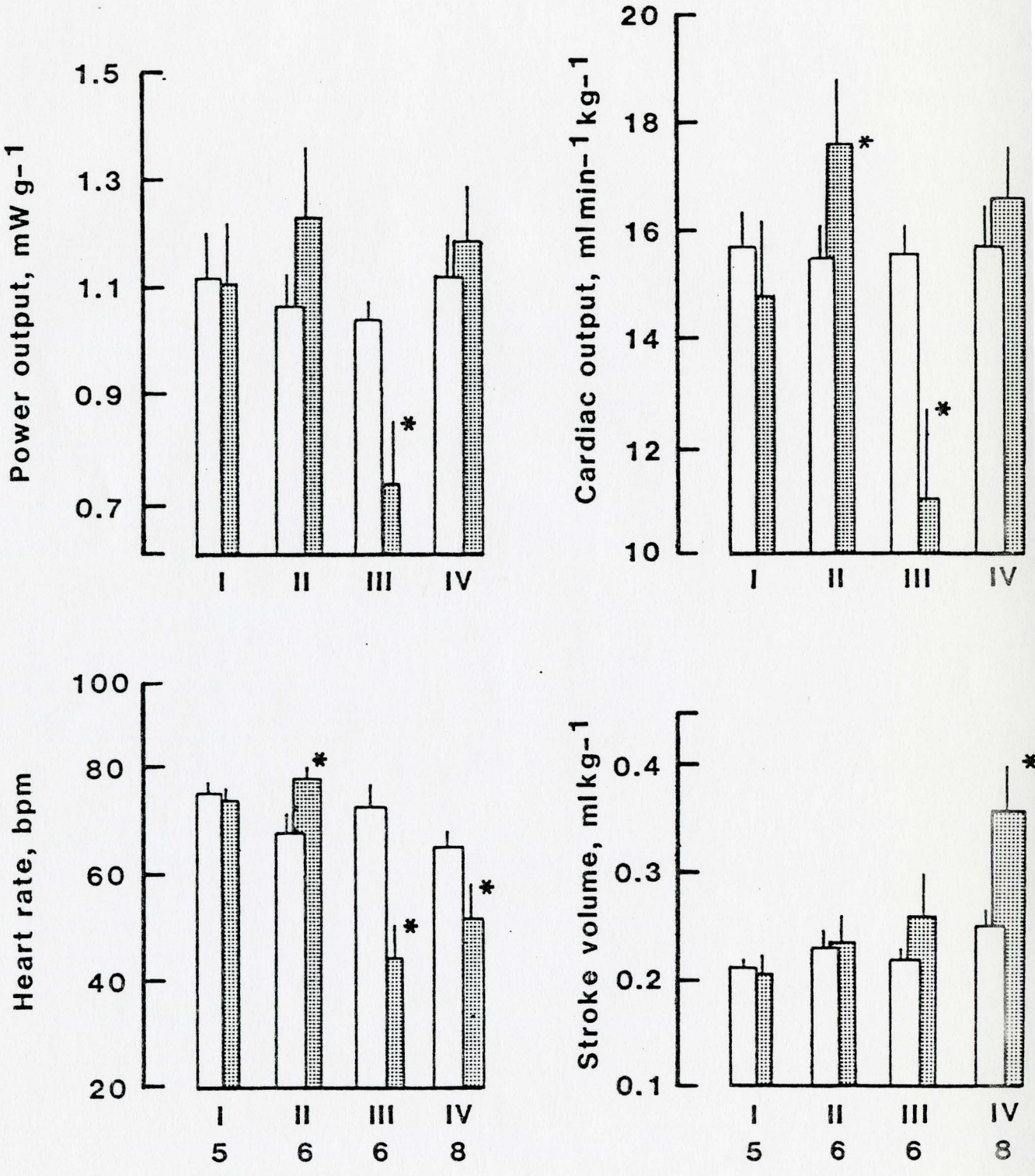
Condition I

Under control conditions (pHe = 8.05 $\pm$ 0.04; P_{CO_2} = 1.67 $\pm$ 0.17 torr, n=5), cardiovascular variables remained unchanged after 20 min of recirculating perfusate, compared to measurements taken at the end of the open circuit circulation (Fig. 1). A net acidic equivalent excretion from the heart occurred in all preparations as the outflow pHe tended to be lower than the inflow pHe by about 0.07-0.10 pH units. There was no evidence of lactate excretion into the perfusate. Under these conditions, mean myocardial

Table 1: Stability of cardiovascular variables during the test period and stability of pH_I measurements as a function of the equilibration time during the test period. Initial and final refer to beginning and end of perfusion period.

	10 min (n=4)		15 min (n=5)		20 min (n=4)		25 min (n=4)	
Variable	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Power output mW g^{-1}	0.89 ± 0.09	0.86 ± 0.12	1.09 ± 0.10	1.08 ± 0.09	1.12 ± 0.10	1.11 ± 0.12	0.96 ± 0.04	0.98 ± 0.03
Cardiac output ml.min kg^{-1}	12.70 ± 1.04	12.15 ± 1.45	14.93 ± 0.74	14.84 ± 0.73	15.69 ± 0.68	14.84 ± 1.34	13.97 ± 0.58	14.00 ± 0.58
Heart rate bpm	47.2 ± 7.9	54.8 ± 10.8	63.4 ± 6.6	57.3 ± 4.7	75.4 ± 1.8	73.5 ± 2.1	70.8 ± 2.9	67.3 ± 5.7
O_2 consumption nMol O_2 s^{-1} g^{-1}	13.9 ± 5.4		21.5 ± 4.8		18.6 ± 3.9		24.3 ± 7.7	
pH_I	7.58 ± 0.01		7.56 ± 0.02		7.53 ± 0.01		7.57 ± 0.02	
DMO_i/DMO_e	0.38 ± 0.05		0.38 ± 0.02		0.35 ± 0.02		0.34 ± 0.03	
Water content l kg^{-1}	0.832 ± 0.008		0.828 ± 0.008		0.830 ± 0.012		0.829 ± 0.011	
ECFV l Kg^{-1}	0.308 ± 0.018		0.252 ± 0.021		0.240 ± 0.015		0.232 ± 0.016	

Figure 1. Initial (open bar) and final (hatched bar) values for the cardiovascular variables of perfused trout hearts under the four test conditions. A significant difference between the initial and final values for a given condition is denoted by an asterix.


Power output, mW g-1
1.5
1.3
1.1
0.9
0.7
I
II
III
IV
Cardiac output, ml min-1 kg-1
20
18
16
14
12
10
Heart rate, bpm
100
80
60
40
20
Stroke volume, ml kg-1
0.4
0.3
0.2
0.1
5
6
6
8

pHi was 7.53 ± 0.01 (Fig. 2).

Condition II

With control perfusate, the presence of 0.5 uM adrenalin (n=6) improved the resting power output and $\dot{V}_b$ through an increase in f_H (Fig. 1). SV_H and systolic pressure were unchanged at the constant preload, even though f_H had increased. While there was a modest stimulation of the cardiac performance compared to condition I, myocardial pHi, P_{O_2} and $\dot{M}_{O_2}$ were unchanged, but mechanical efficiency was improved (Fig. 2., Table 2). No lactate was detected in the perfusate and there was a small, though significant, increase in the ECFV at the expense of ICFV (Fig. 3.)

Condition III

Extracellular acidosis (pHe = 7.39 ± 0.03; P_{CO_2} = 8.7 ± 0.6 torr, n=6) reduced the resting cardiac performance. Under the imposed conditions of constant preload and diastolic afterload, there were significant decreases in $\dot{V}_b$ and power output (Fig. 1). The primary change was a decrease in the intrinsic f_H. Impaired contractility, usually observed with acidosis (see Farrell et al. , 1986), was only indicated by the absence of a compensatory increase in SV_H as f_H decreased (see condition I). The increase in P_{O_2} without a change in $\dot{M}_{O_2}$ reflected the decrease in O_2 delivery (a reduced $\dot{V}_b$). Nevertheless, the heart was less efficient during extracellular acidosis (Table 2).

Myocardial pHi decreased significantly to 7.25 ± 0.04 (Fig. 2). This acidosis was of mixed respiratory and metabolic origin (Fig. 4.). There was no change in fluid distribution (Fig. 3.) compared to

Figure 2. A comparison of intracellular and extracellular pH measurements in the perfused trout heart at the end of the four test periods. An asterix denotes a significant difference from the control condition I.

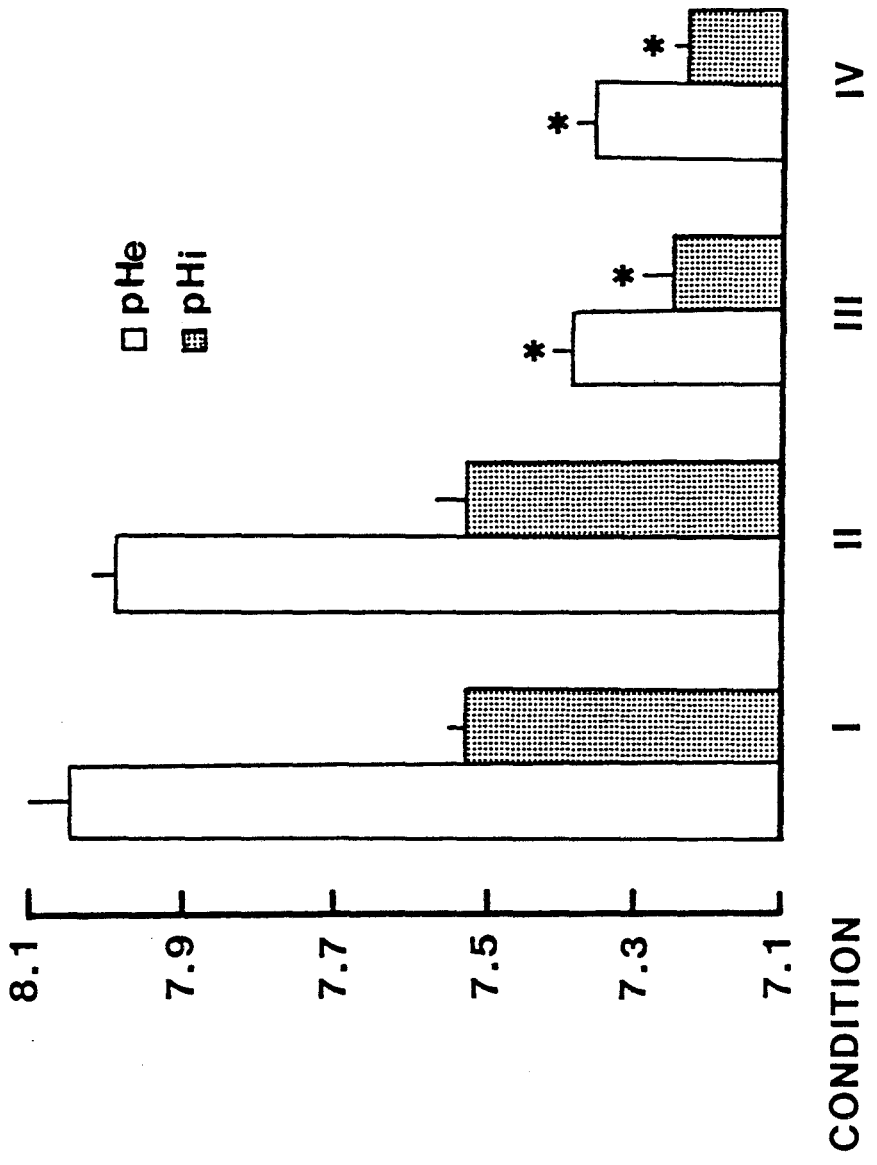

□ pHe
▦ pHi
8.1
7.9
7.7
7.5
7.3
7.1
I
II
III
IV
CONDITION
*
*
*
*

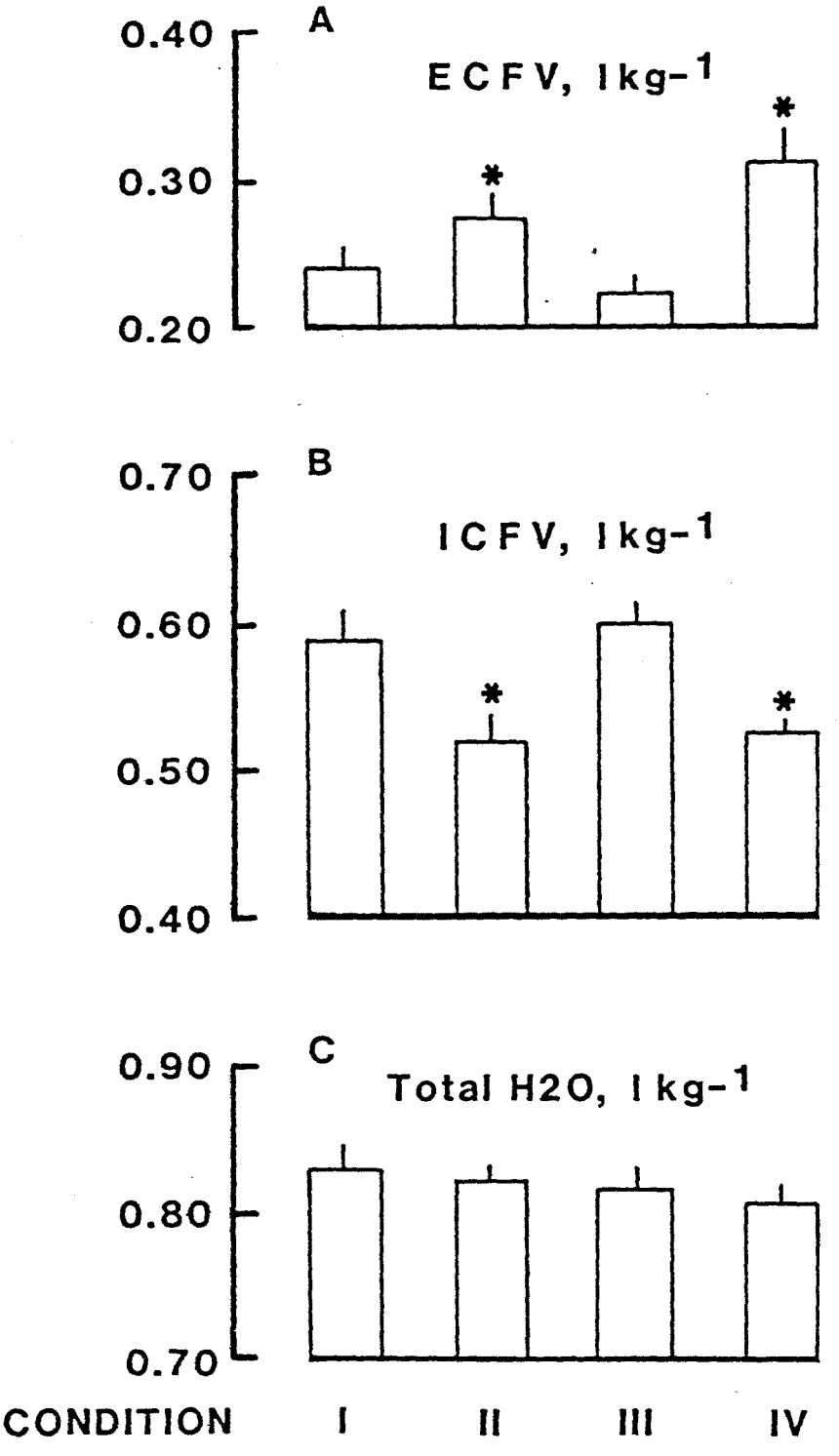
Table 2. Effect of extracellular acidosis with and without 0.5 μM adrenalin on oxygen consumption and mechanical efficiency.

	Perfusion conditions			
	I (n=5)	II (n=6)	III (n=4)	IV (n=8)
Mo_2, nMol s^{-1} g^{-1}	22.0±3.0	22.7±7.7	28.0±7.7	26.9±3.7
Mechanical Efficiency, %	11.0±3.8	16.4±2.6[a]	7.3±1.2[a]	10.3±0.9[b]
ΔPo_2, mmHg,	37.0±3.8	32.2±6.8	63.0±8.7[a]	43.2±3.9[b]

a = significantly different to condition I

b = significant difference between conditions III and IV

Figure 3. Comparisons of fluid distributions in the perfused trout heart under the four test conditions. An asterix denotes a significant difference from the control condition I.


A
ECFV, l kg-1
0.40
0.30
0.20
B
ICFV, l kg-1
0.70
0.60
0.50
0.40
C
Total H2O, l kg-1
0.90
0.80
0.70
CONDITION
I
II
III
IV

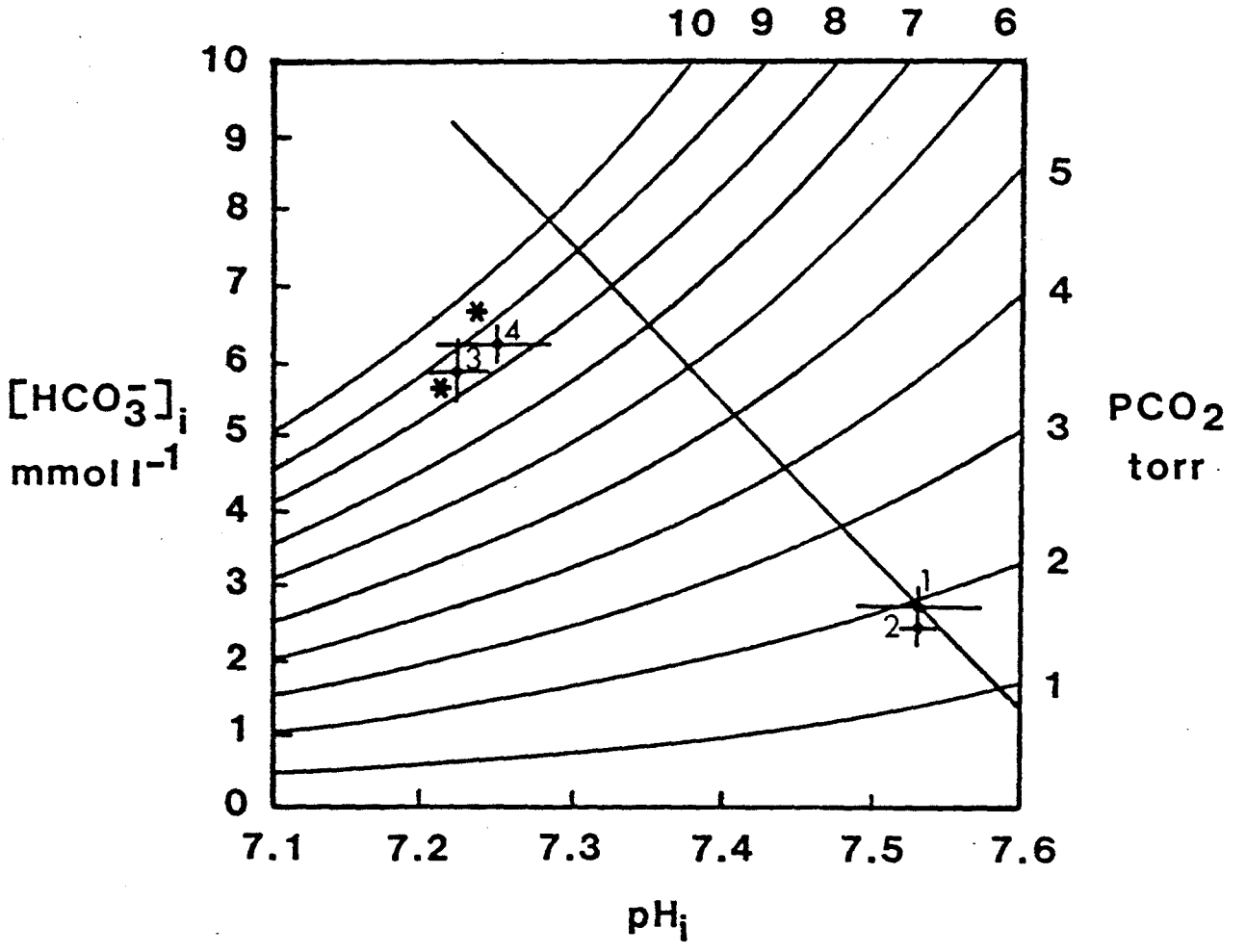
condition I and lactate was detected in only one of the 6 preparations (0.14 mM).

Condition IV

The presence of 0.5uM adrenalin during extracellular acidosis (pHe = 7.37 ± 0.01; P_{CO_2} = 8.53 ± 0.30 torr; n=8) prevented the loss of cardiac performance normally associated with acidosis (condition III) (Fig. 1, Table 2). This was achieved by partially preventing the negative chronotropy associated with acidosis and by improving contractility so that SV_H could increase when f_H was reduced. Systolic pressure was also increased significantly from 49.4 ± 0.4 to 53.6 ± 0.7 cm H_2O.

Myocardial pHi was unchanged compared to condition III (Fig. 2), indicating that pHi regulation was not involved in the myocardial protection afforded by adrenalin under the present conditions. Furthermore, the myocardial intracellular acid-base status was not appreciably different from that of condition III, with again, the response of the heart to the increased P_{CO_2} being passive (Fig 4). While the presence of adrenalin did not affect O_2 consumption, O_2 delivery was enhanced, as indicated by the fall in P_{O_2} to a level comparable with condition I. Mechanical efficiency was also restored (Table 2). No lactate excretion from the heart was detected. There was a 10% increase in ECFV, and, as total water content was unchanged, there was a reciprocal reduction in ICFV (Fig. 3). This increase was slightly greater than that observed in condition II.

Figure 4. A pH-HCO_3^- diagram indicating the intracellular changes under control conditions (1), control plus adrenalin (2), extracellular acidosis (3), and extracellular acidosis plus adrenalin (4). The non-bicarbonate buffer line for the trout heart is indicated. The gradient of this line (B) was -22.58 nnmol/pH/kg wet tissue (r = 0.987) or -27.54 mmol/pH/l intracellular fluid. The points significantly off the buffer line are denoted by an asterix. Means $\pm$ 1SEM are plotted.


10 9 8 7 6
10
9
8
7
6
5
4
3
2
1
0
5
4
3
2
1
[HCO3-]i
mmol l-1
PCO2
torr
1
2
3
4
7.1
7.2
7.3
7.4
7.5
7.6
pHi

DISCUSSION

The stability of the preparation was demonstrated by the control experiments (condition I). $\dot{M}_{O_2}$ has not been measured previously in a perfused trout heart, but the average value for all measurements with control perfusate (n= 34 fish) of 23.20 $\pm$ 2.30 nmol/s/g and a mechanical efficiency of 13.10 $\pm$ 1.20% at an average power output of 1.06 $\pm$ 0.05 mW/g compare favourably with values determined for perfused, sea raven hearts (Farrell, Wood, Hart and Driedzic, 1985). In the sea raven, $\dot{M}_{O_2}$ and mechanical efficiency were, respectively, 15 nmol/s/g and 16% at a power output of 1.1 mW/g. The measurement of $\dot{M}_{O_2}$ in the trout heart may be an underestimate since the ventricle could remove oxygen from the superfusate as well as the perfusate. This underestimate is likely to be small (less than 5%) based on recent experiments (M.S. Graham and A.P. Farrell, unpublished) in which deoxygenated superfusate was found to have a minimal effect on $\dot{M}_{O_2}$ and maximum cardiac performance. This also means that the oxygenated perfusate probably provided an adequate P_{O_2} gradient to supply oxygen to the outer, compact layer of myocardium, which is normally perfused *via* the coronary circulation. Hypoxic and, presumably, acidotic compact myocardium would not, therefore, have biased the pHi measurements appreciably. Indeed, there was good agreement with *in vivo* estimates of trout ventricle pHi. Furthermore, if there was an appreciable amount of hypoxia, it is unlikely that the perfused heart would have been able to generate workloads comparable to maximum levels observed in swimming trout (Farrell *et al.*, 1986).

The equilibration of DMO over the 20 min test period was confirmed by the similar DMO distribution ratios, hence pHi values, for the 15 min and 25 min equilibration times. The experiments with a 10 min equilibration time produced slightly higher pHi values, indicating that full equilibration probably had not been attained after 10 min. Under conditions I and II, the DMO distribution ratio ([DMO]i/[DMO]e) was identical, 0.35 $\pm$ 0.02 (n=4) and 0.34 $\pm$ 0.04 (n=6), respectively. This suggests that DMO was also fully equilibrated after 20 min under condition II. Under conditions III and IV, the DMO distribution ratio increased significantly to 0.75 $\pm$ 0.03 (n=6) and 0.77 $\pm$ 0.04 (n=8), respectively, indicating that in the 20 min equilibration period, DMO was redistributing in response to the acidosis.

Fish myocardial pHi has not been measured previously in perfused heart preparations. The pHi of 7.53 at a pHe of 8.0 is in good agreement with DMO measurements of pHi in the hearts of intact fish at rest (sea raven: pHi = 7.50, pHe= 7.90 (Milligan and Farrell, 1986); trout: pHi = 7.34, pHe =7.70 (Milligan and Wood, 1986)). In isolated ventricle strips from trout, pHi was 7.3 at a pHe of 7.6 (Gesser and Jorgensen, 1982; Nielsen and Gesser, 1984).

There was no effect of adrenalin on pHi of the trout heart during exposure to extracellular acidosis under the present experimental conditions. The fall in pHi was greater than that predicted from the *in vitro* non-bicarbonate buffer capacity. This suggested that a metabolic acidosis accompanied the respiratory

acidosis (Fig. 4.). The presence of adrenalin did not appreciably change the myocardial intracellular acid-base status, although cardiac performance was restored. Gesser and Jorgensen (1982) found that reducing pHe from 7.6 to 6.9 (3% CO_2 to 13% CO_2) reduced the pHi of trout ventricular strips from 7.34 to 6.99, and reversed th pHe-i gradient. The pHe-i gradient was not reversed in the present experiments.

In the presence and absence of adrenalin, an increase in P_{CO_2} led to a shrinkage of the intracellular compartment and a recipro~al expansion of the extracellular compartment. A similar shift of fluid from the intracelllular to the extracellular compartment in response to anoxia has been observed in isolated trout ventricle strips (Nielsen and Gesser, 1984). These responses are opposite to observations on fish erythrocytes, which swell in response to an increased P_{CO_2} and the swelling is exacerbated in the presence of adrenalin (Nikinmaa, 1982). The mechanism(s) responsible for the myocardial cell shrinkage is (are) unclear, as are the reasons for the differences with fish erythrocytes.

Since the present work provides no support for catecholamine mediated myocardial pHi regulation in trout, the absence of major myocardial pHi disturbance in intact fish following exhaustive exercise (Milligan and Farrell, 1986; Milligan and Wood, 1986) remains unexplained. Because there is evidence of active HCO_3^- accumulation in mammalian myocardium which is stimulated by circulating catecholamines (Clancy, Gonzalez and Fenton, 1976;

Gonzalez and Clancy, 1984), the teleost myocardium may, therefore, differ in this regard. An alternative mechanism which might regulate myocardial pHi in teleost myocardium may involve uptake of lactate ions from extracellular fluid and subsequent utilization of the lactate as a fuel with the associated utilization of intracellular hydrogen ions. In trout after exhaustive exercise, the myocardium experiences a significant alkalosis which is accompanied by an accumulation of lactate in the myocardium (Milligan and Wood, 1986). A similar myocardial lactate uptake and oxidation may account for the intracellular accumulation of base observed in the sea raven heart after exercise (Milligan and Farrell, 1986). Both trout and sea raven hearts are capable of using lactate as a substrate to fuel cardiac activity (Lantcin, McMorran and Driedzic, 1980; Driedzic and Hart, 1984). Thus, the present experiments may have prevented active pHi regulation by not including lactate in the perfusate. Whether this hypothesis is true must await further experiments.

Myocardial protection is also afforded by an increase in the intracellular calcium pool, which offsets the negative effects of intracellular acidosis (see Gesser and Poupa, 1983). Passive sarcolemmal calcium influxes, resulting from an increase in extracellular calcium, can improve contractility under control conditions, as well as during acidosis (Farrell et al. , 1986). However, these effects are never as large as those produced by adrenalin with a constant extracellular calcium (Farrell, Hart, Wood and Driedzic, 1984; Farrell et al. , 1986). Catecholamine stimulation

of the acidotic myocardium without pHi regulation may result from adrenergic modulation of sarcolemmal calcium-channels in the trout heart. In mammalian and amphibian hearts, B-adrenoceptors can mediate an increase in the inward calcium current by increasing the probability of a given calcium-channel entering an open state (Reuter, 1983). If this occurs in the trout heart during respiratory acidosis, then the rise in intracellular calcium could protect contractility.

It must be stressed, however, that important species differences exist amongst the teleosts with respect to the recovery of myocardial contractility during acidosis. Flounder ventricular strips, for example, recover spontaneously through a release of calcium from intracellular stores such as mitochondria, without any apparent involvement of trans-sarcolemmal movement of calcium from the extracellular space (Gesser and Poupa, 1981; 1983). This intracellular calcium release does not occur in trout ventricular strips, and while it occurs in the Atlantic cod, it does not restore contractility (Gesser and Jorgensen, 1982). Similar to the sea raven (Farrell et al. , 1984), extracellular calcium is less effective at restoring contractility in the Atlantic cod compared to the trout. Therefore, adrenergic modulation of sarcolemmal calcium-channels may be important in trout, but not necessarily all teleosts.

In summary, catecholamines restored contractility in the trout heart, in the absence of pHi regulation under the present perfusion conditions. It is suggested that the mechansim for this recovery may be related to adrenergic modulation of sarcolemmal calcium-channels.

Further studies on these exchanges are clearly warranted, as are studies on myocardial lactate uptake and metabolism, which might explain the enigma of constant myocardial pHi during the post-exercise respiratory acidosis observed in trout and sea raven.

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