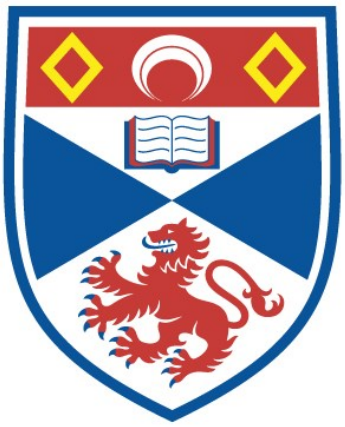
THE EFFECTS OF TEMPERATURE AND HYPOXIA ACCLIMATION ON THE METABOLISM OF FISHES

Irvine P. Aleleye-Wokoma

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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THE EFFECTS OF TEMPERATURE AND HYPOXIA ACCLIMATION ON THE METABOLISM OF FISHES

A thesis submitted to the University of St. Andrews for the degree of Doctor of philosophy

BY

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DECLARATION

I hereby declare that the research reported in this thesis was carried out by me and that the thesis is my own composition. No part of this work has been previously submitted for a higher degree.

The research was conducted in the Department of Biology and Preclinical Medicine, United College of St. Salvator and St. Leornard, University of St. Andrews, under the direction of Prof. I. A. Johnston.

CERTIFICATE

I hereby certify that Irvine Paul Aleleye Wokoma has spent eleven terms engaged in research work under my direction, and that he has fulfilled the conditions of General Ordinance No.2 (Resolution of the University Court No.1, 1967) and that he is qualified to submit the accompanying thesis for the Degree of Doctor of Philosophy.

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I dedicate this thesis to the memory of my late father, Paul Benibo Horsfall

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SUMMARY

CHAPTER 1. The organisation and anatomical design of fish myotomal muscles.

1. The literature on the anatomy, physiology and biochemistry of fish muscle is reviewed.
The responses of fibre types to various environmental stimuli including temperature and hypoxia acclimation show a reorganisation of cellular components in order to maintain metabolic independence. For example, the increase in slow fibres, mitochondria or electron transport enzymes during exposure to low temperature is suggested to supply an increase in ATP and to partially compensate for the adverse kinetic effects of low temperature on oxygen/metabolites diffusion rates. An outline of energy metabolism in fish and the variety of factors which affect metabolism such as starvation, exercise and hormonal activity is given.

CHAPTER 2. The effect of thermal acclimation on the metabolism of the swimming musculature of flounder (Platichthys flesus L.)

1. Flounder (Platichthys flesus L.), were acclimated for 8 weeks to either 5 or 23°C. Activities of enzymes associated with energy metabolism were determined at a common temperature of 15°C.

2. Hexokinase activities (μmoles substrate utilised, g wet wt.$^{-1}$ min^{-1}) increased in both slow (0.40 to 0.62) and fast (0.09 to 0.16) muscle fibres with cold acclimation.

3. Cytochrome oxidase activity was 2.8 and 2.3 times higher in the slow and fast fibres respectively, in fish acclimated to 5°C

than 23°C.

4. Carnitine palmitoyl transferase and B-hydroxy acyl CoA dehydrogenase, were 2.6 and 1.6 times higher respectively, in the slow fibres following cold acclimation, indicating an enhanced capacity for fatty acid oxidation.

5. Activities of glycolytic enzymes, phosphofructokinase (PFK) and lactate dehydrogenase (LDH) were unchanged by temperature acclimation.

6. This shows cold acclimation enhances aerobically generated ATP via electron transport enzymes and fatty acid oxidation, coupled with a decreased reliance on anaerobic glycolysis.

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2. Samples of fast and slow muscles, approximately (0.5 to 1.0 g), were rapidly dissected, homogenised and assayed for phosphorylase activity.

3. Phosphorylase 'a' activity was defined as the ratio of phosphorylase activity in the absence of AMP, to the phosphorylase activity in the presence of AMP. In the control fish phosphorylase activities (μmol g^{-1} min^{-1}), were 4.57 for

slow and 10.87 for fast muscle. Following epinephrine injection the fraction of phosphorylase in the 'a' form increased by 46% for slow and 36% for fast muscles. The results suggest glycogenolysis in flounder due to increased formation of phosphorylase a resulting from epinephrine activation of membrane bound adenyl cyclase.

Chapter 4. Effects of hypoxia acclimation on anaerobic metabolism in the plaice (Pleuronectes platessa L.)

1. Plaice (Pleuronectes platessa L.), 261±12.8 g mean weight and 23.2±3.5 cm standard length were acclimated for 8 weeks to either aerated water PO_2 21.0 KPa, or 5 h periodic exposure to PO_2 2.0 KPa, alternated with 48 h recovery in aerated water. Fish were transferred from holding tanks to respirometers individually or in pairs, 24 h prior to experiments. At the start, fish were quiescent and the respirometers were subsequently sealed. Aerated water PO_2 21.0 KPa was allowed to reduce by respiration (2 h.), followed by the introduction of nitrogen gas (2 min) to PO_2 (KPa) 2.6. Fish were kept in this condition for a further period of 3 h at 10°C and the PO_2 was (KPa) 2.0.

2. Routine oxygen consumption for plaice in aerated water (ml $Kg^{-1}h^{-1}$) was 22.0. Exposure to PO_2 2.0 KPa resulted in the reduction of oxygen consumption from 22 to 7.4 ($P>0.001$). Following 8 weeks acclimation to periodic hypoxia exposure, oxygen consumption increased from 7.4 to 10.2.

3. Glycogen concentrations (μmol g^{-1} glucose equiv.) for liver (213.0), and fast muscle (28.8) were similar for fish acclimated to aerated or hypoxic water. In contrast, that of the slow muscle (108.8) increased 2-fold ($P> 0.05$) with hypoxia

acclimation.

4. Hypoxia acclimation resulted in the increase in lactate concentrations (μmol g^{-1}) (1.7 to 4.1), liver; (2.5 to 4.2), fast muscle; (3.07 to 7.4) ($P > 0.05$), slow muscle.

5. The increase in lactate was accompanied by a parallel increase in the activity of lactate dehydrogenase (μmoles substrate utilised $g^{-1}min^{-1}$) for both fast (123 to 268) and slow (114 to 209) muscles; and liver (4.6 to 9.3). It would appear, an increased dependence on anaerobic glycolysis results in excess acid lactate load which may disturb redox balance. There were no detectable levels of alcohol dehydrogenase activities, matched by the absence of ethanol pathway utilization. This may contribute to the failure of plaice to survive anoxia exposure.

Chapter 5. Effects of acclimation to periodic anoxia exposure on the utilization of the ethanol pathway in goldfish (Carassius auratus L.).

1. Goldfish (Carassius auratus L.), were acclimated for a period of 6 weeks to either aerated water or periodic anoxic exposure (at 15°C).

2. The effects of 6 h anoxia and subsequent recovery on the concentrations, glucose, lactate and ethanol in whole fish were determined.

3. In fish acclimated to aerated water, the concentrations of glucose and lactate, (μmol g^{-1}) were 0.58 ± 0.1 and 0.81 ± 0.11 respectively. Ethanol was not detected in aerated water. Periodic anoxia (6 h) induced-increase in concentrations in the carcasses (μmol g^{-1}wet wt.) were glucose, 2.49 and lactate, 2.87 respectively. Concurrently, anoxia induced the production of

ethanol concentrations (0.38 μmols $g^{-1}h^{-1}$ in the tissue), and the net ethanol excretion after 6 week acclimation to periodic anoxia exposure amounted to 1.10 umol $g^{-1}h^{-1}$. This shows 74% of the total ethanol was excreted to the surrounding water.

4. Oxygen consumption of fish acclimated to aerated water was (ml $kg^{-1}h^{-1}$) 26.8 $\pm$ 2.4. This is equivalent to an ATP production of aerobic metabolism of 7.14 mmol ATP $kg^{-1}h^{-1}$, assuming a P/O ratio of 3.

The ATP yield from lactate and total ethanol (tissue & excreted) is assumed for anaerobic glycolysis of 0.016 mmoles ATP per mg lactate (Bennett & Licht, 1972). Expenditure of energy from lactate and ethanol pathways represents 27% of that from aerobic pathways. This decreased energy needs during anoxia exposure indicates metabolic depression.

5. Acclimation to periodic anoxic exposure resulted in a reduction of maximal enzyme activity of lactate dehydrogenase. In contrast, alcohol dehydrogenase activity increased 2-fold in the fast fibres. This suggests periodic anoxia exposure enhances ethanol pathway utilization which uses lactate as substrate to produce neutral ethanol. The process therefore ensures that glycolysis can continue and redox balance is not disturbed. This contributes to the survival of goldfish during prolonged anoxia exposure.

Chapter 6. Effects of acclimation to periodic anoxia exposure on the ultrastructure of goldfish (Carassius auratus L.).

1. Goldfish (Carassius auratus L.), following acclimation to either aerated water or periodic anoxic exposure, were analysed by electron microscopy.

2. Small bundles of fast and slow myotomal muscles were excised for electron microscopy. In fish acclimated to aerated water, the range of frequency distribution of cross-sectional areas were (μm^2), (20 to 1000) for slow and (500 to 6500) for fast fibres. Anoxia acclimation resulted in an increase in the range of fibre diameters in both slow (20 to 1500) and fast (500 to 9000) muscles.

3. Anoxia exposure resulted in an increase in capillary density NA(c,f) (μm^{-2}) for both fast (234 to 308) and slow (8o6 to 1373) muscle fibres. The surface density of capillaries was 3.5 times greater for slow than for fast muscle.

4. Acclimation to periodic anoxia exposure resulted in an increase in mitochondrial volume densities V_v(mit,f) slow (0.12 $\pm$ 0.01 to 0.20 $\pm$ 0.01); and fast (0.015 $\pm$ 0.09 to 0.03 $\pm$ 0.01) muscles.

5. Anoxia induced the proliferation of mitochondrial volume density V_v(mit,f), (which supports ethanol production, Chapter 5). It is accompanied by a parallel reduction in oxygen and/or metabolite diffusion pathlength (μm) slow muscle (20 to 15); and fast (37 to 32) muscle fibres. This indicates an ultrastructural reorganisation of cellular components partially to reduce the effects of anoxia.

CHAPTER 7.

General Discussion

This section contains a general discussion of the biochemical responses to thermal acclimation and hypoxia. These responses precede ultrastructural changes which are stimulated to partially offset the adverse effects to normal homoeostasis. It includes a critical survey of current techniques and suggestions for future research.

CHAPTER 1

The organisation and anatomical design of fish myotomal muscles:

1.1 General introduction

The myotomal muscles of fish are anatomically separated into discrete zones, distinquished by colour, enzyme composition and functional characteristics. In most teleosts, the red muscle constitutes from 5 to 10% or more of the body mass, contained within a thin, superficial triangular wedge and in close proximity to the lateral line. The individual muscles insert via tendons into myosepta, composed of tissue sheets and vary from "V" to "W" shaped overlapping cones (Bone, 1966). The complex geometry of the design enables each fibre to be inserted on a similar angle with respect to myosepta. Two basic patterns have arisen from this arrangement, one for selachians representing primitive bony fishes such as Anguilla, and the other for advanced teleosts (Alexander, 1969). The superficial slow fibres run parallel to the longitudinal axis whilethe deep fast fibres are arranged in complex patterns so as to make angles 30 to 40^{o} with the lateral axis of the body. Alexander (1969) has concluded from these studies that all the fast fibres would contract simultaneously to the same degree for a given body flexure but would only result in a minimal sacomere shortening. For example, the longitudinally arranged red fibres would only shorten by 10% of their resting lengths during swimming, while the selachians (primitive) fast fibres, by 7.0 to 9% and the helically arranged fast fibres of advanced teleosts by 2 to 3% (Alexander, 1969). This isometric nature of contraction of the

myotomal muscles has been supported by endurance exercise training experiments. For example, prolonged enforced swimming in a flume produced hypertrophy of slow and fast fibres in brook trout (Johnston & Moon, 1980a).

1.1.1. Fibre Types:

The different fibre groups are further sub-divided into sub-population of fibres. Histochemical, biochemical, ultrastuctural and physiological criteria have been used for their differentiation. In brook trout (Salvelinus fontinalis) and mackerel (Scomber scomber), two types have been identified (Johnston & Moon, 1980b; Bone, 1978). In the carp (Cyprinus carpio), three distinct types are present while in the dogfish (Scyliorhinus canicula), five fibre types have been recorded (Bone, 1966). These differences in fibre types may be related to the differences in life-style.

1.1.1. Slow fibres:

The lateral trunk of most fishes contains a superficial triangular wedge of red fibres, which runs along the lateral line from head to tail (Bone, 1978). In comparison to other muscle types, red fibres are highly vascularised, and have a high mitochondrial density as well as high myoglobin (Matsura & Hashimoto, 1954), glycogen and lipid content (George, 1962; Bone, 1966; Bokdawala, 1967; Love, 1980). The activity of markers of aerobic metabolism, including enzymes from the tri-carboxylic acid cycle and electron transport chain, is also greatest in this tissue (Crabtree & Newsholme, 1972; Bostrom & Johanson, 1972), and well developed capillary supply (Mosse,

1978). Mitochondria supply the energy for slow speed cruising (Johnston et al., 1977; Bone, 1978) which depends on the oxidation of lipids as the major source for muscle contraction (Driedzic & Hochachka, 1978). Electrophoretic data have shown that the myosin light chain pattern of red muscle corresponds to that of slow twitch fibres, and consequently this tissue has been termed slow oxidative muscle (Johnston et al., 1977).

The proportion of slow muscle varies along the length of the body and with the activity of the fish. For example, red muscle amounts from 0.5% in the sedentary demersal species, chimaerid (Chimaera monstrosa) to 29% in an active, pelagic swimmer, anchovy (Engraulis encrasicolus) (Greer-Walker & Pull, 1975). This positive correlation between the amount of myotomal red fibres and the swimming ability does not apply to those fishes which use their pectoral fins as prime means of locomotion. For example, the Antarctic Cod (Notothenia rossii) primarilly uses enlarged pectoral fins for labriform swimming. Superficial red fibres in the myotomes of N. rossii constitute 5.5% relative to 85% for the pectoral fin muscles (Walseby & Johnston, 1980).

Some pelagic fish have internalised red muscle. For example, skipjack tuna (Katsuwonus pelamis), where an effective, counter-current vascular, heat exchange mechanism is used to maintain an elevated muscle temperature (Carey & Teal, 1966). This muscle has a significantly increased content of mitochondria and a more highly developed capillary supply than

superficial red muscle (Bone, 1978).

There is an exception to the classification based on muscle pigmentation and aerobic capacity. For example, the haemoglobinless ice-fish, (Chaenocephalus gunnari) has white slow fibres due to the absence of myoglobin, although the fractional volume of the fibre occupied by mitochondria is 45% (Walesby, Nicol & Johnston, 1982).

1.1.3 Fast Fibres

Most of the myotomal musculature (80 to 90%) is composed of white muscle, which is arranged in helical trajectories. This is characterised by poor blood supply (Love, 1980; Johnston, 1981), and has higher activities of glycolytic enzymes (Crabtree et al., 1972) and myofibrillar ATPase than than the oxidative musculature (Crabtree & Newsholme, 1972; Johnston et.al., 1977a). The high myosin ATPase activity of this tissue is paralleled by the speed of muscle shortening (Barany, 1967). Consequently, the white muscle is capable of more rapid contractions than the slow oxidative fibres. The myosin light chain pattern corresponds to that of 'fast twitch' muscle (Johnston et al., 1977a), and these fibres are referred to as 'fast/glycolytic'. Hence the major differences between these two types of musculature would appear to lie in the velocity of contraction and metabolic pathway. The slow oxidative fibres would utilise both carbohydrates and lipids for aerobic generation of ATP, while fast glycolytic fibres appear to rely on anaerobic glycogenolysis (Crabtree & Newsholme, 1972). However, this generalisation is subject to variations between

species. For example, while there is a marked disparity in glycolytic enzyme activities between aerobic and anaerobic fibres in rainbow trout (Salmo gairdneri), this difference is negligible in the corresponding tissues of the carp (Cyprinus carpio L) (Johnston et al., 1977). This may be related to a difference in lifestyle with varying exposure and tolerance to periods of muscular anoxia (Johnston et al., 1977).

The fast glycolytic fibres have a more regular packing of myofibrils probably to allow for rapid development of tension for burst swimming. Furthermore, fast fibres possess a more extensive sarcoplasmic reticulum and tubular system than slow muscle and approach the slow muscles of other vertebrates. It has been shown that the S.R and the T-system are concerned with the transmission of impulses in the fibre and the subsequent release of bound calcium ions which ultimately activate muscle contraction (Peachey, 1965). In addition fish fast fibres contain high concentrations of parvalbumins, low molecular weight cytoplasmic calcium-binding proteins. These may function by operating a shuttle mechanism between the S.R and troponin C (TNC). Calcium ions released by the S.R are mopped up by parvalbumin such that calcium activation of the crossbridges is a transient process and rapid relaxation is ensured (Gerday, 1982). This aids high frequency tail-beats which usually operate at burst swimming speeds.

1.1.4 Intermediate Fibres

There is a third type of fibre in fish myotomal muscle, which is located between the red and the white fibres. Because of its intermediate colouring, it has been termed pink. Only a

little work has been done on intermediate fibre types but generally they possess aerobic capacity that is intermediate between slow and fast fibres (Johnston et al., 1977a; Johnston & Maitland, 1980). These 'pink' fibres may be differentiated from other types on the basis of lipid content, succinic dehyrogenase and myofibrillar ATPase activity, and appear to have characteristics intermediate to oxidative and glycolytic fibres. As the myosin light chain patterns are similar to those of fast glycolytic fibres, but the pink muscle has a greater aerobic capacity, these fibres have been termed 'fast oxidative glycolytic' (Johnston et al., 1977a). While fast fibres fatigue rapidly and slow fibres appear more resistant to this, intermediate fibres have a fatigue threshold that is between that of the other two fibre types (Johnston et al., 1977a). The pink muscle of carp (Carassius carassius) differs from the white and the red muscle not only histologically but also in its myosin heavy chain, as shown by peptide maps of myosin heavy chains, thus reflecting differences in primary structure (Scapolo & Rowlerson, 1987). Since these pink fibres have a myosin with fairly high ATPase activity and have high levels of both oxidative and glycolytic enzymes they are reasonably fast contracting and are capable of deriving energy from both pathways.

1.2 Fibre Innervation & Recruitment.

In terrestrial locomotion the power required for motion is known to increase linearly with speed. In contrast, in order to overcome the drag forces exerted by motion in water, the power required is inverselly related to the velocity (Webb, 1975).

Consequently, the large mass of the musculature of fish is essential in order to generate sufficient power for short periods (1-2 min.) of burst swimming. The buoyancy imparted by the water medium eliminates the need for weight economy. Hence 80-90% of the total body mass is made up of fast fibres capable of rapid recruitment during burst swimming. To a large extent, it is independent of the circulation (Greer Walker & Pull, 1975; Bone, 1978). The limitation of conveying nutrients and oxygen to large masses of muscle is offset by the reliance on anaerobic glycogenolysis and glycolysis, in which glycogen or phosphagen - based substrate provides the fuel source.

The focal pattern of innervation is associated with a sharp transition between sustained and burst swimming speeds (Bone, 1966). For example, Pacific herring are able to swim at 3-4 body lengths s^{-1} almost indefinitely but fatigue within 2 min at > 4.5 body lengths s^{-1} indicating the recruitment of focally innervated fibres (Bone, 1978). In contrast, many teleosts recruit polyneuronally innervated fast fibres at sustainable swimming speeds which utilize either steady (Johnston et al., 1977) or flick - glide (Hudson, 1973) swimming patterns. Capillary and mitochondrial volume densities are considerably higher in the fast muscles with polyneuronal innervation relative to those with focal pattern (Johnston & Moon, 1981; Johnston et al., 1983). Fast fibres of teleosts have varying diameters. The small fibres have a greater mitochondrial density and higher glycogen concentration than the larger diameter fibres (Boddeke et al., 1959). However, the polyneuronal innervation pattern of fish muscle is such that a given axon terminates on fibres of different diameters, which

makes the selective activation of fibre size populations impossible (Altringham & Johnston, 1981). The mean number of end plates on deep fibres is less than that on the superficial fibres, which suggests that the deep fibres are innervated by fewer axons than their superficial counterparts (Altringham & Johnston, 1981). This would appear to cause the superficial fibres to be recruited at lower threshold swimming speeds than those which are localised at a deeper position in the musculature (Johnston & Moon, 1980). In general, in increasing muscular activity, the recruitment of motor units takes place in a fixed order (DeLuca et al., 1982). Hence, it would appear that due to the pattern of innervation of teleost muscle, recruitment occurs according to position, such that the superficial fibres are activated earlier than the deep fibres.

Slow fibre innervation

All fish slow fibres have multiple innervation with small diameter myelinated fibres terminating in "en-grappe" endings (Baret, 1961; Bone, 1978). They are activated by junction potentials although they may also be capable of propagating action potentials (Stanfield, 1972; Altringham & Johnston, 1988). In hagfish, Eptatretus burgeri, the slow fibres are innervated by one axon from each myoseptum while in elasmobranchs and teleosts each fibre is innervated by a minimum of two axons (Bone, 1966). Failure to observe action potentials in earlier studies on sculpin may be related to the inadequacies in the Ringer composition (Hudson, 1969).

Separation of slow and fast fibres:

The separation of slow and fast fibres occurs at the level of the spinal cord (Hudson, 1972). It has been suggested that activation of any of the fibre systems may involve different levels of activation of neuronal pools or neuronal pathways. The fast fibres of dogfish are focally innervated by large diameter axons which pass in the myosepta to produce basket-like "en plaque" end formations of the fibre (Bone, 1964, 1966). A single fibre is usually innervated by two axons which fuse together to form a single endplate (Bone, 1964, 1966). These fast fibres demonstrate an action potential which overshoot zero potential, thus they resemble frog fast twitch fibres.

Fast fibres of advanced teleosts have a different pattern of innervation to these of elasmobranchs, chondrosteans, dipnoans and some taxonomically primitive teleosts (Bone, 1964). Instead of a single motor end plate, a single fibre is overwhelmed by a dense network of innervation. For example, in cod, (Gadus Morhua) each fast fibre may recieve up to 23 motor terminations depending on whether it is situated near the skin or near the vertebral column (Altringham & Johnston, 1981). The fast glycolytic fibres near the skin have higher fractional volume of mitochondria and capillaries (Egginton & Johnston, 1981), than deeper fibres. Westerfield, M^{C}Murray and Eisen (1986) have shown that in the zebrafish motoneurons specifically innervate cell-specific subsets of muscle fibres with a remarkable degree of precision. For example, each myotomal fibre is innervated by only one primary motoneurone, and

these do not cross myosepta into adjacent myotomes. Most fibres are innervated by 1-4 secondary motoneurons but only 8% are innervated by secondaries from adjacent segments. The initial outgrowth of these motoneurons in the embryo unerringly project to regions of the segment appropriate for their adult functions (Meyers, Eisen & Westerfield, 1987). Westerfield et.al. (1986) recorded only action potentials in zebrafish fast fibres in response to spinal nerves stimulation in normal Ringer. These fast fibres are innervated by only 2 - 3 axons in this species compared to about 20 in sculpin (Myoxocephalus scopius) and may function differently. Experiments on sculpin (Hudson, 1969; Altringham & Johnston, 1988a,) suggest that the fast fibres are unlikely to be activated by junction potentials when stimulated through the nerve. Since each spinal nerve contributes only 2 - 5 axons to a muscle fibre, each with a different threshold voltage for recruitment, it would appear likely that in many cases action potentials were elicited by the firing of a single axon. Similar to focally innervated vertebrate muscle fibres, isolated fast fibres from sculpin responded to suprathreshold stimulation with an all - or none - twitch (Altringham & Johnston, 1987, 1988). This is supported by the mechanical studies on opercular muscles (Granzier et.al., 1983; Akster et al., 1985).

Josephson (1985) has shown that power output during locomotion is determined by the frequency, amplitude and phase relation of the mechanical and electrical events during cycling. In a study of synchronous insect flight muscle, Josephson (1985) found that multiple stimuli per

oscillatory work cycle increased power output above that generated from single twitch contractions and may serve as a possible function of polyneuronal innervation (Altringham & Johnston, 1988). In the sculpin, for example, each muscle fibre receives axons from 4 spinal nerves, and the myotomal fields innervated by these nerves overlap (Hudson, 1969). Consequently , a sequential activation of the spinal nerves will lead to multiple stimulation of the muscle fibres. Such an arrangement may enable the number of stimuli to be varied, in a way determined by the neuromuscular circuitry, and the activity patterns of the central nervous system, so as to optimize power output over a range of swimming speeds. The mechanical properties may be modulated, but involving a mechanism different from that proposed by Hudson (1969).

1.3 Effects of hypoxia on fish metabolism:

The most outstanding anaerobic abilities, in terms of duration of anoxia tolerance are expressed by invertebrate groups (parasitic helminths, burrowing annelids, intertidal bivalves). Even amongst the vertebrates there are a few species with surprising tolerance to anoxia such as goldfish and carp of the cyprinid family.

1.3.1 METABOLIC DEPRESSION

The initial problem with the onset of hypoxia is that aerobic energy production ceases to meet metabolic requirements. If, concurrent with the hypoxic insult, there is a large drop in energy requirements, anaerobic energy production may not be needed at all. The limited

supply of aerobic metabolism may be sufficient to satisfy the metabolic demand. If energy demands could not be met with aerobic metabolism, the amount of anaerobic flux would be less than if no metabolic depression had occurred.

Thus depression of metabolism is a process which solves many of the major metabolic problems associated with oxygen limitation. The two major questions are, firstly, to determine how depression is mediated and secondly, to what extent can the metabolism of a cell be reduced without hampering the cell's recovery. The clue suggested is the absence of a Pasteur effect.

1.3.2 THE MISSING PASTEUR EFFECT.

Removal of oxygen causes a cascade of metabolic changes which activate the glycolytic enzymes and cause increased glycolytic flux. However, the best anaerobes do not use this strategy at all. In these animals glycolysis may be slightly activated, unchanged or actually reduced during oxygen deprivation (i.e. the Pasteur effect is either modest or is simply missing). The process of metabolic depression far surpasses all other adaptations in term of relative effectiveness of hypoxia tolerance (Hochachka et al, 1983).

The Pasteur effect observed in the muscles of vertebrates during anaerobiosis does not occur in the adductor muscle of the sea mussels. This results because the energy demand in the anoxic state is strongly reduced to about 5-20% of that in the normoxic state i.e. metabolic depression occurs (Ebberink et al, 1979; Storey, 1985). Pamatmat (1979) observed by a calorimetric method, a

similar anoxia-induced reduction of metabolic rate in the sea mussel, the anaerobic production of heat at a temperature of 20^0C being 13 times less than that under aerobic condition. Measurement of heat production in anoxic goldfish showed a significant reduction in energy consumption from 20% at 18^0C to 30% at 5^0C (Anderson, 1975).

In contrast to trout, goldfish and crucian carp are known to show a high tolerance of anoxia which is dependent on low temperature. For example, crucian carp has been reported by Blazka (1958) to survive for several months in ice-locked lakes during the winter months. Surprisingly, twenty years elapsed before any research was done on anoxia tolerance. Studies have shown that the anaerobic survival of goldfish increased from 16 hours at 20^0C to several days at 4^0C (Van den Thillart, 1977). Thillart et al (1980) has shown that the survival rate of goldfish is enhanced by prior conditioning to hypoxia. For example, when goldfish are transferred from one tank to another without prior conditioning, they survived for only two hours (Van den Thillart, 1982).

Accumulation of lactate from glycogen depletion indicates increased conventional anaerobic metabolism which utilises the Embden Meyerhof pathway. Recent studies have shown that in the red muscle of some species, modified pathways exist. These provide additional energy to glycolysis by coupling carbohydrate and amino acid catabolism reactions, thus yielding succinate and alanine as end products with concomitant increase in ATP production, as well as the maintenance of redox balance

(Hochachka et al, 1973; Hochachka et al, 1975; Johnston, 1975; De Zwaan & Wijman, 1976). Other intriguing findings have been made on these species with high hypoxic tolerance. For example, Kutty (1972) obtained a higher concentration of ammonia production than could be calculated from oxygen consumption; the production of metabolic carbon dioxide during anaerobiosis has been reported by several authors (Ekberg, 1962; Hochachka, 1961; Kutty, 1968, 1972; Dejours et al, 1968).

Metabolic studies on goldfish (Van den Thillart et al., 1978) suggest a reduction in metabolic rate or the presence of an alternative metabolic pathway to glycolysis, in which molecular oxygen is replaced by an unknown electron acceptor, during long term anoxia. This elusive substance therefore operates to provide a continuous supply of NAD^+ and so redox balance is maintained (Hochachka et al, 1973; Hochachka, 1975; Van den Thillart & Kesbeke, 1978).

Prosser et al (1957), failed to detect lactate in the water of hypoxic fish, however these authors postulated that an excretory product which they were unable to identify was the result of glucose fermentation. Shoubridge & Hochachka (1980), following the hypothesis that the unidentified product might be the result of further lactate catabolism. In experiments in which the goldfish were made anoxic by carbon monoxide poisoning, and measuring the end products in both tissues and surrounding water, they found lactate and ethanol in equal proportion. These studies provided two pieces of evidence which suggest a concerted metabolic strategy in which lactate, produced

in glycolytic tissues is metabolised to ethanol in muscle. Firstly, ^{14}C - ethanol is produced from ^{14}C-lactate at a rate which is four to five times greater than that from ^{14}C-glucose. This shows blood lactate is a better substrate for alcoholic fermentation than blood glucose. It was also observed that sixty percent of the ethanol produced was excreted to the surrounding water following 12 hours of anoxia. Secondly, the greatest increase in whole body lactate occurred during the first 12 hours of anoxia, after which further increase was slow or negligible. Since lactate is not excreted the most likely fate is conversion to ethanol, a novel end product of vertebrate anaerobic metabolism.

The operation of the alternative anaerobic pathway has been localised to the anaerobic red muscle mitochondria (Mourik et al, 1982). Recently, Johnston & Bernard (1983) have shown that in crucian carp, ethanol produced in the white muscle is fifty percent that of the red. The mechanism is based on a co-operation of mitochondrial and cytoplasmic enzymes. Acetaldehyde and carbon dioxide are released from the pyruvate dehydrogenase complex in the red muscle mitochondria (Mourik et al, 1982). Acetaldehyde diffuses into the cytoplasm, where it functions as an electron acceptor to produce ethanol as the end product of skeletal muscle metabolism. In the red muscle there is a high activity of alcohol dehydrogenase which shows a preference for acetaldehyde reduction to ethanol at physiological pH's and consequently maintains redox balance of the fish (Van den Thillart, 1982; Mourik et al, 1982). This suggests that the conversion of lactate by the ethanol

pathway enables glycolysis to continue to provide ATP without an excessive accumulation of lactate.

1.4 Metabolic Responses To Cold Acclimation.

In temperature zones many species of fish are exposed to temperatures ranging from 1 to 35^{0}C during their annual cycle. The thermal dependence of contractile properties and energy metabolism would apparently require the locomotory capacities of these fish to be greatly reduced at low temperatures. However, certain species achieve considerable thermal independence increasing their sustained swimming capacity after acclimation to cold temperatures (Brett, 1967; Fry & Hart, 1948; Griffiths & Alderdice, 1972). This has been attributed to a variety of strategies including increases in the proportion of red muscle in the swimming musculature (Smit et al, 1974; Johnston & Lucking, 1978; Sidell, 1980; Jones & Sidell, 1982), by increases in the aerobic capacity of the red and white fibres (Jankowski & Korn, 1965; Johnston & Maitland, 1980; Tyler & Sidell, 1984; Johnston et al, 1985; Johnston & Wokoma, 1986; Jones & Sidell, 1982; Kleckner & Sidell, 1985; Shaklee et al, 1977; Sidell, 1977,1983; Wilson, 1973), and in the case of carp by adjustment of the contractile properties of muscle fibres (Johnston et al, 1985). For example, acclimation of goldfish from 31 to 2^{0}C over several months results in an increased activity (catalytic efficiency) and reduced activation enthalpy (H) of myofibrillar ATPase at low temperature. In vertebrates activation of myosin cross-bridges is controlled at least in part by a Ca^{2+} - sensitive

regulatory protein complex (tropomyosin - troponins) located on the actin filaments. These proteins are involved in adaptations in the temperature dependence of goldfish actomyosin following acclimation. For example, cross-hybridisation of regulatory proteins from cold acclimated fish to desensitised actomyosin (devoid of its proteins) from warm acclimated fish alters the ATPase towards that of cold acclimated natural actomyosin and vice versa (Johnston, 1979). Furthermore, the properties of desensitised and Mg^{2+}, EGTA-activated natural actomyosin are similar despite differences in acclimation temperature.

Recent studies show that myosins from cold-water fish have a much more "open" tertiary structure than those from warm water species. These structural differences are genetically fixed and possibly reflect selective changes at the level of primary amino acid sequences.

In contrast to goldfish, the kinetic properties of brook trout actomyosin ATPase are not altered following several months acclimation to either +4 or 24^{0}C (Walesby & Johnston, 1980). Salmonids tolerate narrow temperature range of (18 - 24^{0})C (McCauley, 1958). The myosin ATPase of brook trout (Salvenilus fontinalis) would appear to be a compromise between the optimal kinetic forms of summer and winter temperatures and this species relies more on migration to regulate body temperature within its preferential zone (Walesby & Johnston, 1980).

Interestingly, certain sternothermal fish are exposed to a broad range of environmental temperatures. For example, the Lake White-fish (Coregonus clupeaformis), experience a temperature range of 1 to 25^{0}C (Elliot, 1981).

In this fish, cold acclimation during six months does not increase the proportion of red muscle in the swimming musculature or the levels of cytochrome oxidase and citrate synthase, indicating a lack of thermal compensation of the aerobic capacity of the swimming muscles. By contrast, the glycolytic potential of the swimming musculature, as assessed by the activities of hexokinase PFk, displayed positive thermal compensation (Pierre et at., 1988). This may facilitate the conservation of locomotory capacity at low temperatures.

The acclimation capacity of an animal may not be the same in all parts of its temperature range. For example, studies of ATPase activity and Ca^{+}-sensitivity of myofibrillar suspensions from muscles of the mummichog (Fundulus heteroclitus) which experience rapid temperature changes between $15^{0}C$ and $30^{0}C$ show this species has a low thermal dependance over the range 12 - $35^{0}C$ (Sidell, Johnston & Moerland, 1983). Acclimation to low and high temperatures is influenced by a variety of factors. For example, when the photoperiod is long metabolism of sunfish fails to compensate for cold, but it does compensates when the photoperiod is short (Roberts, 1964).

Pretch (1958) provided a classification of the patterns of adaptation. Resistance adaptations would modify the upper and/or lower limits which would change the temperature over which normal function is maintained. For example, arctic and antarctic fishes use glycerol or specific proteins as antifreezes to avoid freezing (Diamond, 1989). Capacity adaptations modify the rate processes (such as enzyme reaction rates) in order to

compensate for temperature effects over the normal thermal range of an organism. The variety of strategies utilised by vertebrates and invertebrates in adaptations include synthesis of proteins, membranes, mitochondria; tolerance of anoxia and very low metabolic rates (Diamond, 1989).

In summary, mechanisms underlying changes in enzyme activity and pathway utilization with temperature acclimation are:

1. Factors influencing enzyme microenvironment (eg $_{p}H$, substrate and modulator concentrations and membrane composition).

2. Conversion of inactive to active form of enzyme (eg phosphorylation).

3. Changes in enzyme concentration.

4. Conformational changes (eg pre-existing genes can give rise to instantaneous isoenzymes).

5. Altered gene expression-isoenzymes.

6. Altered post translatimal processing.

1.5 METABOLISM.

The metabolic pathways involving carbohydrates, amino acids and fat in omnivorous mammals are known. The means and mechanisms of regulation of these pathways are also known to a large extent. The same basic pathways are found in salmonid fish. But there are some differences.

The carnivorous salmonids, like many other fish, have a much greater protein need than omnivorous animals. This, together with the limited ability to utilize carbohydrate, constitutes the basis for the differences.

A key role in the regulation of carbohydrate

metabolism in mammals is played by insulin. In contrast, insulin seems to have a different function in carnivorous fish than in rats (Walton & Cowey, 1982). It has been shown that the measurement of the maximal activities of non-equilibrium enzymes, in vitro, changes in flux through a metabolic pathway (Zammit & Newsholme, 1979). Such enzymes catalysing non-equilibrium reactions can be identified by determination of their mass action ratios and as they are associated with the control points of a metabolic pathway, they tend to have the lowest activities in that pathway. The estimation of such activities has formed the basis of many studies. Newsholme and coworkers have employed an extensive comparative study of carbohydrate and lipid metabolism in the musculature and other tissues of mammals, insects, amphibia and fish (Sugden and Newsholme, 1973, 1975; Zammit & Newsholme, 1976, 1979). In addition, the use of this approach has been useful in the study of teleostean biochemistry. For example, this has been useful in the study of the effects of nutritional status (e.g. Cowey et al, 1979), environmental factors such as temperature and pressure (e.g. Hochachka, 1975), stress (e.g. Nakano and Tomlinson, 1967) and exercise (e.g. Johnston & Moon, 1980a,b; Wokoma & Johnston, 1981) on metabolism. Studies on the regulatory enzymes of glycolysis and gluconeogenesis have led to a greater understanding of the metabolic activities of different tissues (Johnston, 1975b; Hubbert & Moon, 1978; Knox et al, 1980; Moon & Johnston, 1980).

1.5.1 Carbohydrate Metabolism.

Recent reviews include Bilinski, 1974; Driedzic & Hochachka, 1978; Walton & Cowey, 1982. Carbohydrates appear to play a less important role in fish than mammals. Fish generally have lower levels of glycogen stores than mammals. Although glycogen is found in all tissues it is stored predominantly in the liver and the concentration in the slow fibres is three times that of the myotomal fast fibres (Johnston, 1973). There are differences in the rate of utilization of glycogen by the muscle types due to differences in aerobic capacity and differences in the species: elasmobranch or teleosts. In general glycogen is utilized by both fibre types during sustained swimming in advanced teleosts (Pritchard et al, 1971; Johnston et al, 1973). The glycogen thus provides fuel for muscle contraction through aerobic metabolism by slow fibres or via anaerobic metabolism by fast fibres resulting in the production of lactate.

The role of the liver in catabolism of glucose seems to be that of providing intermediates for biosynthetic processes. In contrast to the rat, the activities of the fish enzymes indicate a low capacity of phosphorylation of glucose in muscle and liver. According to Newsholme and Start (1973) the rate limiting step in the utilization of glucose is phosphorylation. In fish hexokinase has a relatively low activity (Nagayama & Oshima, 1974; Newsholme and Start, 1973). Glucokinase which has a high k_m and v_{max} for glucose is also absent in carnivorous cats, (Ballard, 1965).

In most mammals an increased glucose concentration

will lead to induction of glucokinase activity (Conwey et al, 1977; Nagayama and Oshima, 1974).

The enzymes of the glycolytic pathway are similar to each other with respect to substrate specificity and regulatory mechanisms in fish and mammals. One exception is pyruvate kinase (EC2.7.1.40) which in contrast to mammals is not considered as a regulatory enzyme in carp (Cyprinus carpio) (Johnston, 1975) or the eel (Anguilla anguilla) (Roberts & Anderson, 1985).

In mammals, glycolysis is regulated by energy change, nutritional status and hormonal modulation. It has been shown in trout that all three potential endogenous fuels of anaerobic metabolism [glycogen, phosphocreatine (PCr) and adenosine triphosphate] are utilized during white muscle work (Dobson & Hochachka, 1987).

Glycogen phosphorylase catalyses the first step in glycogen breakdown. It has been shown that phosphorylase b is only active in the presence of AMP while the phosphorylase a is fully active in its absence. Krebs and Fischer et al., (1955) demonstrated that the inactive phosphorylase b could be converted to the active phosphorylase a by a kinase enzyme requiring ATP as a cofactor; and the reverse reaction is catalysed by a phosphate. Furthermore, the interconversion of the a and b forms proceeds by a system of multi-site phosphorylation controlled by a cyclic AMP-dependent protein kinase. The complex sequence of events ultimately allows the activation of glycogen phosphorylase.

In rabbits, glycogen breakdown depends on enzyme cascade mechanism initiated by the activation of muscle

phosphorylase kinase. In contrast, the dogfish enzyme depends only on Ca^{2+} for its activity (Fischer et al, 1975). This may be due to relatively poor capillary supply and consequently low blood circulation in the white fibres of the species.

1.5.2 Phosphofructokinase (E C 2.7.1.11)

Catalyses the phosphorylation of fructose 6-phosphate (F6P) to fructose,1,6,bis-phosphate (FDP). PFK is activated by various substrates including AMP, ADP, Pi and NH_4 and inhibited by ATP, citrate and creatine phosphate (Mansour, 1972). PKF from goldfish (Carassius auratus) fast fibres has been found to have similar characteristics (Freed, 1971). Some fish fast fibres display high activities, even exceeding those found in other vertebrates (Crabtree & Newsholme, 1972). It has long been suggested that substrate cycling operates between F6P and FDP, resulting in amplification of enzyme response to variation in AMP concentration (Moon & Johnston, 1980). Newsholme et al (1978) have shown that the higher the initial ATP/AMP ratio, the greater the AMP and the consequent increase in stimulation of glycolysis.

Newsholme et al, (1973) have proposed futile cycles may provide an amplification mechanism for the allosteric control of metabolic flow. This theory stipulates that a small change in the concentration of an effector may provide much larger variations in the net flux through the cycle. The extent of amplification depends on the rate of recycling (Newsholme & Start, 1973). Stein and Blum (1978) have, however, noted that this theory does not take into

account the influence of the concentration of the product of the forward reaction on the rate of the back reaction, so that the amplification obtained is less than was previously thought.

It appears the futile cycles may have other functions in the control of metabolism. For example, the orientation of flux at metabolic crossroads. The orientation of the metabolic flow in the direction of glycolysis or gluconeogenesis depends on the activity of PKF and PK. The glucose / glucose-6-phosphate cycle is involved in the control of glucose output and uptake and the pyruvate cycle may participate in the complete oxidation of amino acids. Newsholme and Start (1973) emphasised that futile cycles offer a sensitive way of controlling the concentrations of metabolites or ions. The buffering of the concentrations of Ca^{2+}, the maintenance of small concentrations of glycogen in the livers of fasted animals, the proportion of an interconvertible enzyme in one of its forms reflect, at least in part, a dynamic equilibrium between antagonistic reactions. Indeed, by the simultaneous operation of the extrahepatic glycolysis, it helps to maintain the blood concentrations of lactate and glucose at a relatively constant level. In this respect, it certainly is not considered futile.

1.5.3 Pyruvate Kinase (EC 2.7.1.40)

Pyruvate phosphotransferase, PK is the final regulatory (not in fish) enzyme of glycolysis. It catalyses the irreversible conversion of PEP and ADP to

pyruvate and ATP. The integration of the activities of PFK and PK involves adenylate coupling since ADP which is a product of PFK reaction serves as a substrate for PK (Driedzic et al, 1978).

The activity of PK is closely linked with two opposing gluconeogenic enzymes, pyruvate carboxylase and phosphoenolperuvate carboxykinase (PEPCK),which operate a futile cycle. PEP is converted to pyruvate and further to oxaloacetate, catalysed by pyruvate kinase and pyruvate carboxylase respectively. The cycle is completed by the conversion of oxaloacetate back to PEP by PEPCK. This futile cycle is similar to the PFK/FDPase substrate cycle, and is thought to allow a greater control of metabolic flux, such that a simultaneous activation of one enzyme and inhibition by another leads to an amplification of enzyme response to changes in substrate concentrations (Newsholme & Start, 1973). The consequence of this phenomenon and the Ca^{++} release from SR during burst swimming produce a rapid generation of energy anaerobically through increased glycolytic flux. Anaerobic generation of energy is also useful when immediate muscular activity is needed, such as escape from predators or in feeding.

1.5.4 The Pentose Phoshate Pathway (PPP) provides an alternative pathway to carbohydrate metabolism from G6P. The pathway generates NADPH for fatty acid synthesis and pentose phosphates for nucleic acid synthesis. Lin et al (1977) have provided evidence that in teleosts such as coho salmon (Oncorhynchus kisutch Walbaum) and in rats, reducing equivalents for lipogenesis are channelled through the

intensively studied in fish and it is found to have identical properties to that of mammals (Tarr, 1972).

1.6 Lipid metabolism.

The major storage form of lipid is triglyceride which consists of glycerol backbone with 3 fatty acid groups attached. There are a variety of fat deposit sites in fish. In teleosts, TG storage sites are the adipose tissue, the musculature and specific sites in the viscera. In contrast, the storage site in elasmobranchs is the liver (Love, 1970). During spawning migrations or sustained swimming speeds lipid serves as the preferential fuel for aerobic metabolism (Bilinski, 1974). High activities of carnitine transferase and triacyl glycol lipase have been found in the slow fibres of teleosts. In contrast, these enzymes are absent in elasmobranchs (Zammit & Newsholme, 1979). Slow muscle contains a higher concentration of lipid deposits than fast (Bone, 1966; Bilinski, 1974), which in salmonids enables oxidation of long-chain saturated fatty acids at about 10 times the rate of that in fast muscle (Jonas & Bilinski, 1964; Bilinski, 1974). During starvation teleosts rely on fatty acid oxidation for their source of fuel while elasmobranchs depend on the utilisation of ketone bodies (Zammit & Newsholme, 1979). It is clear that the slow muscle is better adapted to utilise lipid than the fast muscle fibres.

1.7 Amino Acid Metabolism.

Unlike carbohydrates or lipids, amino acids cannot be stored in specific organs. Excess amino acids or proteins are either deaminated and oxidised to CO_2 and H_2O via the TCA cycle, or alternatively converted into carbohydrates or lipids. In mammals the major end product of nitrogen metabolism is urea. In contrast, end products in fish include mainly ammonia with lower concentration of uric acid, trimethylamine oxide, creatine and creatinine. In the carp for example up to 80% of nitrogen is in the form of ammonia (Creach & Serfaty, 1974).

1.7.1 In aquatic species, ammonia can be excreted directly into the surrounding medium without prior conversion to other energetically expensive compounds such as urea, hence energy is conserved. It has been suggested that this adaptation is particularly important for species with low intake of dietary carbohydrate, and high fat or protein intake.

Proteins as well as all other cellular constituents, are in a state of continuous turnover. This phenomenon enhances the organism's ability to adapt to changes in its environment (Dice et al., 1976). Protein turnover is a function of the rate of synthesis and the rate of degradation (Odessey et al., 1974).

1.7.2 In fish muscle the two major free amino acids are glycine and histidine, form a great proportion of the free amino acid pool in some species and are utilised extensively in starvation (Fontaine & Marchelidon, 1971).

It has been suggested histidine and other amino acids are utilised directly for energetic purposes (Driedzic & Hochachka, 1978).

Fish muscle contains a large number of transaminating enzymes. Alanine and aspartate aminotransferase are quantitatively the most important (Walton & Cowey, 1982; Siebert et al, 1965). The major oxidative deamination enzyme found in fish is glutaminate dehydrogenase which is ultimately involved in the production of ammonia.

1.7.3 During starvation all animals utilise their muscle proteins as an energy source. Amino acids play a major role in muscle metabolism. Following induced starvation in the laboratory, the order of catabolism of substrates in fish are free amino acid < glycogen < and/or lipids (see Creach & Serfaty, 1974). Johnston and Goldspink (1973d) reported that fast muscle fibres are preferentially mobilised before the slow muscle. Several experiments have shown that the relative anaerobic capacities and metabolism of the slow and fast muscle fibres can be modified by environmental factors such as temperature acclimation, seasonal variation, fish migration (Hazel & Prosser, 1974), exercise training (Johnston & Moon, 1980a), nutritional state such as starvation (Moon & Johnston, 1980) and development (Bostrom & Johansson, 1972). For example, HK was as expected increased in rainbow trout given a carbohydrate rich diet, while a high protein diet led to decrease both in HK and in the PFK activities.

In starvation, HK, PFK and PK showed decreased activities and interestingly, Moon and Johnston (1980)

found only small changes in the activities of these enzymes in livers of plaice (Pleuronectes platessa) starved for four months while the activities of muscle enzymes were depressed. In the rat starvation will quickly lead to 100-fold increase in G-6-PDH activity (Potter & Ono, 1961), while the reverse appears to occur in fish.

In rainbow trout submitted to physical stress, Morata et al (1982b) observed a rapid fall in liver glycogen, with a maximal reduction of 40% after 30 minutes. Subsequently, de novo synthesis brought the glycogen level back to the control level within 45 minutes. In muscle, the breakdown during stress was more rapid and no replacement synthesis could be observed during sixty minute experimental period. This indicates that the liver glycogen is an emergency store and is only mobilized during the first minutes of a critical situation. The muscle glycogen however is more readily available and especially the white muscle will quickly breakdown the glycogen to lactate during burst swimming activity or escape from predators (Stevens & Black, 1966).

In contrast to mammals, in fish the role of hormones in the regulation of glycogen metabolism is less well understood and both an increase (Yanni, 1964; Plisetskaya et al, 1984) and a decrease (Ince & Thorpe, 1976; Carneiro & Amural, 1983; Ottolenghi et al, 1984, 1985) in glycogen content of the liver have been reported as a result of insulin administration. Some hormones are capable of utilizing both the cyclic AMP and Ca^{2+} second messenger systems. For example, adrenaline can bind to at least three types of receptors α_1, α_2 and β_1, causing

stimulation of PIP_2 (diacylglycerophospho(1)inositol 4,5-bisphosphate) hydrolysis, inhibition of adenylate cyclase and activation of adenylate cyclase respectively. These multiple interactions between the cyclic AMP and Ca^{2+} second messenger systems greatly increase the flexibility and versatility of hormone action (Berridge et al, 1984; Joseph, 1984).

Cohen (1983) has shown that phosphorylation of phosphorylase kinase by cAMP-PK increases its sensitivity to Ca^{2+} as well as its catalytic efficiency , and plays an important role in the mobilisation of muscle glycogen by adrenaline.

The present study was to investigate the various aspects of the metabolism of myotomal muscles of fishes in relation to thermal acclimation and hypoxia.

CHAPTER 2

The effect of thermal acclimation on the metabolism of the swimming musculature of flounder (Platichthys flesus L.)

2.1. Introduction

Fish are adapted to different thermal conditions of their habitat ranging from 0-1.9^{o}C in Antactic species to 25-40^{o}C in Tilapia of the hot lakes of Kenya (Reite et al., 1974). The behaviour and metabolism of many fish varies with the length of time they are exposed to a particular temperature. The effects of temperature acclimation are more marked for species which live in environments of marked seasonal variation in temperature, e.g. inland waterways and shallow seas. Some species become relatively inactive and cease feeding as temperature is reduced and are thought to enter a torpid or dormant state (Crawshaw, 1984). These are characterized by low metabolic rates. Some species of fish have evolved strategies for reducing this metabolic dependence and for sustaining locomotory ability at decreased temperatures (Prosser, 1973, Dean, 1969). Johnston & Dunn (1987) have shown that muscle contraction is modified by low temperature acclimation in some freshwater fish of the cyprinid family. For example, the Mg^{2+} Ca^{2+} ATPase activity of myofibrils at 1^{o}C was found to be 3-4 times higher in goldfish acclimated to 1^{o}C than 26^{o}C (Johnston et al., 1975). It has been shown, cold acclimation in goldfish increased catalytic efficiency but decreased thermal stability (Johnston et al., 1975). Myofibrillar ATPase activity is related to the speed of shortening of muscles (Barany, 1967) and hence

qualitative alterations in cold acclimated fish would appear to offer a mechanism for enhanced locomotory performance at lower temperatures (Loughna et al., 1983). Changes in ATPase activity with temperature acclimation require several weeks for completion and probably involve the synthesis of new proteins (Johnston, 1979). It is suggested that the calcium regulatory proteins of muscle play a significant role in the acclimation of actomyosin system to an altered cell temperature and are capable of influencing the kinetics of the ATPase enzyme (Johnston, 1979).

Studies with skinned muscle fibres from common carp have shown that cold acclimation increases force production, contractile velocity (Johnston et al., 1985), and the economy of isometric contraction (Altringham & Johnston, 1985).

Temperature acclimation is also reported to affect the potential for ATP production in fish skeletal muscle (Hazel & Prosser, 1974; Sidell, 1983). Cold acclimation results in an increase in myoglobin content and the activities of mitochondrial enzymes, particularly in the slow myotomal fibres, while the glycolytic enzymes remain unchanged or decline slightly (e.g. Lepomis cyanellus, Shaklee et al., 1977; Carassius auratus, Sidell, 1980). The flounder (Platichthys flesus), a marine fish is a common coastal fish occurring from 55 metre to the tide line. It also penetrates into rivers and lakes which flow into the sea. It is a relatively eurythermal species and tolerates temperatures ranging from 0 to 25^{o}C (Duthie and Houlihan, 1982). The aim of the present study was to utilize quantitative measurement of enzyme activities to determine the effect of temperature acclimation on the metabolism of flounder.

2.2 Materials and Methods

2.2.1 Fish

A group of 12 flounders (Platichthys flesus L.) (303 ± 38 g body weight, 25.9 ± 1.20 standard length, mean ± S.E.) were acclimated in tanks of filtered sea water to either 5°C or 23°C for 8 weeks. They were subjected to a photoperiod of 12h light:12h light:12h dark, and fed daily on live ragworms and lugworms.

2.2.2 Preparation of tissue homogenates

Fish were killed by stunning followed by transection of the spinal cord. The entire red muscle strip from the ventral myotomes was dissected, minced and approximately 0.5 g taken for enzyme analysis. White muscle was sampled from the deeper fibres adjacent to the red muscle.

Tissue samples were homogenised in a ground glass homogeniser with ice-cold extraction buffer 0.4°C 1:10 w/v) which contained 100 mmol L^{-1} Tris aminomethane-HCl (Tris-HCl), 1.0 mmol L^{-1} EDTA; 0.5 mmol L^{-1} dithioerythritol (DTE), pH 7.4. DTE was omitted for assays of carnitine palmitoyl transferase and citrate synthase due to the presence of 5', 5' dithiobis 2-nitrobenzoic acid (DTNB) in the reaction medium.

2.2.3 Assay of enzyme activities

Assays were performed at 15° C ± 0.2°C using a Cecil CE599 dual-beam spectrophotometer which had been fitted with a constant temperature cell. Preliminary determinations were

carried out in order to ensure substrate concentrations, cofactor concentrations and pH produced optimal activities. Changes in absorbance at 340 nm were used in calculation of enzyme activities for hexokinase, 6-phosphofructokinase and lactate dehydrogenase; at 412 nm for carnitine palmitoyl transferase and citrate synthase; and 550 nm for cytochrome oxidase. Calculations of enzyme activities were based on millimolar extinction coefficients of 6.22 (for NADH, NADPH at 340 nm) and 13.6 for reduced DTNB at 412 nm).

Hexokinase (EC.2.7.1.1):

The assay media were as follows (in mmol L^{-1})
Tris-HCL, 75; $MgCl_2$, 7.5; EDTA, 0.8; KCl, 1.5; $NADP^+$, 0.4; ATP, 2.5; glucose 10; creatine phosphokinase, 0.9 units ml^{-1}; glucose phosphate dehydrogenase, 0.7 units ml^{-1}; pH 7.6.

6-Phosphofructokinase (E.C.2.7.1.11)
The assay medium contained (in mmol L^{-1}): Triethanolamine, 75; $MgCl_2$, 7.5; KCl, 200; KCN, 1.0; AMP, 2.0; ATP, 1.0; NADH, 0.15; fructose-phosphate, 0.4; aldolase, 2 units ml^{-1}; trisphosphate isomerase, 10 units ml^{-1}; glycerol-phosphate dehydrogenase, 2 $units^{-1}$; pH 8.2.

Lactate dehydrogenase (E.C.1.1.27)

The assay medium contained (in mmol L^{-1}): Immidazole, 50; KCN, 2.5; NADH, 0.15; pyruvate, 10; pH 7.3

Carnitine palmitoyl transferase (E.C.2.3.1.23)

The assay medium contained (in mmol L^{-1}): Tris, 7.5; EDTA, 1.5; DTNB, 0.25; carnitine, 1.25; palmitoyl Coa, 0.035; pH 8.0.

Hydroxy acyl CoA dehydrogenase (E.C.1.1.1.35)

The assay medium contained (in mmol L^{-1}): imidazole, 50; EDTA, 1.0; NADH, 0.15; acetyl CoA, 0.1; pH 7.5.

Cytochrome oxidase (E.C.1.9.3.1)

The assay medium contained (in mmol L^{-1}): K_2HPO_4 / KH_2PO_4 10; 0.065% reduced cytochrome C, pH 7.0 (obtained by adding sodium dithionite to a solution of cytochrome C to yield an absorbance of 1.0), then dialyzed overnight against several volumes of the same phosphate buffer, pH 7.0. Enzyme activity was assayed by following the oxidation of reduced cytochrome C at 550 nm.

2.3 Statistical analysis

Results obtained for fish acclimated to either $5^{o}C$ or $23^{o}C$ were compared using one-way analysis of variance.

2.4. Results

Activities of enzymes of energy metabolism from myotomal muscles of flounder, acclimated to either 5°C or 23°C are shown in Figs 2.1, 2.2.

Hexokinase activity of the slow fibres was four times that of the fast fibres ($P<0.001$). The activity was 50-70% higher with cold acclimation in both slow and fast fibres.

Carnitine palmitoyl transferase, thought to be the rate limiting step for fatty acid oxidation and 3-hydroxyacyl CoA dehydrogenase, a marker for beta-oxidation of fatty acids were 3 times ($P<0.001$) and 1.6 times ($P<0.05$) greater in cold than warm acclimated slow myotomal muscle. The concentrations of CPT and 3 HACDH of the slow muscle were 11 times ($P<0.001$) and 2 times ($P<0.05$) higher than the corresponding enzymes in the fast skeletal muscle respectively.

Cytochrome oxidase, a respiratory chain enzyme was 2.8 times higher in the cold than warm acclimated slow muscle ($P<.0001$). The activity of the slow muscle was 5 times higher than the corresponding enzyme of the fast muscle ($P<0.001$).

When assayed under a constant temperature, PFK, a prime regulator of glycolysis and LDH, the last step in glycolysis were found to be independent of acclimation temperature.

2.5 Discussion

2.5.1. Aerobic enzymes

In flounders, following a period of cold acclimation, the activities of enzymes associated with aerobic ATP turnover and fatty acid catabolism increase. For example, COX, 3-HACDH and CPT were 2.8, 1.6 and 2.6 times higher in the cold than warm acclimated fish. (Fig. 2.2). This effect occurs to a greater extent in slow than fast muscle, when enzyme activities are measured at a common test temperature (Figs. 2.1,2.2) Similar results were found in green sunfish (Shaklee, Christiansen, Sidell and Prosser, 1977), common carp (Johnston, Sidell & Diedzic, 1985), striped bass (Jones & Sidell, 1982) and chain pickerel (Kleckner and Sidell, 1985). For example, Wilson (1973) found that acclimation of goldfish from 25°C to 5°C resulted in respectively, a 66 and 45% increase in concentration and activity of muscle cytochrome oxidase. Cold acclimation in fish is also associated with an increase in cytochrome C concentration (Wilson, 1973) which is an indication of mitochondrial activity. The mechanisms underlying these changes in enzyme concentrations result in part, from the different thermal sensitivities of the rates of synthesis and degradation. For example, Sidell (1977) used (δ^{-14}C) aminolaevulinic acid to determine the loss of specific radioactivity of cytochrome C during acclimation of green sunfish (Lepomis cyallenus R.) from 25°C to 5°. The net increase in cytochrome C with low temperature acclimation results from changes in the synthesis/degradation ratio (Sidell., 1977). This suggests

changes in tissue mitochondrial density with temperature acclimation could result from direct effects of temperatre on mitochondrial turnover. The effect of higher mitochondrial densities is to increase the rate of aerobic ATP generation relatively for that of acutely cooled or warm tissues. Mitochondrial proliferation also partially offset the reduced diffusion rates of oxygen and metabolites at low temperatures (Hazel & Sidell, 1987). This is supported by increase in the concentrations of mitochondrial succinic dehydrogenase (Sidell, 1980), myoglobin (Johnston et al., 1985) and oxygen consumption with cold acclimation (Johnston & Maitland, 1980).

In flounder the activities of 3 Hydroxy acyl CoA dehydrogenase (3 HACDH) and CPT are 2-4 fold greater in cold- than warm- acclimated slow fibres (Table 2.1, Fig. 2.2) which suggests an increased capacity for fatty acid oxidation. These activities are similar to the correesponding enzymes in carp (Johnston et al., 1985) following cold acclimation. In striped bass (Morone saxitilis) the volume density of lipid droplets in slow muscle is 15-fold higher in 5^{o}C than 25^{o}C-acclimated fish (Egginton and Sidell, 1986). Teleosts primarily utilise fat fuels such as triacylglycerol, fatty acids or acetoacetate, and have a very low capacity for ketone body oxidation (Crabtree and Newsholme, 1972; Zammit & Newsholme, 1979). It has been demonstrated that half the maximum in vitro activity of carnitine palmitoyl transferase is sufficient to account for the calculated rates of lipid utilisation of intact muscles (Crabtree and Newsholme, 1972).

Cold acclimation does not change the density of enzymes on the inner mitochondrial membrane. For example, the stereological analysis of Tyler and Sidell (1984) found no difference in the surface densities of inner mitochondrial membrane between warm-and cold-acclimation in goldfish muscle fibre. Another possibility is that the changes in mitochondrial enzyme activity occur as a result of changes in membrane phospholipid composition which accompany thermal acclimation (Hazel 1972, a, b; Van den Thillart and Modderkolk, 1978; Van den Thillart and Bruin, 1981). In membrane-bound enzymes, the physical state of associated lipids may play an important role in stabilizing protein structure (Cossins et al., 1981). There is generally an increase in the unsaturation of phospholipid fatty acids with membranes at lower environmental temperatures, and this is associated with maintaining membrane fluidity (Johnson and Roots, 1964; Knipprath and Meads, 1968; Sinensky, 1974; Cossins et al., 1977). Some membranes, such as those from mitochondria seem more responsive to change than others, such as sarcoplasmic reticulum (Cossins et al., 1981). The importance of the composition of the membrane environment for enzyme activity was shown by Hazel (1972 a, b,). For example, the mitochondrial enzyme, succinic dehydrogenase (SDH) isolated from the expaxial muscle of goldfish (Carassius auratus) acclimated to 5°C had 1.3 - 2.2. times the activity of SDH from 25°C acclimated goldfish.

Interestingly, although both oxygen consumption and cytochrome oxidase activities are higher in muscle mitochondria isolated from cold compared to warm acclimated goldfish, there are no consistent changes in the degree of unsaturation of membrane phospholipids (Van den Thillart and Modderkolk, 1978). For example, it has been shown that mitochondria from cold-acclimated carp contain more phosphatidyl ethanolamine and less phosphatidyl choline, suggesting the membranes are more negatively charged at low acclimation temperature; the fatty acids of phosphatidyl choline are more unsaturated (Wodtke, 1981).

In contrast to the increase in aerobic enzymes, the activities, and hence the concentration of glycolytic enzymes PFK and LDH in muscle are generally unchanged in temperature acclimation (Fig. 2.1). Similar results have been obtained by other teleosts (green sunfish, Shaklee et. al. 1977; goldfish, Sidell, (1980); Common carp, Johnston et. al. 1985; chain pickerel, (Kleckner and Sidell, 1985). The degree of activation of individual enzymes is determined by the concentrations of substrates and modulators and by the rate of product removal. One possibility is that the concentrations of these modulators change following an acute drop in body temperature such as to reduce the direct effects temperatures on enzyme reaction rate (Walseby and Johnston, 1980; Walsh and Somero, 1982). For example, Walseby and Johnston (1980) acclimated brook trout (Salvelinus fontinalis) to either 4 to 24°C in a water

tunnel at a constant swimming speed of 2.5 body lengths sec^{-1}. It was found that parameters of metabolic control such as the adenylate energy charge [ATP] + 0.5 [ADP]/[ATP] + [ADP] + [AMP]. phosphorylation state ([ATP/ [ADP] [Pi] and the ratio [ATP]:[AMP] were all significantly lower in cold than warm - acclimated white muscle. These changes are consistent with a greater degree of activation of mitochondrial respiration and glycolysis at cold acclimation temperatures.

Marked tissue differences occur in the responses to temperature acclimation. For example, increase in the activity of glycolytic enzymes are observed in the brain at low temperatures (Shaklee et.al., 1977). In the hepatic system of striped bass (Morone saxitilis) in contrast, cold acclimation results in an increased reliance on carbohydrate substrates for energy metabolism, while lipids are utilized more at warm temperatures (Stone & Sidell, 1981). It is suggested changes in the patterns of fuel usage between the liver and muscle tissue at low temperatures allow the partition of energy reserves for the seasonal activities of migration and reproduction (Stone & Sidell, 1981). Interestingly, low winter temperatures induce high lipid concentrations in the liver, which may be utilized during migration or prolonged swimming activity in the spring.

Recent studies (Johnston and Wokoma, 1986) on contractile characteristics of flounder muscle show that they are unaltered by several months acclimation to either 5°C or 23°C. They appear to represent a compromise between those optimal for the extremes of its thermal range. The force-velocity (p-v) relationship of flounder fibres was found to become progressively less curved with decreasing temperature. A decrease in curvature represents a higher power output and this provides an intrinsic mechanism for stabilising muscle power output. Such characteristics would not require the need for further changes in contractile proteins with acclimation a this may constitute an evolutionary strategy for fish capable of tolerating sudden changes in body temperature.

FIGURE 2.1. Histograms showing maximal enzyme aciivities (measured at 15°C) of carbohydrate metabolism (HK, PFK and LDH) in slow and fast myotomal muscles from either 5°C- (striped) or 23°C- (clear) acclimated flounders (Platichthys flesus). Results represent (μmoles substrate utilised g^{-1} min^{-1}, n=6)

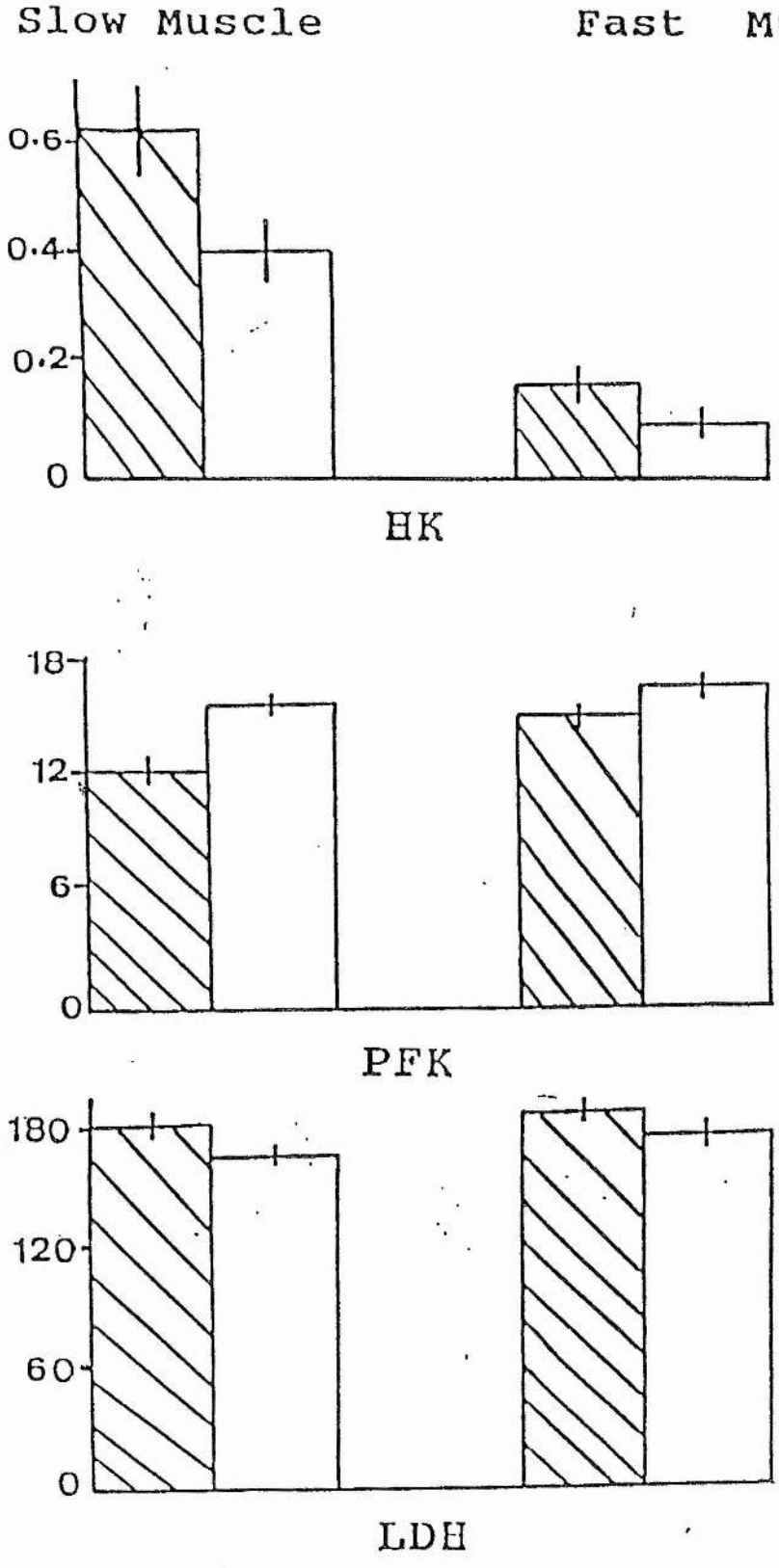
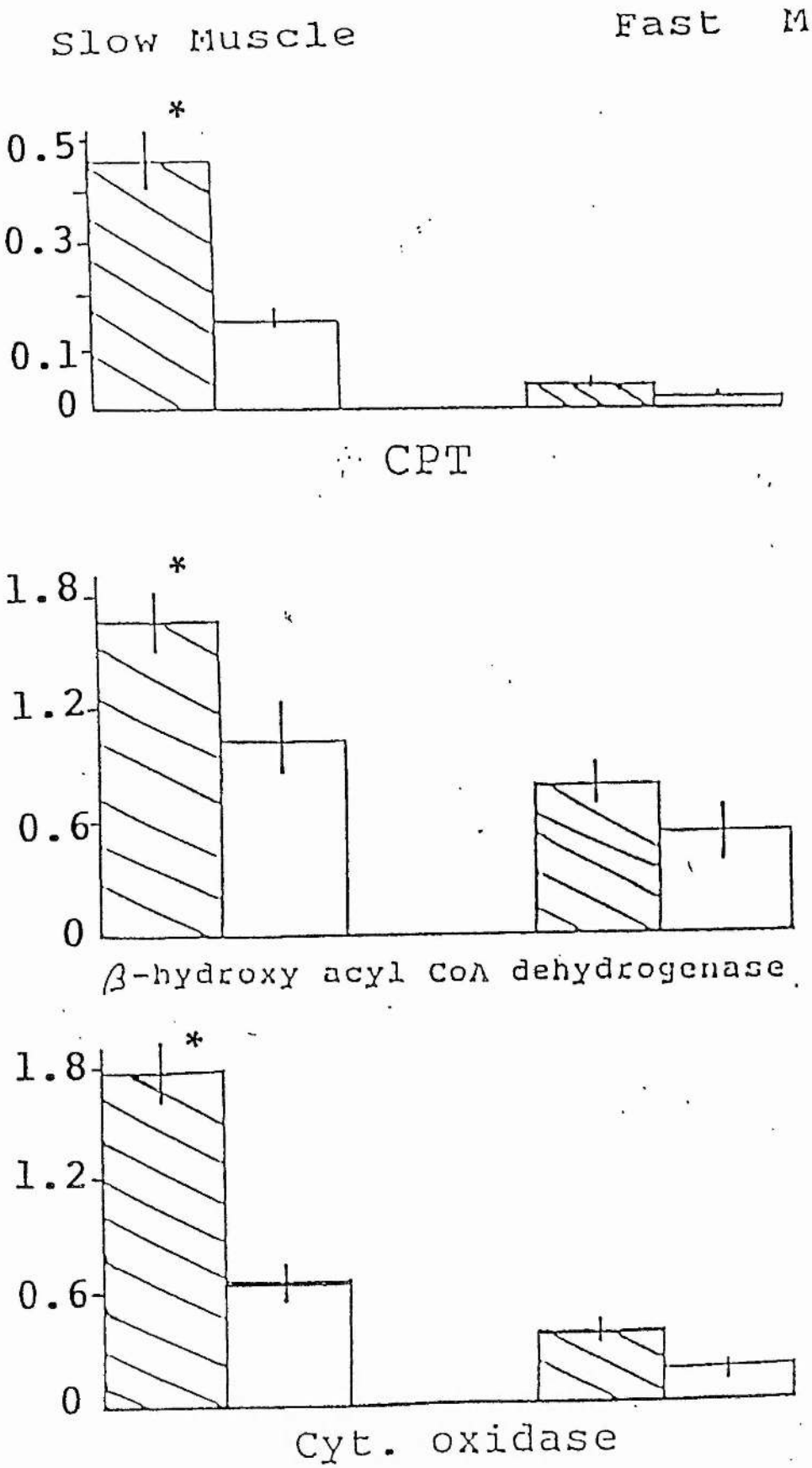

Slow Muscle
Fast Muscle
0.6
0.4
0.2
0
HK
18
12
6
0
PFK
180
120
60
0
LDH

FIG.2.1.

FIGURE 2.2. Histograms showing maximal activities (determined at 15°C) of marker enzymes of fatty acid oxidation, carnitine palmitoyl transferase (CPT), β-hydroxy acyl CoA dehydrogenase (β-HACDH) and mitochondrial metabolism, cytochrome oxidase (COX) from either 5°C- (striped) or 23° C- (clear) acclimated flounders (Platichthys flesus). Results represent (umoles substrate utilized g^{-1} min^{-1}, Means ± S.E., n=6)


Slow Muscle
Fast Muscle
*
0.5
0.3
0.1
0
CPT
*
1.8
1.2
0.6
0
β-hydroxy acyl CoA dehydrogenase
*
1.8
1.2
0.6
0
Cyt. oxidase

CHAPTER 3

The effect of epinephrine administration on phophorylase activity in the slow and fast muscles of flounder (Platichthys flesus L.).

Introduction

In mammals epinephrine treatment results in hepatic glycogenolysis and consequently hyperglycaemia by stimulation of specific activity liver glycogen phosphorylase. In skeletal musculature epinephrine causes glycogenolysis resulting in a rise in lactate concentration in blood. The increased glycogenolysis is due to the stimulation of adenylate cyclase system, which leads to an activation of glycogen phosphorylase through an increase in cyclic-AMP synthesis (Sutherland and Cori, 1951).

The hyperglycaemic effect of epinephrine in teleost fish is well documented by in vivo experiments (Maseaud, 1964; Nakano and Tomlinson, 1967; Murat and Serfaty, 1975; Ottolenghi et al., 1984a). Some authors found that the increase in blood glucose is accompanied by a decrease in hepatic glycogen concentration (Stimpson, 1965; Demael-Suard and Garin, 1974; Ottolenghi et al., 1984a), but others (Kumar et. al., 1966; Young, 1968) did not observe changes in glycogen levels. Differences of hormonal effect on fish glycogen metabolism have also been observed in in vitro experiments, and has been related to fish species. For example, Birnbaum et al., (1976) have shown that hepatocytes isolated from goldfish (Carassius auratus) respond to epinephrine treatment by increasing glucose release to the medium via the glycogenolytic pathway. On the other hand,

experiments by Morata et. al., (1982), showed that liver slices of rainbow trout (Salmo gairdneri) respond to epinephrine treatment by increasing glucose release to the medium during incubation with no decrease, and sometimes with an increase in the glycogen content.

In catfish, epinephrine induced rapid hyperglycaemia accompanied by a decrease in glycogen of the liver, while that of the red and white muscles remained unaffected (Ottolenghi et al., 1984). In vitro, epinephrine induced an increase in glycogen phosphorylase activity, a decrease in glycogen level and increase in glucose output in liver slices (Ottolenghi et. al., 1986). These effects have been ascribed to the prevailing beta-adrenergic activation of liver phosphorylase (Brighenti et. al. 1987) which is specifically blocked by the beta-adrenergic antagonist, propranol but is unaffected by phentolamine, an alpha-adrenergic blocker nor prozosin, yohimbine, which are blockers for varieties of alpha-receptors (Brighenti et. al. 1987; Janssens et. al., 1983) respectively.

However, on the basis of lack of glycogen phosphorylase in Cyprinus carpio, and of the presence in the liver homogenates of an important gamma-amylase activity, an amylolytic pathway was suggested as an alternative pathway to glycogen degradation (Murat et. al., 1972, Murat, 1976). Interestingly, Picukans and Umminger (1979), demonstrated that glycogen phosphorylase activity was in fact, present in addition to the gamma-amylase. However, the presence in the liver of tench (Tinca tinca) of an alpha-amylase activity which is influenced by fasting and season (Brighenti and Callegarini, 1968) gives some support to an amylolytic pathway.

In fish the skeletal muscle forms a larger part of the body mass

than other vertebrates (Bone, 1978) and catecholamines are liberated under stress, during attack escape reaction, and during muscular exercise (Nakano and Tomlinson, 1967). Therefore in the present study an *in vivo* administration of epinephrine was employed to determine its effects on phosphorylase activity in both the slow and fast muscles of flounder (*Platichthys flesus* L.).

3.2. Materials and Methods

Fish:

Two groups of 12 flounders (*Platichthys flesus* L.) 141.2 ± 7.37 g body wt, 16.3 ± 2.40 cm standard length; Mean± S.E.) were maintained in holding tanks of recirculated filtered sea-water at 10°C. Fish were transferred individually to the experimental tank at least 2 days prior to the experiment in order to remove the effect of stress. Fish were lightly anaesthesised with tricaine methanesulphonate (MS.222), which was slowly introduced to the experimental tank to a final concentration of 0.1 g, L^{-1}. Following an intravenous injection of epinephrine (10 µg kg^{-1}) fish were returned to the experimental tank for a further 15 minutes before being sacrificed. The control group had no epinephrine treatment. Another group was injected with propranolol (10 µg Kg^{-1}) before the administration of epinephrine.

3.2.2. Preparation of homogenates

Fish were killed by a blow to the head and spinal

transection. Tissue samples of fast and slow muscles (0.5 to 1 g) were dissected and clamped between tongs pre-cooled in liquid nitrogen ($-196^{o}C$).

Muscle samples were ground and homogenised in 5-10 vol. ice-cold buffer at $1^{o}C$ with a polytron homogeniser. Glycogen phosphorylase extraction buffer was 100 mM tris-malate, 20 mM NaF, 1 mM EDTA, 0.5 mg, ml^{-1} bovine serum albumin, and 10 mM dithiothreitol at pH 6.8.

3.2.3. Enzyme Assay

The assay medium contained 100 mMol phosphate buffer, pH7.0, 1 mM EDTA,10 mMol NaF, 8 mmol $MgCl_2$, 2 mg ml^{-1} fructose 1, 6-bisphosphate, 0.4 mmol $NADP^{+}$, 1 mg ml^{-1} purified muscle glycogen, excess coupling enzymes phosphoglucomutase and glucose-6-phosphate dehydrogenase (1 ml final volume). The medium was used in the presence and absence of 1 mmol AMP.

3.3. Statistical Analysis

The results were compared using a Student's t-test.

3.4. Results

The effect of epinephrine on phosphorylase activity of slow and fast skeletal muscle is shown in Table 3.1.

In the control fish, phosphorylase activities ($\mu mol\ g^{-1}\ min^{-1}$) were 4.57 for slow and 10.87 for fast. Following administration of epinephrine 15 minutes were allowed for in vivo activation of phosphorylase (i.e. conversion of phosphorylase b to a).

Phosphorylase a activity is taken as the ratio of phosphorylase activity in the absence of AMP, to the phosphorylase activity in the presence of AMP. The results show that phosphorylase a activity increased from (59-86%) for slow and (64-87%) for fast muscle. In contrast to flounders with epinephrine, the group with propranolol treatment showed no increase in phosphorylase activity, suggesting the involvement of B-adreno-receptors.

3.5. Discussion

Glycogen is the major source of energy for anaerobic metabolism, and is found to be depleted rapidly in the fast muscle, during burst swimming. For example, in rainbow trout (Salmo gairdneri Richardson) approximately 50% of the fast muscle glycogen is depleted during the first 15 seconds burst swimming (Stevens & Black, 1966). The catalytic enzyme is phosphorylase, the activity of which is the result of hormonal action, such as adrenaline or via the influence of Ca^{2+}.

In the slow muscle which is well vascularised, hormonal control is likely to be more important, whereas in fast muscle Ca^{2+} activation is thought to be of greater importance. Under normoxic conditions the glycogen content in the flounder is of the same magnitude as the coalfish (Pollachus virens) white muscle (Johnston & Goldspink, 1973) and eel (Anguilla anguilla) white muscle (Phillips & Hird, 1977). However, the glycogen concentration in flounder muscle is far below that of the white and especially the red muscle of carp.

The mechanism of activation of phosphorylase involves a cascade series of enzymatic reactions. An initial receptor

stimulation of the membrane - bound adenyl cyclase leads to the activation of c-AMP-dependent protein kinase (Robinson et. al., 1971). This enzyme in turn catalyzes phosphorylase b kinase whose substrate is phosphorylase b and requires ionized Ca^{2+} (from the S.R.) for activity (Hansford & Sacktor, 1970).The latter enzyme finally catalyzes the formation of phosphorylase from phosphorylase b with the concomitant degradation of glycogen to glucose-1- phosphate.

However, the studies of Sacktor et. al., (1966) and Childress and Sacktor (1970), have demonstrated that phoshorylase b was too low to account for the rate of glycogenolysis during flight of the blow fly (Formia regina). They calculated that activity of phosphorylase a under simulated in vivo conditions was adequate to account for glycogenolysis during flight provided at least 50% of the total enzyme was in the 'a' form. Their data indicate that phosporylase a levels in the resting flies was 18% of the total. After 30 seconds of flight, phosphorylase a levels rose to 72% of total. Thus glycogenolysis is facilitated by phosphorylase b to a conversion (Childress & Sacktor, 1970).

The ratio of phosphorylase activity without AMP to phosphorylase with AMP represents the percentage of total phosphorylase present in the active form. In the present study on flounder, the percentages of phoshorylase a levels in the resting state are 59 and 64 for slow and fast muscle fibres respectively. Following epinephrine stimulation, enzyme activities increased by 46 and 36% respectively (Table 3.1). Other teleosts have been shown to have phosphorylase a activities. For example, the percentage of phosphorylase a in the normoxic catfish were 10 and 34 in the fast and slow muscle fibres respectively, which increasd by 5 and 57% respectively

following epinephrine stimulation (Ottolenghi et. al., 1986).

The liver appears to have the highest concentration of phosphorylase a activities. For example, these were 77, 78 and 85% of total phosphorylase activities in the liver of catfish, carp and goldfish respectively (Ottolenghi et. al., 1986; Picukans & Umminger, 1979; Table 3.1).

This indicates there are differences in the rate of utilization of glycogen which is dependent on several factors including, substrate availability, the amount of phosphorylase a available at the initial state and B-receptor sites on the membrane. It has been shown that in contrast to the red muscle in catfish (Ottolenghi et. al., 1986), epinephrine enhanced phosphorylase activity in flounder (Table 3.1). This is supported by the depletion of glycogen in the swimming muscles of flounder (Jorgensen & Mustafa, 1980a). The red muscle of flounder does not appear to contain glucose-6-phosphatase activity which catalyzes the hydrolysis of G-6-phosphate to glucose (Newsholme & Start, 1973). Consequently, all glycogen mobilized in flounder is metabolized within the tissue. The aerobic potential is 3 to 4 times that of the white muscle (See Chapter 2, Table 2, and also Johnston & Moon,1981). In the fast fibres, the effect of epinephrine was an increase of phosphorylase a from 64 to 87% (Table 3.1).

Morata et al., (1982a) found in liver slices of rainbow trout, incubated in the presence of epinephrine, an increase in glucose release. These authors presumed that this effect was related to activation of gluconeogenesis, because they found no corresponding decrease in glycogen content, and because, in the presence of an inhibitor of gluconeogenesis, they observed a

reduction of liberated glucose.

On the other hand, it has been reported that epinephrine *in vitro* stimulates liver glycogen phosphorylase both in killifish, (*Fundulus heteroclitus*), (Umminger et. al., 1975) and in bullhead, (*Ictalurus nebulosus*), (Umminger and Benziger, 1975).

The role of glycogen stores during hypoxia is uncertain. While decline in liver glycogen content during hypoxia have been measured, in various species, it is not a universal response. Johnston (1975) did not observe a decline in carp liver glycogen content during hypoxia. Similarly, Demael-Suard et al. (1974) reported that tench liver glycogen was stable for 3 h following the first hour of hypoxia. However, in flounder, Jorgensen and Mustafa (1980a, b) reported depletion of glycogen in the liver and skeletal muscles.

Interestingly, there are discrepancies in glycogen metabolism in some fish species regarding phosphorylase activity. Studies with fish have indicated there is no correlation beteen glycogen breakdown and phosphorylase activity. Janssens (1964, 1965) found no changes in phosphorylase activity with hepatic glycogenolysis in aestivating lungfish. Stimpson (1965) reported that hepatic glycogenolysis in starved goldfish was not accompanied by an associated increase in phosphorylase activity. Killifish, (*Fundulus heteroclitus*) (Umminger et al., 1975) as well as goldfish (Rush & Umminger, 1978) subjected to handling stress showed hepatic glycogenolysis with no changes in the specific activity of hepatic glycogen phosphorylase. These studies indicate that regulation of glycogen metabolism may involve enzymes other than glycogen phosphorylase.

In some species of fish, phosphorylase activity is very low

or absent. For example, phosphorylase activity seems to be lacking in carp liver, Murat (1976) has detected enzymatic defect which bears a resemblance to glycogen storage disease type VI described in humans. Interestingly, gamma-amylase activity found in the hepatic homogenate of carp has been suggested as a possible alternate enzyme in regulating glycogenolysis in teleosts. This is supported by similar observation in the livers of tench (Brighenti and Callegarini, 1968) and goldfish (Murat et al., 1973, 1976). It seems likely that gamma-amylase pathway can provide a synergistic coupling to low reactive phosphorylase activity in glycogen metabolism of some fish species.

It is known that the physiological responses of target tissues to catecholamines are mediated by alpha- and/or beta-receptors (Ahlquist, 1948). While there is much evidence the alpha-receptors are associated with the glycogenolytic action of catecholamines in rat liver (Sherline et al., 1972; Exton, 1979), epinephrine appears to act predominantly via beta-receptors in adult rabbit (Rufo et al., 1981) and man (Rizza et al., 1980). The findings that either alpha- or beta-receptors mediate the regulation of catecholamine action in different animals must be related to the density of alpha- and/or beta-receptors in the tissues or organs. These receptors have been quantified only in the rat liver (Schmelck and Hanoune, 1980). This makes further study necessary.
In conclusion the results of this study suggest that epinephrine causes an increase in activity of phosphorylase which utilises glycogen as substrate, during anaerobic metabolism.

TABLE 3.1. Phosphorylase activity in the slow and fast skeletal muscles of flounder following epinephrine administration.

	µmoles wet wt.$g^{-1}min^{-1}$		Ratio	
	-ve AMP	+ve AMP	$\frac{-AMP}{+AMP}$	% increase phos a
FLOUNDER[1]				
Slow muscle/ control	4.57 ± 0.66	7.61 ± 0.1	0.59 ± 0.02	
Slow muscle/ epinephrine	17.82 ± 1.12	21.84 ± 0.52	0.86 ± 0.04	46
Fast muscle/ control	10.87 ± 0.89	17.64 ± 1.71	0.64 ± 0.04	
Fast muscle/ epinephrine	25.12 ± 0.22	29.34 ± 0.42	0.87 ± 0.3	36
CATFISH[2]				
Slow muscle/ control	5.48 ± 0.08	15.82 ± 0.7	0.34	
Slow muscle/ epinephrine	5.76 ± 0.9	15.88 ± 0.6	0.36	5
Fast muscle/ control	1.18 ± 0.22	12.36 ± 0.51	0.10	
Fast muscle/ epinephrine	1.96 ± 0.45	12.75 ± 0.48	0.15	57
Control Liver	7.03 ± 0.7	9.12 ± 0.8	0.77	
Liver/ epinephrine	10.19 ± 0.6	11.40 ± 0.7	0.89	16
CARP[3]	72.53 ± 6.3	92.69 ± 8.9	0.78	
GOLDFISH[4]				
Liver Trained	335.53 ± 37	395.57 ± 51	0.85	
Liver Untrained	406.74 ± 60.43	452.21 ± 60	0.90	

References: 1. This study

2. Ottolenghi et. al. 1986

3. Picukans & Umminger, 1979

4. Murat et.al. 1973, 1976.

FIGURE 3.1. Effect of epinephrine administration (striped bars), on phosphorylase activity in slow(R) and fast(W) skeletal muscles of flounder (Platichthys flesus). Note: Contol (clear bars), frog (F_1).

Reference 1. Postner, Stern and Krebs, 1965.

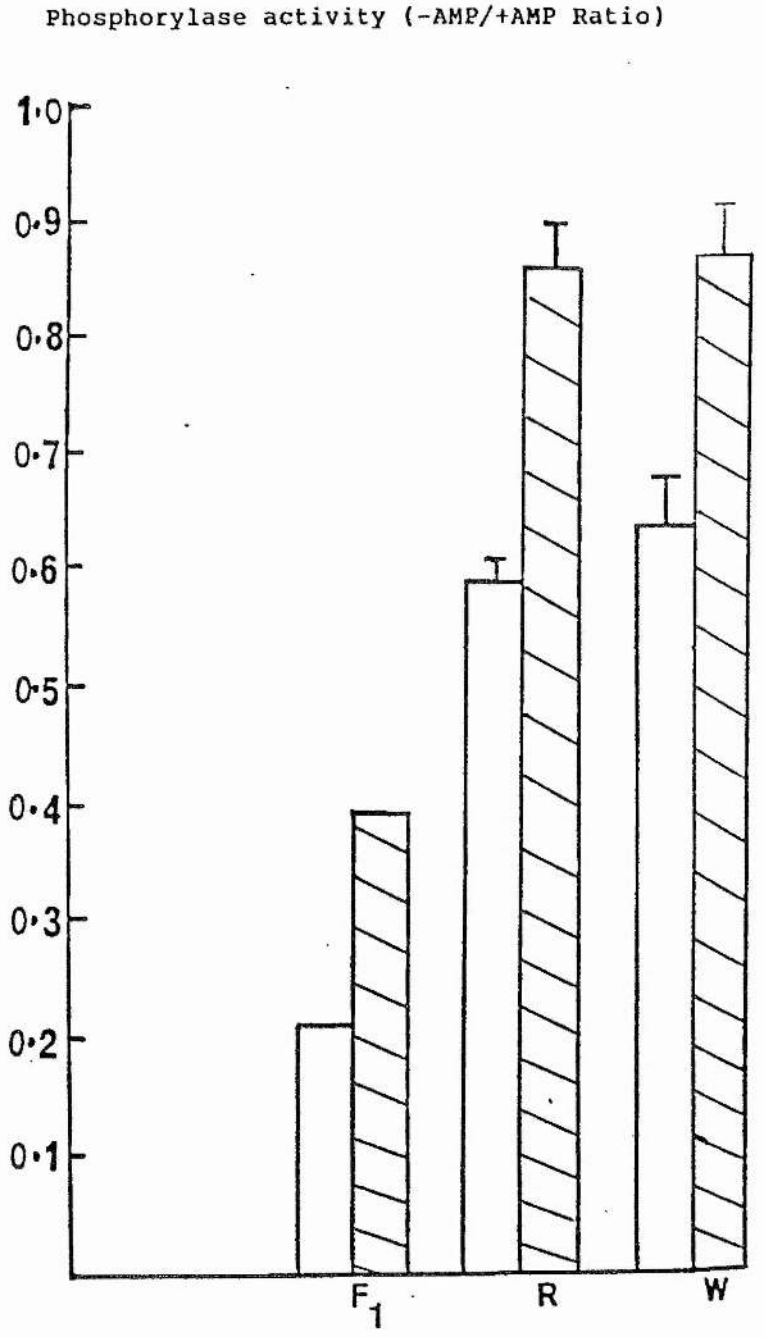

Phosphorylase activity (-AMP/+AMP Ratio)
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
F1
R
W

FIG.3.1.

CHAPTER 4.

Effects of hypoxia acclimation on anaerobic metabolism in the plaice (Pleuronectes platessa L.)

4.1 Introduction

Fish species differ considerably in their tolerance of environmental hypoxia (Davis, 1975). Some species maintain their oxygen uptake until some critical Po_2 is reached (regulators) e.g. rainbow trout (Hughes & Saunders, 1970; Hughes, 1973). In other species oxygen consumption falls in parallel with decreases in water oxygen tension (conformers) e.g. carp (Hughes, 1973; Lomholt and Johansen, 1979). These two responses are not mutually exclusive and can occur in the same species depending on the PO_2 (Lomholt and Johansen, 1979).

The reduced rate of oxygen delivery during hypoxia results in a reorganization of metabolic processes which are designed to compensate for the adverse effects. The compensatory effects are shown by either an increased rate of anaerobic ATP production (i.e, pasteur effect) and/or reduced ATP turnover (DeZwaan & Wijsman, 1976; Dunn, 1985; Hochachka and Somero, 1984; Pamatmat, 1979; Robin, 1980; Van den Thillart, 1982).Interspecific differences are also thought to be involved in the compensatory strategies among the different fish species. For example, species tolerant of anoxia/hypoxia often do not accumulate large concentrations of lactate.

Goldfish survive anoxia by metabolic rate depression coupled with the ethanol pathway metabolism of lactate (Shoubridge, 1980), resulting in reduced lactate accumulation. Species such

as tench, eel and trout lack alcohol dehydrogenase activities and are unable to utilize the ethanol pathway, resulting in their failure to survive anoxia (Johnston & Bernard, 1982; Van den Thillart, 1983; Dunn and Hochachka, 1986). There is evidence that increased utilisation of anaerobic pathways is primarily an "emergency" response to acute hypoxic stress. For example, Wood and Johansen (1973) reported a high concentration of lactate in eels following 2 days of hypoxia but not after 7 days. The life-style of plaice involves periodic hypoxia exposure. The aim of the present study was to determine the effect of acclimation to periodic hypoxia exposure on muscle metabolism.

4.2 Materials and Methods

4.2.1. Fish:

Plaice (Pleuronectes platessa L.) (n= 20), were obtained from local fishermen (Firth of Forth, Scotland). Fish, 261.2 $\pm$ 12.8g mean weight; 23.2 $\pm$ 3.5 cm standard length, were acclimated for 8 weeks to either recirculated aerated water or periodic hypoxia exposure for 5 h at 15^{o}C. They were fed daily with chopped liver and commercial fish pellets.

4.2.2 Acclimation to low oxygen tension

Fish were transferred individually or in pairs to open respirometers (22cm x 22cm x 6cm) 24h before analysis. At the start of the experiment, air and sampling outlets were sealed and the respiration of the fish was allowed to reduce the PO_2 for 2h. Nitrogen was then introduced to the respirometers for

1.0 to 2.0 minutes to reduce the PO_2 to 2.6±0.2 KPa. Following a further period of 3h hypoxia (PO_2 2.2 to 2.0 KPa), fish were allowed to recover in aerated water, and experiments were repeated after every 72 h. Other groups of fish were maintained in holding tanks of well aerated sea-water at the same temperature.

4.2.3. Metabolite determination

Fish were killed by a blow to the head and decapitated. Tissue samples of slow and fast skeletal muscles and liver were immediately dissected. Frozen samples (200-300 mg) were pulverized in mortar and pestle cooled in liquid nitrogen. The powder was extracted in 2.1 ml of 0.6 N perchloric acid for 4 minutes at 0°C. Tissue debris was immediately removed by centrifugation (5 min at 2000 g) and an aliquot of the clear supernatant, neutralized by approximately 400 ul of 2.2 M Potassium carbonate in the presence of methyl orange indicator. Samples were divided into a small number of glass vials and stored frozen (-20°C) until analysis.

Glycogen:

Ethanol (3.5 ml) was added to the neutralized supernatant and the solution re-boiled, cooled, and left overnight. The precipitate was collected and washed in 3ml absolute ethanol. Ethanol was removed by heating in a water bath and the glycogen was hydrolised to glucose by boiling for 2h in 2ml 2N H_2SO_4. The solution was neutralised using 2M K_2CO_3 and 100 ul aliquots removed for glucose determination. Glucose was assayed at 450

nm, following reaction with anthrone reagent (500 mg anthrone in 720 ml of 70% H_2SO_4(Carroll, Longley and Roe,1956).

Lactate:

Lactate was determined enzymatically from the neutralised extract based on the reduction of pyridine nucleotide at 340 nm in a medium containing (mM L^{-1}) hydrazine, 150; glycine buffer, pH 9.5,NAD^+, 2.5; lactate dehydrogenase, 0.02 (Hohorst, 1965).

Glucose:

The glucose content of the neutralised extract was determined spectrophotometrically at a wavelength of 450 nm in a buffer-enzyme medium containing 120 mM phosphate buffer pH 7.0, 40 ug peroxidase/ml; 250 ug glucose oxidase/ml to which was added 5 mg O-dianisidine hydrochloride (Bermeyer and Bernt, 1965).

4.2.4. Enzyme Assays

Lactate dehydrogenase (E.C.1.1.1.27): LDH activity has been determined by the method described in chapter 2.

Alcohol dehydrogenase (EC.1.1.1.1.): ADH was determined at 340 nm, in a medium containing (mM L^{-1}) phosphatebuffer, pH 7.0, 100; acetaldehyde, 3.6; NADH, 0.20; reduced glutathione, 1.0.

4.3. Statistical Analysis

The results obtained for fish, acclimated to either aerated water or hypoxia were compared using Student's t-test.

4.4. Results

Effects of acclimation to low oxygen tension:

Acute hypoxia exposure resulted in a decrease in the oxygen consumption of plaice (ml 0_2 $kg^{-1}h^{-1}$) from (22 to 7.4) ($P>0.001$). After 8 weeks periodic hypoxia exposure, the resting VO_2 ($ml^{-1}kg^{-1}h^{-1}$) was 20 $\pm$ 0.5 oxygen. This value was only slightly lower than that on aerated fish. Oxygen consumption of hypoxia acclimated fish following acute hypoxia was slightly higher at 10.2$\pm$ 1.5 (38%). This value was not statistically significant.

The concentrations of lactate,
glycogen and glucose in myotomal muscles and liver of plaice acclimated to either aerated water or periodic hypoxia exposure, are shown in (Table 4.1, Figs. 4.1, 4.2). The activities of lactate dehydrogenase from the corresponding tissues are shown (Fig. 4.3).
Glycogen concentrations (μmol g^{-1} glucose equivalent) for liver (213.0) and fast muscle (28.8) were similar for fish acclimated to either aerated water or to periodic hypoxia exposure (Fig. 4.1). In contrast, glycogen concentration in slow muscle increased 2-fold with hypoxia acclimation from (108.8 to 268.4)

Lactate:

Acclimation to hypoxia exposure resulted in the significant increase in lactate concentrations (μmol^{-1}) (2.5 to 4.2) fast muscle; (4.2 to 7.4) slow muscle and (1.7 to 4.1) liver.

Lactate dehydrogenase:

Acclimation to hypoxia resulted in an increase in LDH activities in all tissue examined. Hypoxia-induced increase in the activities of lactate dehydrogenase (μmol substrate utilised wet wt. $g^{-1}min^{-1}$) were (123 to 268) ($P>0.05$) for fast muscle, (114 to 209) slow muscle, and (4.6 to 9.3) ($P>0.05$) for liver (fig. 4.3). Alcohol dehydrogenase activities were not detected in the tissues of plaice.

4.5 Discussion

In response to decreasing oxygen concentrations, organisms employ metabolic rate depression, in order to balance declining ATP production with reduced utilization. In the present experiment, plaice (Pleuronectes platessa L.), could not survive anoxia. Respiration of plaice in aerated water was reduced from (ml $kg^{-1}h^{-1}$) 22.0 to 7.4 on exposure to low PO_2 of 2.0 kPa. But the resting VO_2 of hypoxia acclimated fish declined less steeply from (ml $kg^{-1}h^{-1}$) 20.5 $\pm$ 1.0 to 10.2. $\pm$ 1.5. Thus indicating a reduction in oxygen consumption with hypoxia acclimation.

The value of hypoxia acclimated fish represents 45% of that in aerated water suggesting acclimation to hypoxia exposure enhances metabolic depression. Similar results have been obtained in other teleosts. For example, the VO_2 in hypoxia acclimated flounder and tench declined to 44 and 48% respectively, compared to the values in aerated water (Kerstens, Lomholt and Johansen, 1979; Johnston & Bernard, 1982). Enhanced oxygen extraction during hypoxia acclimation may be due to increased blood oxygen-carrying capacity and/or circulatory and ventilatory responses which favour O_2 loading at the tissues.

Some species utilise the strategy of metabolic depression, coupled to enhanced lactate concentration, indicating glycolytic activation (e.g. catfish, Marvin & Heath, 1968). Tench (Tinca tinca) are also able to survive indefinitely at an O_2 tension of 1.5 KPa (Johnston & Bernard, 1982). In contrast, the African lungfish exhibits a reduced metabolic rate (Lahiri, Szidan & Fishman, 1970) with no glycolytic activation in the fast muscle

during hypoxia (Dunn, Hochachka, Davidson & Guppy, 1983). Goldfish have adopted a different or modified strategy by incorporating metabolic rate depression with ethanol pathway utilization (Chapter 5, Shoubridge and Hochachka, 1980).

The major energy source for hypoxic excursion is glycogen. In contrast to the fast muscle and liver, the glycogen content of the slow muscle increases with hypoxia acclimation. Glycogen concentration was 3.8 times that of the fast muscle (Fig. 4.1), and constitutes an adaptive strategy.

The capacity of fast muscle to maintain a high energy status during decreasing oxygen concentration is common in fish. For example, this phenomenon has been observed in lungfish, flounder, goldfish and eel (Dunn et. al., 1983; Jorgensen & Mustafa, 1980; Van Den Thillart, 1980; Van Waarde et. al., 1983).

In control fish, lactate concentration in the slow muscle, 4.2 μmol g^{-1}, was higher than that of fast muscle (Fig. 4.1), as reported for other teleosts (Johnston, 1975; Johnston & Goldspink, 1973; Wokoma & Johnston, 1981). Hypoxia-acclimated fish retained a higher concentration of glycogen and lactate accumulation in the slow muscle, relative to fish in aerated water (Fig. 4.1). The major anaerobic end product produced by plaice during hypoxia is lactate (Fig. 4.1). A similar response has been shown in flounder, eel and trout (Jorgensen & Mustafa, 1980; Van Waarde et. al., 1983; Dunn & Hochachka, 1986). Wardle (1978), suggests lactate retention in the fast muscle of plaice appears to be under the catecholaminergic control. There is evidence that lactate may be actually taken up against a concentration gradient and incorporated into glycogen by muscle cells (Batty & Wardle, 1979). These authors suggested that

removal by metabolic process *in situ* is the principal fate of endogenously produced lactate. Interestingly, the low level of gluconeogenic enzymes in the fast fibres is against such a scenario (Johnston & Moon, 1980). However, lactate could be transported to the liver and other peripheral tissues which have high lactate oxidative capabilities (Bilinski & Jonas, 1972). Isotopic studies in support of this includes the Australian eel (Phillips & Hird, 1977) the American eel (Renaud & Moon, 1980), rainbow trout (Cowey et. al. 1977 a, b; Walton and Cowey, 1979 a, b), and cod (Knox *et. al.*, 1980), which indicate that lactate and certain amino acid precursors can be converted to glucose in fish liver. This is in agreement with the present experiment (Fig. 4.1)

The hypoxia-induced increase in LDH activities (Fig. 4.3), provides a continuous supply of reduced NAD^+ to match the increased flux of lactate during hypoxia. Similarly, glycolytic enzymes, PFK, PK and LDH activities were significantly higher in slow muscle and liver of tench acclimated to hypoxia (Johnston & Bernard, 1982). The results suggest an increased dependence on anaerobic glycolysis for ATP production with hypoxia acclimation. The resulting increase in acid end-product, lactate may disturb redox balance, decouple metabolic and membrane functions (Hochachka, 1980) and contribute to the failure of plaice to survive anoxia. This is supported by the absence of alcohol dehydrogenase activity in the skeletal muscles of plaice. In contrast, prolonged anoxia survival in goldfish is maintained by a decline on anaerobic glycolysis, coupled with ethanol pathway utilisation during anoxia (Chapter 5).

The liver is the major reservoir for glycogen (Fig. 1). Metabolism is reorganised in a tissue-specific manner during hypoxia. As in mammalian liver, plaice liver glycogen, supplying blood glucose, becomes the primary fuel reserve supporting metabolism. This is indicated by 4-fold increase ($P>0.001$) in glucose concentration. There is evidence, this is matched by high hexokinase activities (Moon & Johnston, 1980), which allow the liver to compete for glucose and maintain its role as a 'glucostat' supplying various peripheral tissues.

The response of liver phosphorylase to hypoxia seems to be variable. Metabolites measurements from livers of lungfish (Dunn et.al., 1983) and flounder (Jorgensen & Mustafa, 1980) indicate a decline in liver glycogen content, carp show no change (Johnston, 1975 a); while in tench liver, glycogen content declines for a short duration (Demael-Suard et.al., 1974). In conclusion, in comparison to salmonids the active metabolic rates and critical swimming speeds of flatfish are low. Thus the total amount of oxygen available to the flatfish is relatively small anaerobic glycolysis is necessary to provide ATP during hypoxia.

Acclimation condition	Tissue	Goldfish[1]	Carp[2]	Plaice[3]
Control (Aerated)	Fast muscle	0.58	3.12	2.5
	Slow muscle	0.84	3.07	4.2
	Liver	0.88	0.99	1.70
Hypoxia	Fast muscle	3.74	12.54	4.2
	Slow muscle	5.92	11.89	7.4
	Liver	6.19	5.54	4.1

TABLE 4.1

Lactate concentrations in fish species acclimated to either aerated (control) water or hypoxia exposure ($\mu mol\ g^{-1}$)

References: 1. Shoubridge, 1980

2. Johnston & Bernard 1983

3. This paper.

FIGURE 4.1

The effect of acute hypoxia exposure on the concentrations of glycogen (a, b) and lactate (c, d) in the fast muscle ( ), slow muscle ( ) and liver ( ) of plaice (Pleuronectes platessa L.) acclimated to either aereted water (a,c), or periodic hypoxia exposure (b,d).

Note: (F, S and L) represent fast muscle, slow muscle and liver; and (C) controls.

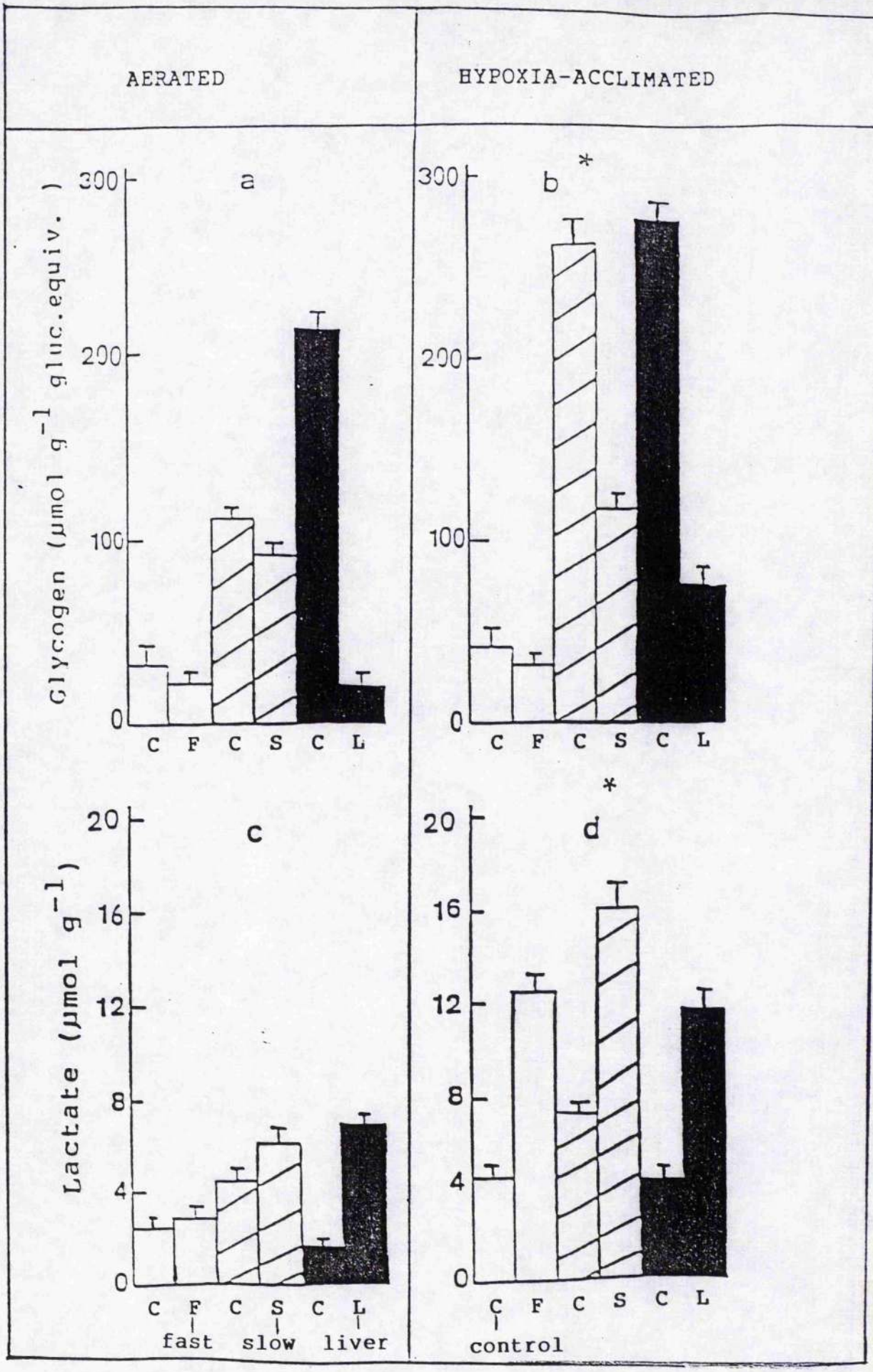

AERATED
HYPOXIA-ACCLIMATED
a
b
c
d
*
*
Glycogen (µmol g⁻¹ gluc.equiv.)
Lactate (µmol g⁻¹)
300
200
100
0
20
16
12
8
4
C F C S C L
fast
slow
liver
control

FIG.4.1.

FIGURE 4.2.

The effect of acute hypoxia exposure on the concentrations of glucose in fast muscle (□), slow muscle (▨) and liver (■)of plaice (Pleuronectes platessa L.) following acclimation to either aerated water (a), or periodic hypoxia exposure (b) .

Note: (F, S and L) represent fast muscle, slow muscle and liver; and (C) controls.

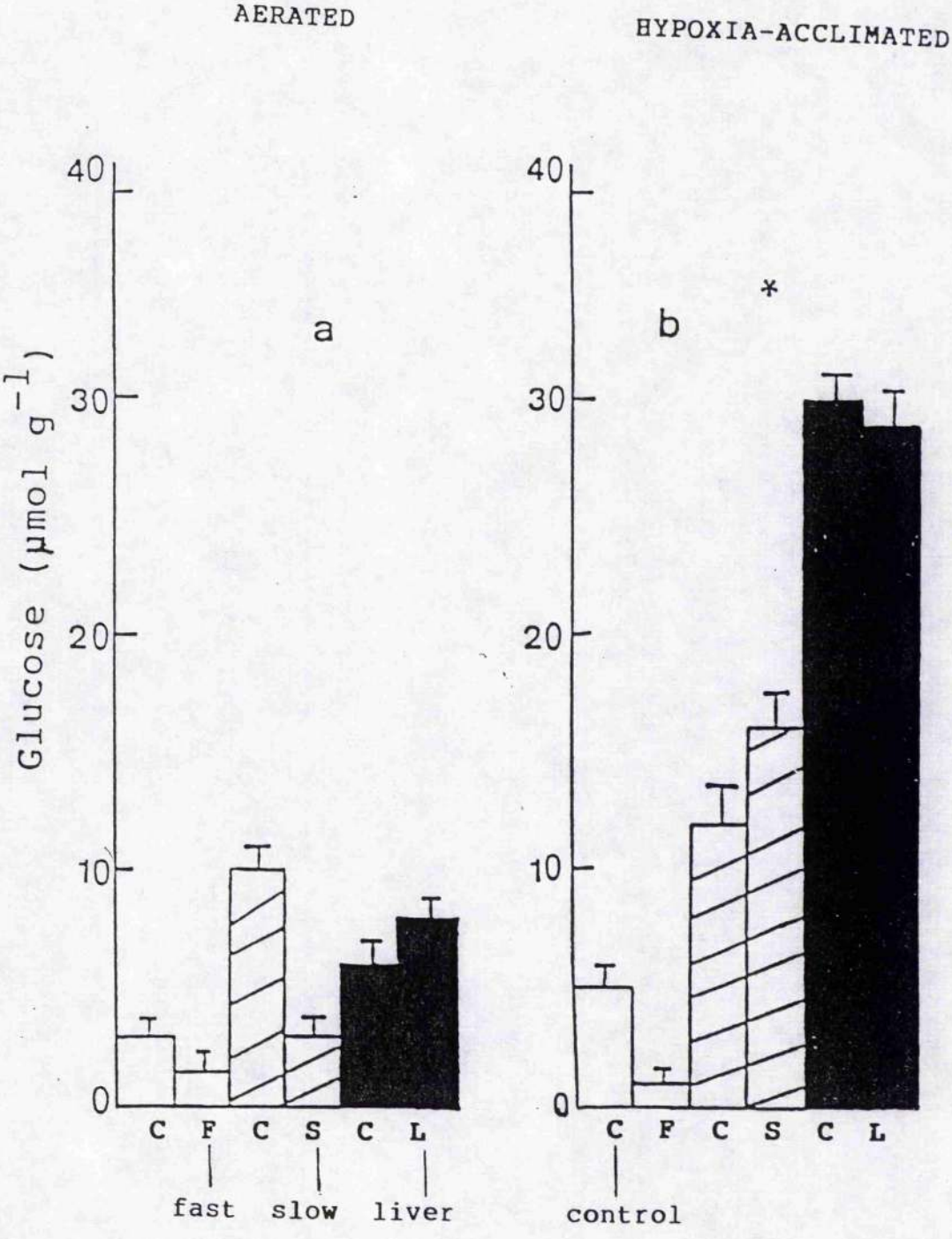

AERATED
HYPOXIA-ACCLIMATED
Glucose (µmol g⁻¹)
40
30
20
10
0
a
b
*
C F C S C L
fast slow liver
control

FIG.4.2.

FIGURE 4.3. Changes in maximal enzyme activities of lactate dehydrogenase in fast muscle ( ), slow muscle ( ) and liver ( ) of plaice (Pleuronectes platessa L.) following acclimation to either aerated water (a), or hypoxia (b).

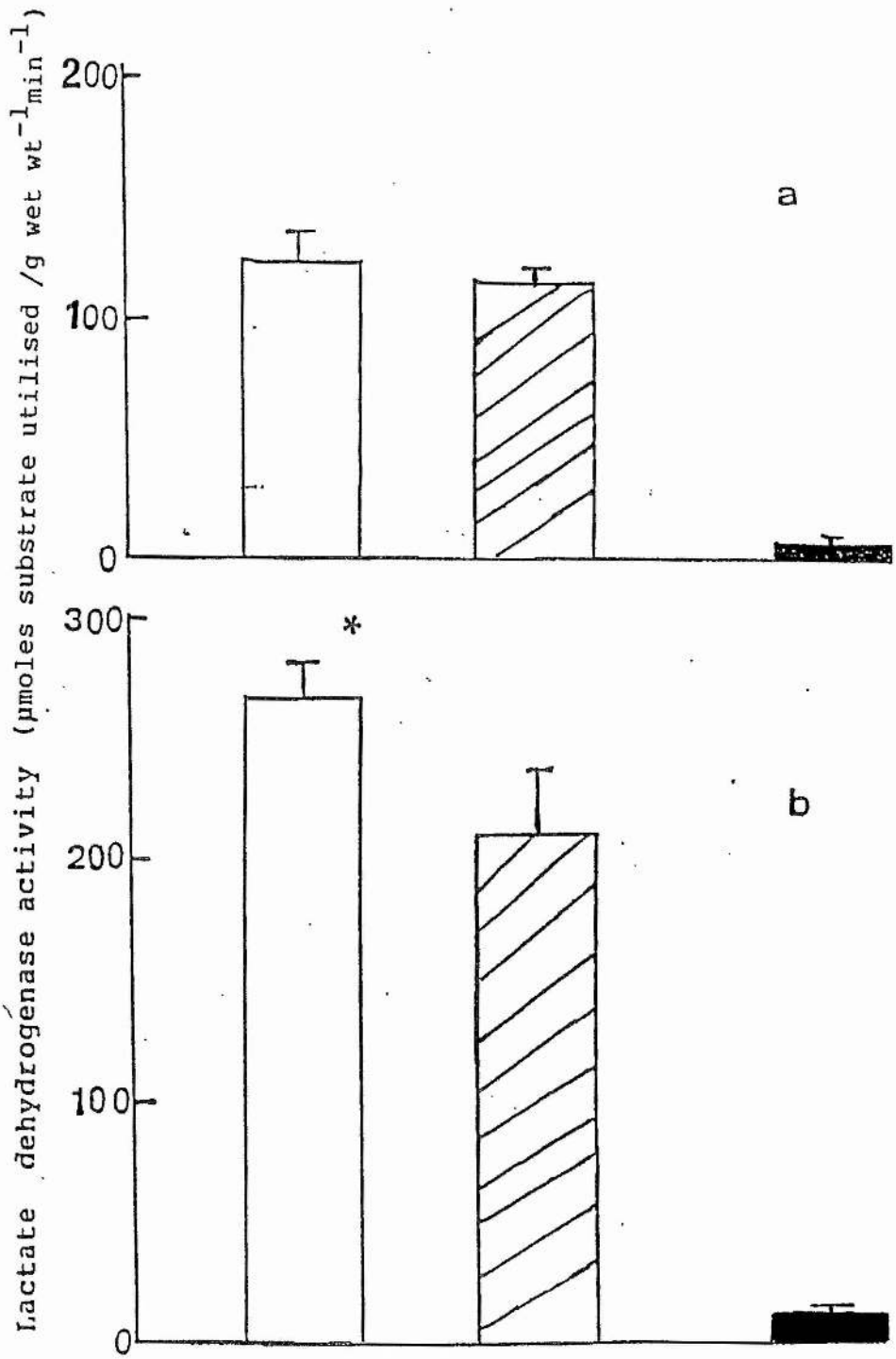

Lactate dehydrogenase activity (µmoles substrate utilised /g wet wt^{-1}min^{-1})
200
100
0
a
300
200
100
0
*
b

FIG.4.3.

CHAPTER 5.

Effects of acclimation to periodic anoxic exposure on the utilisation of the ethanol pathway in goldfish (Carassius auratus L.)

5.1 Introduction

Three important observations have provided the impetus for the study of the metabolism of goldfish during anoxia :(1) lactate accumulation was less than expected, (2) true metabolic CO_2 was produced during anoxia and; (3) the extraordinary ability of goldfish to withstand prolonged anoxia. The production of lactate from glycogen is indicative of the activation of glycolytic pathway. Species such as rainbow trout and plaice are dependent only on anaerobic glycogenolysis for ATP production during hypoxia, resulting in a large lactate load (Dunn & Hochachka, 1986., Chapter 4). In contrast, carp and goldfish are shown to be highly tolerant of anoxia without producing high lactate concentrations (Johnston & Bernard, 1983; Van Den Thillart, 1977). Shoubridge et. al., (1980), discovered that the decreased lactate production from glycogen depletion, observed in anoxic goldfish was due to further metabolism of pyruvate to ethanol. Unlike anaerobic glycolysis, the ethanol pathway does not result in accumulation of an acid end product but instead, neutral ethanol is excreted to the surrounding water without disturbing redox balance (Shoubridge, 1980; Mourik et.al.,1982; Johnston & Bernard, 1983).The ethanol pathway requires the co-operation of mitochondial and cytosolic enzymes involving the pyruvate dehydrogenase reaction complex (Mourik et.al.,1982).

The mechanism is based on the presence of alcohol dehydrogenase activity in the red muscle which catalyses the reduction of acetaldehyde to ethanol (Mourik et. al., 1982). The aim of the present study was to determine whether periodic exposure to anoxia can induce ADH and alter the balance between the proportions of energy needs met by the lactate and ethanol pathways.

5.2. Materials and Methods

Fish:

Goldfish (Carassius auratus L.), body weight 7.2 ± 0.4 g, and standard length 5.8 ± 0.2 cm (Mean ± S.E., n = 47) were obtained from commercial supplies. One group was maintained in aerated water at 15°C and the other, acclimated to periodic anoxia exposure.

Holding Tank:

The holding tank for fish consisted of a 10 litre tank of recirculated aerated water at 15°C. Waste products were removed by circulating the water through a charcoal external filter containing layers of coarse gravel, glass wool and activated charcoal. Temperature was maintained by pumping antifreeze glycol through a series of glass coils.

Respirometer:

Opaque, open respirometer boxes (6 x 7 x 10 cm) were used for the experiment. Each box had a perspex lid with two small holes,

(one for delivery of nitrogen and the other for sampling the water). Respirometer boxes were immersed in the holding tank to maintain a temperature of 15 ^{o}C.

5.2.1. Acclimation to anoxic conditions

Fish were transferred to the respirometer boxes in groups of 4, 24 hours before the measurement of oxygen consumption in order to reduce stress. Subsequently, the respirometers were sealed, and the respiration of the fish was used to reduce the oxygen tension (2 to 3h). Serial water samples were collected for measurement of PWO_2 with Rank Brothers Oxygen Electrode (Bottisham, Cambridge, England). Nitrogen was then bubbled through (10 to 15 minutes) to further reduce the PWO_2 to zero. One group of fish were held under anoxic condition for a further period of 6 h and later returned to aerated water for recovery. The experiment was repeated every 48h.

5.2.2. Dissection of muscles:

Fish were stunned by a blow to the head, followed by spinal transection and decapitation. In a separate series of experiments samples of superficial red and deep white myotomal muscles were rapidly excised from anaesthesized goldfish (6 fish, mean body wt. 7.0 g) and freeze-clamped in brass tongs pre-cooled in liquid nitrogen (-196^{o}C)

5.2.3. Metabolite determination

The methods used for determining glycogen, lactate and glucose are described in chapter 4.

Ethanol:

Ethanol was measured from the perchloric acid neutralized tissue extract or water sample. The method is based on the reduction of pyridine nucleotide in a medium containing (mmol L^{-1}), nucleotide adenine dinucleotide, (NAD^{+}), 1.8; Alcohol dehydrogenase, 150 units in glycine buffer pH 9.0 at a wavelength of 340 nm using a double beam spectrophotometer.

5.2.4. Enzyme Assays

The method for the measurement of lactate dehydrogenase activity has been described in chapter 2.

Alcohol dehydrogenase (EC.1.1.1.1.)
ADH activity was measured by the method described in chapter 4.

5.3. Statistical Analysis

Data from fish acclimated to aerated and hypoxic water were compared using a one way analysis of variance for unequal numbers.

5.4. Results

Fish remained quiescent during acclimation to periodic exposure to hypoxia and anoxia. They appeared to be disinterested but ate avidly on return to aerated water. Oxygen consumption of the goldfish (7g wt.) acclimated to aerated water at 15°C (ml $kg^{-1}h^{-1}$) was (26.8) (Fig.5.1, Table 5.2). This is equivalent to an ATP production from aerobic metabolism of 7.14 mmol ATP Kg^{-1} h^{-1}, assuming a P/O ratio of 3.

The effects of 6 h periodic anoxia exposure and subsequent recovery on the concentrations of glucose lactate and ethanol in whole fish were determined (Fig 5.1, Table 5.1). In fish acclimated to aerated water, the concentrations of glucose and lactate were (µmol g^{-1}, Mean $\pm$ S.E) 0.58 $\pm$0.1 and 0.81 $\pm$ 0.11 respectively. Ethanol was not detected in aerated water. Acclimation to anoxia exposure resulted in the increase in concentrations in the carcasses (µmol g^{-1}wet wt.) glucose, (2.49); lactate, (2.87) respectively. Concurrently, production of ethanol (µmol g^{-1} h^{-1}) was 0.38 during 6 h periodic anoxia exposure. (Table 5.1, Fig. 5.1). The net ethanol excretion after 40 days acclimation to periodic anoxia exposure was 1.10 µmol g^{-1} h^{-1}(Fig 5.2.). This indicates 74% of the total ethanol was excreted to the surrounding water (Fig. 5.3). The ATP yield from lactate and total ethanol (tissue and excreted) indicated the energy requirements for anoxia acclimation. The ATP turnover from the lactate and ethanol pathways represents 27% of that from aerobic pathways under normoxic condition.

At the termination of anoxia exposure, the rate of

recovery was faster for ethanol, followed by glucose and finally lactate (Fig. 5.1).

The alcohol dehydrogenase activity in slow muscle (μmoles substrate utilised g wet wt.$^{-1}$min^{-1}) was 31.7 ± 2.75. This was 3.5 times that of the fast muscle (Table 5.3). Acclimation to periodic anoxia exposure resulted in a 2-fold increase in alcohol dehydrogenase activity in the fast fibres. This was accompanied by a reduction of maximal enzyme activities of lactate dehydrogenase (Table 5.2). The results suggest anoxia enhances metabolic depression and ethanol pathway utilisation.

5.5 Discussion

Anoxia tolerance in the goldfish involves a tissue specific re-organization of metabolism and metabolic rate depression (Shoubridge and Hochachka, 1983). The primary adaptive strategy supporting facultative anaerobiosis is a profound depression of metabolic rate, lowering ATP requirements to a level that can be supported over an extended period by less efficient fermentative pathways of catabolism (Storey, 1985; 1987). As a result of these modified pathways, the major anaerobic end-products produced by goldfish during periodic anoxia exposure are lactate and ethanol (Table 5.1, Fig.5.1). This confirms the results of recent studies on goldfish (Shoubridge and Hochachka, 1980; Van den Thillart, 1981), and crucian carp, (Johnston & Bernard, 1982).

Following 6 h periodic anoxia exposure tissue ethanolaccumulation (μmol gm^{-1}wet wt. fish h^{-1}) was 0.38. This is equivalent to the utilization of 0.72 umoles glucose equivalents g^{-1}wet wt. fish h^{-1}(Fig. 5.1, Table 5.1). After 6 weeks periodic anoxia exposure the net excretion rate for ethanol (μmol g^{-1} h^{-1}) was 1.10. This indicates 74% of the total ethanol produced during this time appeared in the surrounding water (Figs.5.2; 5.3; Table 5.1). However, on return to aerated water ethanol maintained an early recovery, whereas lactate recovery was slow (Fig. 5.1) as was also reported by Shoubridge & Hochachka, (1980). The net lactate accumulation after 6 h anoxia was 2.87 μmol gm^{-1}wet wt. and accounts for 60% of the glycogen breakdown (Table 5.1). Shoubridge and Hochachka, (1980) utilized carbon monoxide poisoned goldfish to demonstrate that a proportion of ^{14}C-lactate injected into anoxic goldfish could be recovered in the ethanol fraction. This provided the evidence for the conversion of lactate to ethanol. Previous investigators have shown that the primary energy source during anoxic excursion is glycogen stored in the slow muscles and liver (Van den Thillart et al., 1976, 1980; Jorgensen & Mustapha, 1980; See also chapter 4).

The mechanism of operation of the ethanol pathway is based on pyruvate dehydrogenase complex of goldfish mitochondria which is able to decarboxylate pyruvate to acetaldehyde under anaerobic conditions. The acetaldehyde produced is subsequently reduced to the non-toxic end-product, ethanol in the cytoplasm by alcohol dehydrogenase particularly in the red muscle (Mourik et al. 1982) (Fig. 5.3). This is consistent with the present findings. Alcohol dehydrogenase activity in the present

experiment is 3.5 times greater in the red than white muscle (Table 5.3; Fig. 5.2). However, following anoxia acclimation, ADH activity in the fast muscle increased significantly ($P>0.05$).

It would appear that the enhancement of the ethanol pathway is accompanied by metabolic rate depression. For example, the energy expenditure from lactate and ethanol concentrations during 6h anoxia, represents 27% of that of the routine activity in aerated water at 15^{o}C (Table 5.3). The result is consistent with decreased energy requirements or decreased metabolic rate during anoxia. Previous studies have shown goldfish respond to anoxia with a drop in metabolic rate to a level of 20 to 33% that of the normoxic basal rate (Anderson,1975; Van den Thillart et. al., 1976, 1982). Other cyprinid species have a significant anaerobic potential which increase their survival chances in environments with poor oxygen content. For example, Rasbora daniconius, a cyprinid fish found in small ponds, ditches and streams of India is known to survive in a sealed jar for more than 100 days (Mather,1967). Crucian carp has similar capacity to withstand anoxia as goldfish (Johnston & Bernard,1982).

Hypoxia induced an increase in the LDH activities in the skeletal muscles in plaice (Chapter 4). Recently, anoxia - induced changes in the kinetic properties of PFK and PK was investigated in goldfish (Rahman and Storey,1988). Treatment of the aerobic forms of both glycolytic enzymes with cAMP dependent protein kinase altered the the enzyme kinetic properties to those typical of the anoxic enzymes, which show less active

enzyme form. These results support a reduction of activity of LDH in anoxic goldfish (Table 5.2).

It has been shown that functional coupling exists between the $CO_2$and ethanol production (See Fig.5.3). For example , following injection of anoxic goldfish with an inhibitor of ADH activity such as carbon monoxide, CO_2 excretion is depressed in direct proportion to the reduction in ethanol excretion. The physiological importance of this is that it circumvents the problem of metabolic acidosis by generating ethanol (Table.5.2.,Fig 5.2.). Minimizing the accumulation of acidic end - products is crucial to prolonged survival in anoxic goldfish , since it has a poor bicarbonate buffering capacity (Hochachka, 1980).

Rates of ammonia excretion were found to be relatively independent of water PO_2 (Johnston & Bernard, 1982). The origin of the anaerobic ammonia production is obscure, but may be derived from deamination of the adenylate pool and from certain free amino acids (Van den Thillart & Kesbeke, 1978; Van den Thillart, 1982; See also Fig. 5.3.). Ammonia is available for acid-base regulation.

Anoxia tolerance is highly temperature dependent, ranging from 16 h at 20^{o}C to several weeks at 0^{o}C (Van den Thillart, 1977; Walker & Johansen, 1977). The survival of goldfish under this condition is related to the frequency of switching (on/ off), of the ethanol pathway. At higher temperature there would be a faster rate of delivery of lactate to the pyruvate dehydrogenase reaction and result in an early switching of the anaerobic ethanol pathway. At a lower temperature, in contrast, there

would be a reduction in metabolic rate, which in turn lower the rate of catabolism of lactate. This delay in the utilization of the ethanol pathway as a result of low temperature and the resultant depressed metabolic rate is an important factor in extending the anoxic tolerance of goldfish and carp.

In conclusion this is the first experimental evidence that periodic anoxia exposure resulted in the induction of alcohol dehydrogenase activity which catalyses the ethanol pathway. The result is the excretion of 74% of the ethanol produced to the surrouding water (Table 5.1, Fig.5.2). This prolongs the ability of goldfish to survive extended periods of anoxia, in contrast to species lacking the genes for the expression of alcohol dehydrogenase activity.

Finally, there is only one other report in the the literature other than fish and yeast of the accumulation of ethanol as a major anaerobic end-product in a free living organism, the midge larva, chironomus (Thummi thummi), produces small quantities of alanine, succinate and lactate coupled with large quantities of ethanol and acetate (Wilps & Zebe, 1976; Wilps & Schottler, 1980).

	Tissue lactate	Tissue ethanol	ethanol excreted
Control	0.81	0	0
6h anoxia	2.87	2.26	6.57

TABLE 5.1

Metabolite changes in whole goldfish following acclimation to either aerated or periodic hypoxia/anoxia exposure.
Values represent (μmol g^{-1}, means $\pm$ S.E. n = 6)

Whole goldfish	Aerated Water	Periodic Anoxia Exposure
$\dot{V}O_2$ (ml kg^{-1} h^{-1})	26.75 ± 2.35	34.8 ± 2.20
Lactate dehydrogenase	61.12 ± 2.15	37.34 ± 3.47 $P < 0.05$
Alcohol dehydrogenase	2.0 ± 1.40	3.40 ± 0.15 N.S.

TABLE 5.2

Oxygen consumption (under normoxic / or anoxia) and changes in maximal enzyme activities of lactate dehydrogenase and alcohol dehydrogenase in goldfish following acclimation to either aerated water or progressive anoxia exposure.
Values represent (μmol substrate utilised g^{-1} min^{-1})

	Aerated water	Periodic anoxia exposure
Slow muscle:		
Alcohol dehydrogenase	31.66 ± 2.75	37.70 ± 0.76 N.S.
Fast muscle:		
Alcohol dehydrogenase	8.59 ± 1.52	18.37 ± 2.69 P > 0.05

TABLE 5.3

Changes in maximal enzyme activities of alcohol dehydrogenase in slow and fast myotomal muscles of goldfish. Values represent (μmoles substrate utilised g^{-1} min^{-1}, means ± S.E.)

FIGURE 5.1. Metabolite changes in whole goldfish, lactate (○), glucose (□) and ethanol (●), during periodic anoxia and subsequent recovery periods of 5, 24, 48 and 72 h. Values represent Mean ± S.E. n=6.

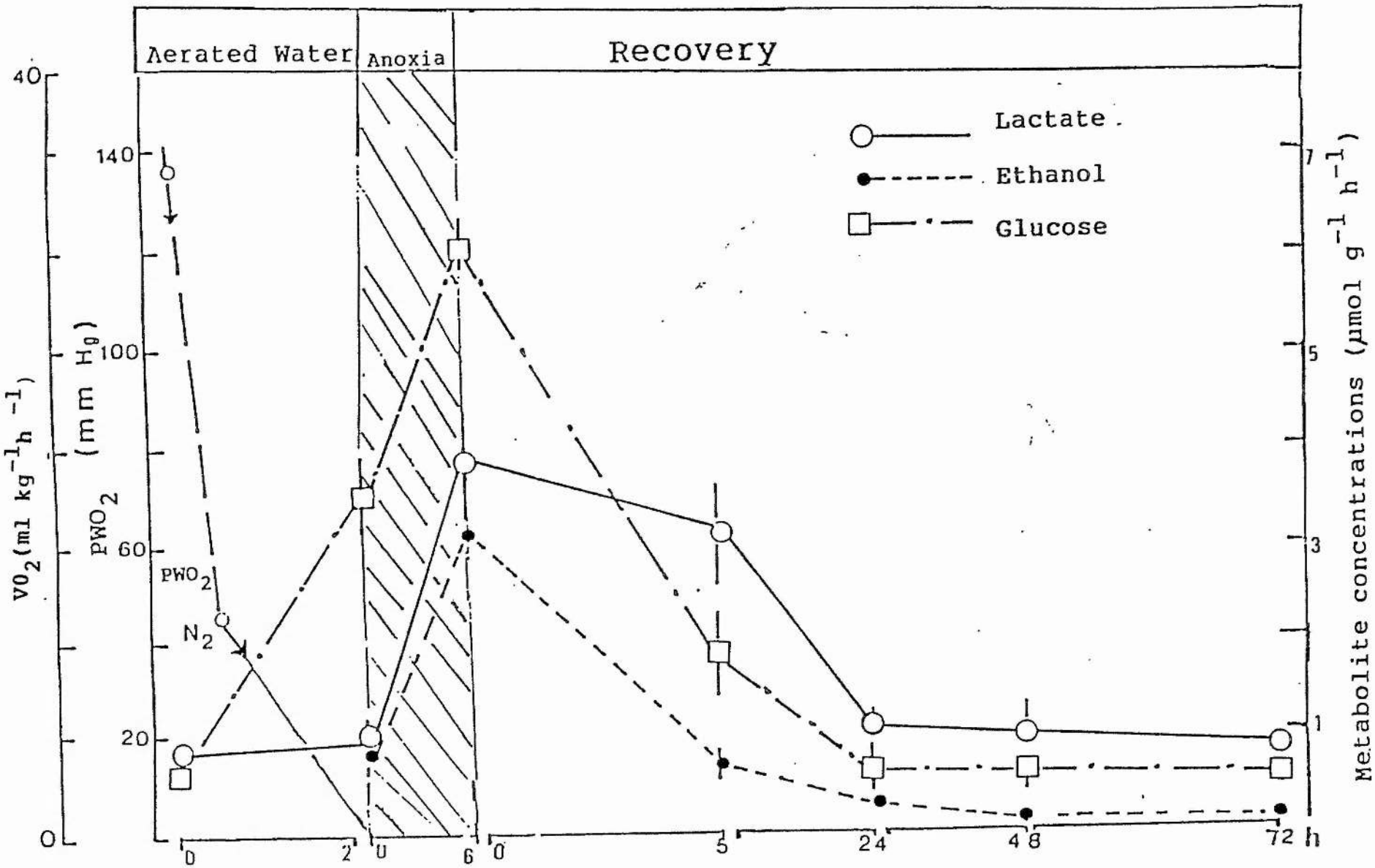

Aerated Water
Anoxia
Recovery
Lactate
Ethanol
Glucose
VO_2(ml $kg^{-1}h^{-1}$)
PWO_2 (mm Hg)
Metabolite concentrations (µmol g^{-1} h^{-1})
PWO_2
N_2
40
140
100
60
20
0
7
5
3
1
0
2
0
6
0
5
24
48
72 h

FIG.5.1.

Figure 5.2

Effect of acclimation of goldfish to periodic anoxia exposure on ethanol excretion during 6h anoxia exposure.

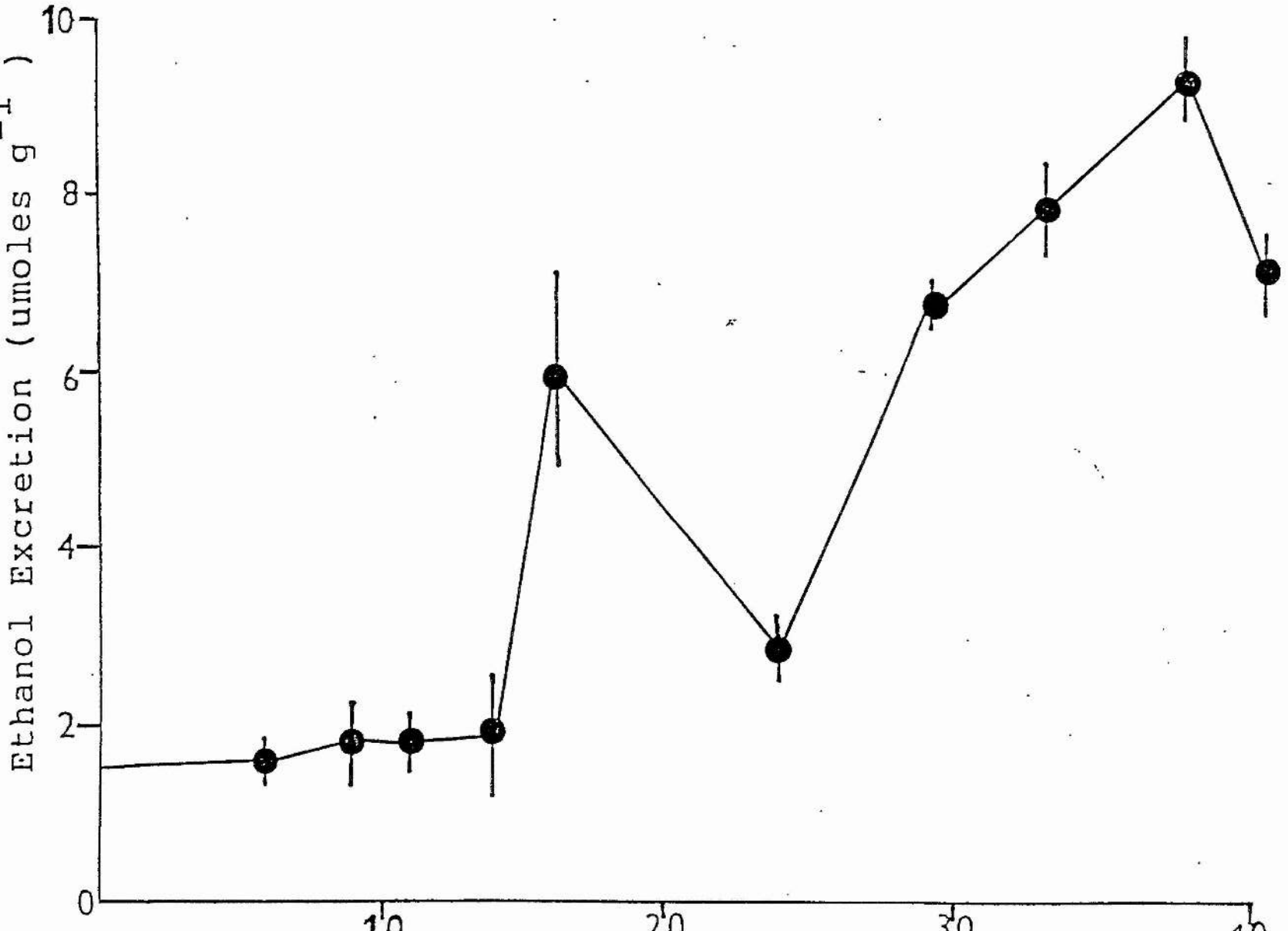

Ethanol Excretion (umoles g^{-1})
10
8
6
4
2
0
10
20
30
40
Acclimation Period (days)

FIG.5.2.

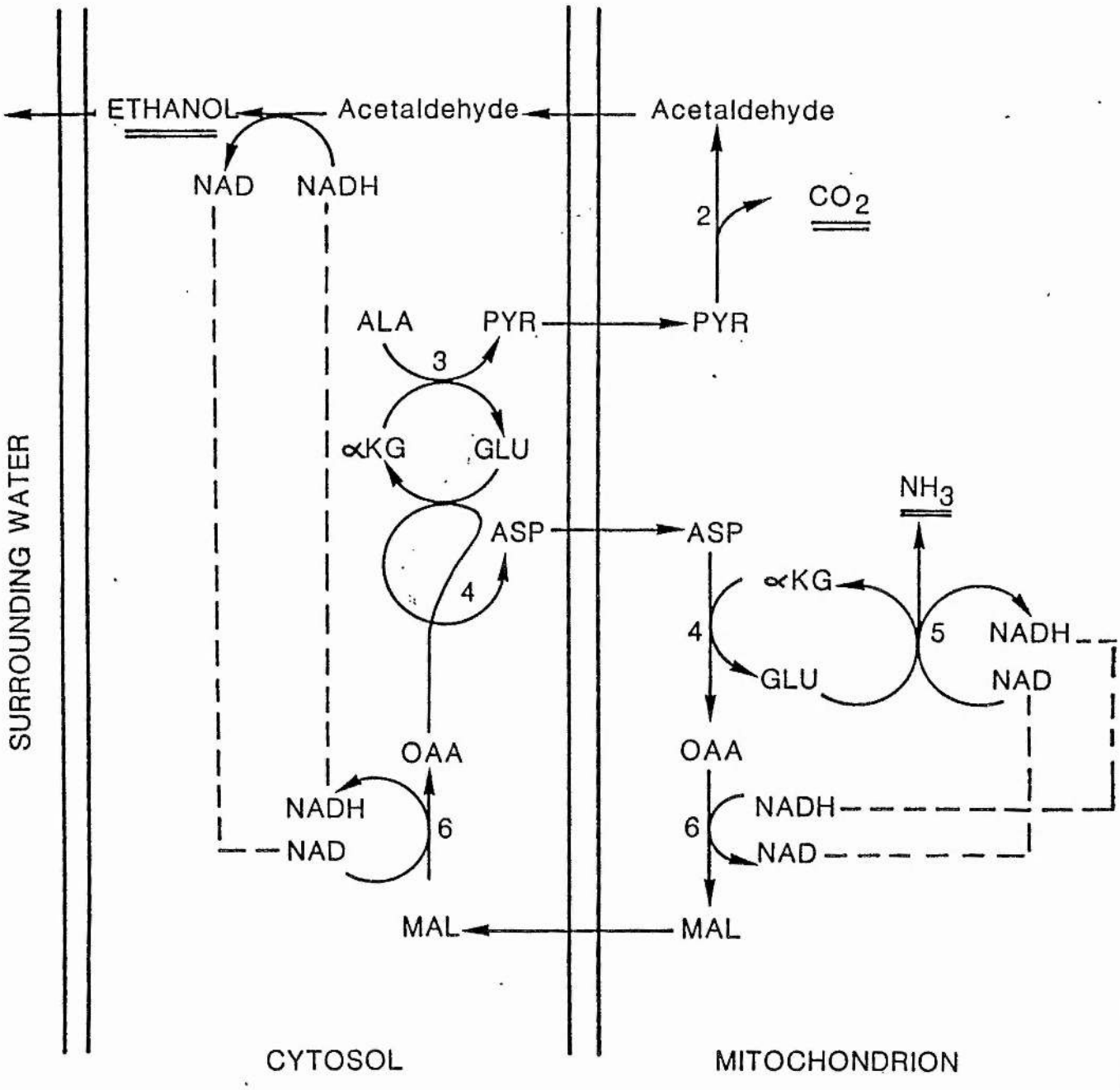


FIG.5.3. Coupling of mitochondrial and cytosolic mechanisms in goldfish showing the evolution of ammonia, carbon dioxide and ethanol.

CHAPTER 6

Effects of acclimation to periodic anoxic exposure on the ultrastructure of goldfish (Carassius auratus L.) muscles

6.1. Introduction

Factors which affect oxygen delivery to muscle fibres under different physiological states, include myoglobin concentrations, blood flow, perfusion distribution, variables affecting the oxygen-haemoglobin equilibrium and the rate of mitochondrial respiration. Information on the structural limits for gas and/or metabolite exchange can be obtained by measurements of the density and surface area of skeletal muscle capillaries.

A variety of environmental factors are been known to produce modifications in mitochondrial volume density and capillary supply to skeletal muscle fibres. For example, the capillary supply to mammalian muscles has been correlated with metabolic fibre type (Romanul, 1965), mitochondrial density (Hoppeler et al., 1981), and body size (Schmidt-Neilsen & Pennycuik, 1961; Pietschmann et al., 1982). In fish, increase in capillary supply (Johnston, 1982b), or mitochondrial density (Crucian carp, Johnston & Maitland, 1980; goldfish, Tyler & Sidell,1984; striped bass, Egginton & Sidell, 1986), has been associated with acclimation to lower temperatures (Johnston, 1982b) or hypoxia (Johnston & Bernard, 1983). For example, in tench (Tinca tinca), subjected to hypoxic acclimation,

slow muscle V_v(mit,f), decreased by 7.9% compared to normoxic value (Johnston & Bernard, 1982a). Similarly, acclimation of crucian carp from winter to summer temperatures results in a decrease in V_v(mit,f) in slow fibres from 31.4% to 14.7% (Johnston, 1982b). The reduction in mitochondrial fractional volume may either suggest an enhanced capacity of mitochondrial respiration or a depression in metabolic rate.

In mammals, capillary supply to the tissues is complicated by the fact that one capillary may supply three or more, metabolically distinct fibre types. In fish the fibre types are separated into anatomically discrete layers, and presents a simplified model to determine the relationship between capillary and fibre types. By means of stereological approach, 3-dimensional measurements could be obtained from fibre sections (Weibel, 1979; Mathieu, Cruz-Orive, Hoppeler & Weibel, 1983). This has simplified the study of the relation between the surface and volume, the size of the mitochondrial compartment and capillary supply. The aim of the present study was to investigate the effect of periodic hypoxia acclimation on mitochondrial content, capillary density and oxygen diffusion distances.

6.2. Materials and Methods

Fish :

Goldfish (Carassius auratus L.), 7.0 ±0.4 g, and standard length 5.8 ± 0.2 cm (Mean ± S.E., n = 47) were obtained from commercial suppliers. One group was maintained in circulated

aerated water at 15°C and the other, acclimated to periodic anoxic exposure. The details of the holding tank, respirometer and acclimation to anoxic conditions have been described in Chapter 5.

6.2.1. Preparation of samples

Small bundles of superficial slow fibres were dissected from an area adjacent to the dorsal fin. Fast fibres were dissected from deeper part of the muscle in the region just below the dorsal fin (Fig 6.1A). Fibre bundles were pinned to cork strips via their myoseptal insertions, and immersion-fixed over-night at their resting length in situ with 2-3 changes in 3% glutaraldehyde, 0.15 M phosphate buffer pH 7.2 at 20°C. Samples were subsequently washed in phosphate buffer, postfixed for 1 h in 1% osmium tetroxide, 0.15 M phosphate buffer pH 7.2, washed in distilled water, dehydrated in a series of alcohols up to 100%, cleared in 1,2 epoxy propane prior to embedding in Aradite Resin CY212 (EM-Scope, Trent, England).

6.2.2. Semi-thin Sections

Semi thin (0.5 -to 1.0 um) sections were cut using a Reichert OM U2 Ultra mitocrotome and stained with either 1.5% p-phenylene diamine in 1:1 isopropanol:methanol (Hollander and Vaaland, 1968)(PPDA) or 1% toluidine blue. Ultrathin (60nm) sections were mounted on 150-mesh proxyline coated copper grids, double stained with a uranyl acetate and Reynolds lead citrate, and viewed with a Phillips 301 transmission electron microscope at 60 Kv.

6.2.3. Blocks - orientation of fibres

Blocks were prepared of slow and fast fibres from 6 goldfish acclimated to aerated water and 6 goldfish acclimated to periodic hypoxia. For each fibre type 6 transversely orientated blocks were prepared from each fish. Sections were cut from 3 to 4 blocks per fish, each containing 50 to 80 slow or 15 to 20 fast fibres.

6.2.4 Analysis by light and electron microscopy

Low power electron micrographs (magnification x 900) were projected (x 2.5) using a photographic enlarger and the outlines of fibres and capillaries traced onto cartridge paper. Fibre cross - sectional areas, perimeters, numbers of capillaries and fibres, and capillary contact length were determined directly from p-phenylene diamine or toluidine blue stained semithin sections (magnification x 500 to 1200) using a microscope drawing-arm, or electron micrograpghs and digital planimeter interfaced to a Hewlett packard 86 B micro-computer. Measurements were made from areas chosen at random which contained (50 to 200) fibres.

6.2.5. Determination of capillary density

Capillary density was determined as the number of capillaries per unit cross-sectional area NA(c,f). The length density of capillaries per unit volume, $J_V(c,f)$, can be calculated from NA(c,f) provided the degree of anisotropy of the capillary network is also known (Hoppeler et. al., 1981; Mathieu et al,

1982; Egginton & Johnston, 1983).

$$Jv(c,f) = y^{-1}\ NA(c,f)$$
$$Vv(c,f) = a(c)\ Jv(c,f)$$
$$Sv(c,f) = b(c)\ Jv(c,f)$$

The value y^{-1} can be estimated assuming a Fisher distrbution of capillaries (Mathieu et. al., 1982). estimates of immersion-fixed coger eel (Egginton & Johnston, 1982,) and two species of Antarctic fish (Egginton & Johnston, 1982, Fitch et al., 1984) were found to be 1.016 (Conger-eel, Notothenia neglecta) and 1.015 (Chaenocephalus aceratus Lonnberg), indicating a high degree of capillary anisotropy (i.e most capillaries run parallel to the longitudinal axis of the fibre). If these values of y^{-1} are representative for fish myotomal muscles, the capillary length per unit volume can be calculated by multiplying NA(c,f)by a factor of 1.02. This enables the volume Vv(c,f) and surface Sv(c,f) densities to be calculated providing the mean capillary cross-sectional areas and perimeters are known. Values for the mean radius (R) of the tissue cylinder surrounding a capillary (Krogh's cylinder) can also be calculated from the length of capillaries per unit volume of muscle fibres (Jv(c,f)).

The capillary volume density is proportional to the volume of the volume of blood within the capillary network and the capillary surface area of capillaries available for gas and/or metabolite exchange. Cross - sectional areas and perimeters of transversely orientated capillaries and fibres were measured from either light (magnification 1200 x) or electron micrographs

(magnification x 2000) using a digital planimeter.

6.2.6 Determination of mitochondrial volume density

The volume density of mitochondria V_v(mit,f) was measured from electron micrographs of whole fibres from each subgroup using a point counting method (Weibel, 1980). Quarter-plate negatives (magnification x 1500 to 4500) of 4 whole fibres, selected from each block sectioned (see Quantitative analysis) were projected onto a 1.0 cm square-lattice grid at varying magnifications (2.3 to 4.5 x), such that the grid spacing was equivalent to >1 to 1.5< times the average mitochondrial diameter. There were approximately 200 test points on the grid, and the volume density of mitochondria was calculated as the ratio of the number of test points falling in mitochondria to the total number of test points (Weibel, 1980). Mitochondria were scored separately as to whether they are located outside a perimeter bounding the myofibrils (sarcolemmal) or intermyofibrillar.

6.3. Statistical Analysis

Measured parameters for fish acclimated to either aerated water or periodic hypoxia exposure were compared using a one-way analysis of variance.

6.4. Results

Electron micrographs were obtained of muscle fibres of fish in either aerated water or following acclimation to periodic anoxia exposure. The frequency distribution of cross-sectional

areas for slow and fast myotomal fibres are shown (Tables 6.1, 6.2; Figs 6.1, 6.2). Fast fibres are heterogeneous, showing a great variety of fibre sizes, while the slow fibres are more homogeneous. The cross-sectional areas for normoxic fish range from (μm^2) (20 to 1000) and (500 to 6500) for slow and fast fibres respectively (Figs 6.1, 6.2). Acclimation to periodic anoxia exposure resulted in an increase in the range of fibre diameters in both slow (20 to 1500) and fast (500 to 9000) muscles.

The numerical capillary density of muscle fibres was higher in fish acclimated to anoxic relative to normoxic. The anoxia-induced increase in capillary density NA(c,f) (μm^{-2}) for both slow (806 to 1373) and fast (234 to 308) muscle fibres are shown (Tables 6.1, 6.2). In fish acclimated to aerated water 34% of slow and 40% of fast fibres had no direct capillary contact (Figs 6.3, 6.4). Acclimation to periodic anoxia exposure resulted in a decrease of non-vascularised slow fibres from 34-30% (N.S). Concurrently, clusters of 2 capillaries/fibre were increased from 10 to 28% ($P>0.05$) in the slow fibres, while those of the fast muscle were reduced from 19 to 15% (Figs 6.3, 6.4). The surface density of capillaries was 3.5 times higher for slow than for fast muscle (Table 6.1, 6.2, Plates 6.1, 6.2).

In fish acclimated to aerated water, mitochondrial volume densities V_v(mit,f) were 0.12 for slow and 0.015 for fast muscles, 83 and 67% of the mitochondrial volume fractions were located in the subsarcolemmal region of slow and fast fibres respectively (Table 6.3; Plates 6.1, 6.3, 6.4, 6.5). Acclimation to periodic anoxia exposure resulted in an increase, 83% for slow and 100% for fast fibres (Tables 6.1, 6.2; Fig 6.5). Increase in mitochondrial volume was accompanied by a

corresponding reduction in diffusion pathlenth of 13 and 23% for fast and slow fibres respectively (Table 6.1, 6.2).

6.5 Discussion

A variety of factors contribute to tissue oxygenation which include mitochondrial density, myoglobin concentrations, blood flow, perfusion distribution and various factors influencing the haemoglobin-oxygen equilibrium such as PCO_2 and ATP concentrations (Prosser, 1973; Weber, 1983). Morphometric parameters such as V_v(mit,f) and capillary density only set certain structural limits to oxygen demand and supply. Capillary density provides only part of the information needed to assess muscle perfusion and blood flow (Granger and Shepherd, 1973). Hoppeler and co-workers (1988), have pointed out the importance of the estimation of total capillary length in relation to the oxidative capacity in skeletal muscles.

A reduction in swimming activity in goldfish during anoxia is matched by a corresponding decline in oxygen requirements, for ATP production. In goldfish metabolic depression has been shown previously (Chapter 5) as an important strategy for surviving anoxic conditions. Interestingly, the energy requirements for anoxic processes amounted to 27% of that of aerobic pathway generation.

Fish skeletal muscle displays a considerable plasticity in response to physiological stimuli. Interestingly, both low temperature and anoxia have produced similar effects such as mitochondrial volume increase in order to compensate for oxygen and metabolite reduced diffusion. For example, in goldfish

anoxia-induced increase in volume density of mitochondria Vv(mit,f) 83% for slow and 100% for fast fibres, was associated with the induction of alcohol dehydrogenase activity which converts lactate to neutral ethanol (74% of the ethanol was excreted to the the surrounding water (Tables 6.1, 6.2; 6.3; Chapter 5). Thus redox balance is maintained and glycolysis is allowed to continue. Interestingly, mitochondrial increase is also matched by an increase in electron transport enzymes (Sidell, 1980), myoglobin (Johnston et.al., 1985) and oxygen consumption with cold acclimation (Johnston & Maitland), with the resultant increase in ATP turnover. Similar reports have been obtained previously (Goldfish, Mourik et.al., 1982; crucian carp, Johnston & Bernard, 1983). Acclimation to cold temperature also induces marked increases in populations of muscle mitochondria (goldfish, Johnston & Maitland, 1980; Tyler & Sidell, 1985; striped bass, Egginton & Sidell,1986), and in the volume of sarcoplasm reticulum (Penney & Goldspink,1980). Both responses will decrease the mean cytoplasmic diffusion pathlength for small molecles and increase the surface area of membrane interface between the cytoplasmic and organelle compartments (Sidell & Hazel, 1987) or sarcoplasmic reticulum (Egginton & Sidell, 1986). Mitochondrial increase has been shown to reduce mitochondrial spacing within muscle fibres in proportion to the reduction in metabolic diffusion (Tyler & Sidell, 1984; Sidell & Hazel, 1984). In striped bass, proliferation of mitochondria resulted in cellular hypertrophy with concomitant increased intracellular lipid deposits. It is suggested intracellular lipid content may accelerate oxygen flux and act as an oxygen store (Egginton and Sidell, 1989),

Higher mitochondrial volume densities Vv(mit,f) following

anoxia acclimation may be associated with the utilization of circulating oxygen stores, reduction in venous PO_2 gradient and ultimately increases the rate of oxygen transfer across the gills. Mitochondrial increase in anoxic goldfish is accompanied by a reduction in oxygen/and or metabolite diffusion pathlength (Table 6.1, 6.2). Mahler, (1978) has shown that the diffusion coeficient for oxygen in frog skeletal muscle decreases by more than 40% between 23°C and 0°C. This is similar to the reduction in diffusion constant of lactate with decreasing temperature (Hazel & Sidell, 1987).

Goldfish show an increase in subsarcolemmal mitochondria, 67% slow; 100%, fast; following periodic anoxic exposure (Table 6.3). Sarcolemmal mitochondria will experience significantly higher oxygen tension due to their proximity to capillaries and show well-defined cristae than intermyofibrilar mitochondria (Plates 6.1.A,B; 6.3, 6.4, 6.5). The high density of sarcolemmal zone (Table 6.3; Plate 6.1.A,B.) further decreases the oxygen/nutrients gradients towards the centre of the fibre due to their own metabolism (Hoppeler et al., 1988).
When blood supply was limited and muscles were contracting the glycogen depleted fast glycolytic fibres were the only fibres showing damage (Hoppeler et al., 1985). It is possible that glycogen depletion leads to an accumulation of lactate in fibres poorly supplied by capillaries. This would increase intracellular pH and possible activation of lysosomal enzymes with consequent degeneration. Hudlicka et al., (1984), have suggested this would result in the synthesis of oxidative enzymes and proliferation of mitochondria.

In goldfish, capillary density was 3.5 times greater in

slow than fast fibres (Tables 6.1, 6.2). Acclimation to periodic anoxia exposure resulted in an increase in capillarisaion in both the slow and fast muscle fibres (Figs. 6.3, 6.4;). Increase in capillaries may be associated with increased blood flow with faster removal of metabolites such as lactate (Hudlicka et al., 1984). There have been a number of comparable studies on the capillary supply to fast and slow muscle fibres in fish (Kryvi et al., 1980; Totland et al., 1981; Egginton and Johnston, 1982 a; Johnston, 1982 b,c; Fitch & Johnston, 1985). Hypoxia acclimation has produced opposite results in different species which probably reflect differences in their oxygen consumption rates. For example, capillary volume density NA(c,f) is increased in goldfish (Tables 6.1, 6.2) and crucian carp (Johnston & Bernard, 1984); decreased in tench (Johnston & Bernard, 1982); but unchanged in catfish (Johnston & Bernard, 1983).

There is a good correlation between capillary density NA(c,f) and the volume density of mitochondria V_v(mit,f) for slow muscle fibres of various species (Fig 6.5). This indicates that the surface area of the capillary network available for gas-exchange is a limiting factor in determining the size of the mitochondrial compartment and probably aerobic capacity.

However, capillary density estimated from muscle tissue cross-section is inadequate for modelling radial oxygen diffusion from capillaries into cells because, for a given capillary density the distrbution distances may vary considerably depending on the actual arrangmeent of capillaries (Kayar et al., 1982 a; Hoofd, 1987). Diffusion of substances to and from mitochondria, depends not only on mitochondrial volume density but also on mitochondrial distribution within fibres and relative to capillaries (Mainwood & Rakuson,1982; Kayar et al., 1986 a).

TABLE 6.1. Effects of acclimation to periodic anoxia exposure on the capillary supply to slow muscle fibres of the goldfish (*Carassius auratus* L.).

Parameter+	Unit	Symbol calculation	Acclimation Condition	
			Aerated water	Hypoxia
Number of fibres		A	100	67
Number of capillaries		B	37	56
Fibre per cross-sectional area	μm^2	C	458.77 ± 7.87	608.55 ± 57
Fibre perimeter	μm	D	100.32 ± 5.59	90.66±4.64
Fractional volume occupied by mitochondria	%	E	0.12	0.22*
Number of capillaries per muscle fibre		F	0.37	0.84**
Capillary contact length per fibre	μm	G	17.68 ± 0.5	17.16 ± 0.68
Percentage of fibre perimeter in direct capillary contact	%	$H = \frac{G \times 100}{D}$	17.74	18.9
Mean perimeter served by one capillary	μm	$I = \frac{D}{F}$	217	107.9**
Capillary contact length per um^2 fibre cross-sectional area	μm^2	$J = \frac{G}{C}$	0.038	0.028
Capillary surface (μm^2) supplying 1 μm^3 of mitochondria	μm^2	$K = \frac{J}{E}$	0.32	0.14**
Maximum hypothetical diffusion distance	μm	$L = \frac{A \times C}{B}$	19.9	15.22
Number of capillaries per unit volume of muscle fibres (mm^{-2})		NA (c,f)	806	1373

N.S. Not significant at P

* $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

\+ Values represent mean ± SE

TABLE 6.1.

TABLE 6.2. Effects of acclimation to periodic anoxia exposure on the capillary supply to fast muscle fibres of the goldfish (Carassius auratus L.).

Parameter+	Unit	Symbol calculation	Acclimation Condition	
			Aerated water	Hypoxia
Number of fibres		A	100	100
Number of capillaries		B	24	33
Fibre per cross-sectional area	μm^2	C	1024.66±58.0	1070.97±74.07
Fibre perimeter	µm	D	133.4 ± 4.06	133.27 ± 4.77
Fractional volume occupied by mitochondria	%	E	0.015	0.03*
Number of capillaries per muscle fibre		F	0.24	0.33
Capillary contact length per fibre	µm	G	16.77 ± 0.48	14.11 ± 0.60
Percentage of fibre perimeter in direct capillary contact	%	$H = \frac{G \times 100}{D}$	17.54	10.59
Mean perimeter served by one capillary	µm	$I = \frac{D}{F}$	556	403.84
Capillary contact length per um^2 fibre cross-sectional area	μm^2	$J = \frac{G}{C}$	0.016	0.013
Capillary surface (μm^2) supplying 1 μm^3 of mitochondria	μm^2	$K = \frac{J}{E}$	1.07	0.43**
Maximum hypothetical diffusion distance	µm	$L = \frac{A \times C}{B}$	36.86	32.13
Number of capillaries per unit volume of muscle fibres (mm^{-2})		NA (c,f)	234	308

N.S. Not significant at P

* P < 0.05 ** P < 0.01 *** P < 0.001

\+ Values represent mean ± SE

TABLE 6.2.

	Aerated Water		Periodic Anoxic Exposure	
	Slow	Fast	Slow	Fast
Subsarcolemmal mitochondria	0.10 ±0.01	0.01 ±0.09	0.16 ±0.01	0.02 ±0.001
Inter myofibrillar mitochondria	0.02 ±0.001	0.005 ±0.001	0.04 ±0.003	0.01 ±0.01

TABLE 6.3. Effects of acclimation to periodic anoxia exposure on mitochondrial volume density $V_v(m,f)$ (μm^2) of slow and fast muscles of goldfish (Carassius auratus L.).

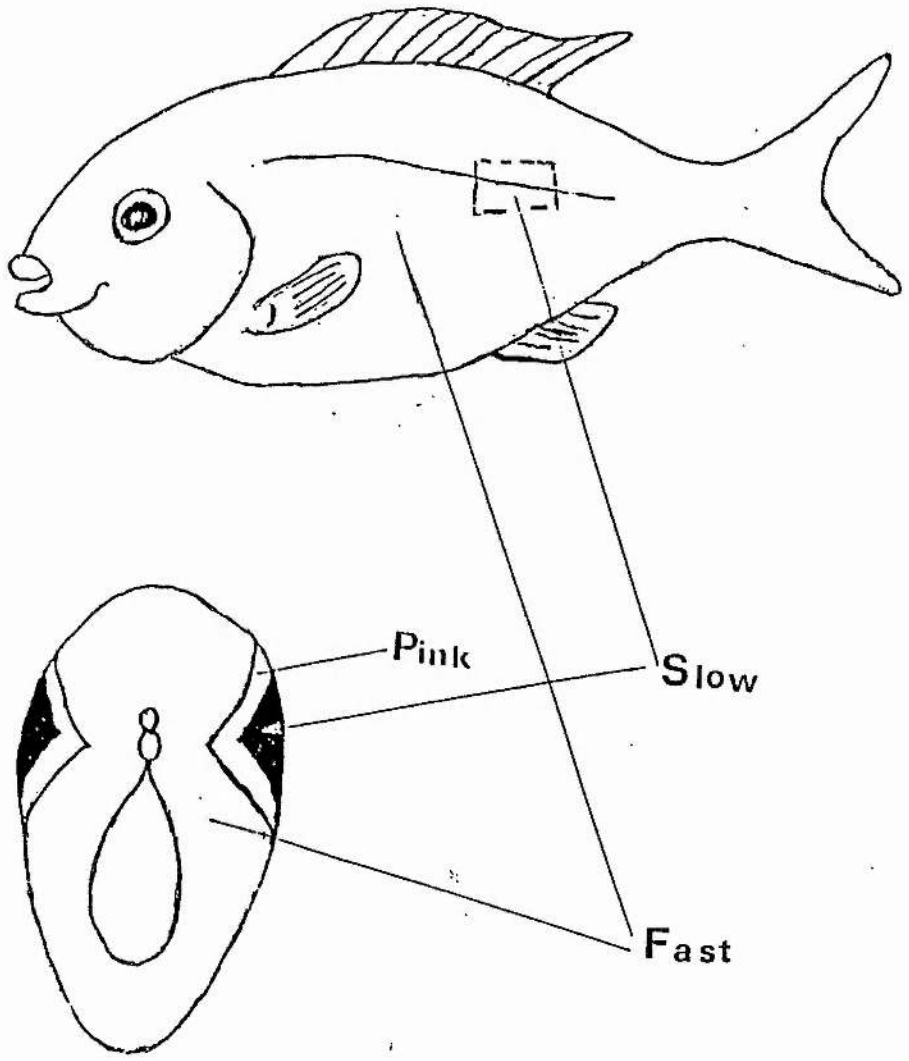


FIG.6.

Sites for the dissection of myotomal muscles in goldfish (Carassius auratus L.).

FIGURE 6.1. Histogram showing frequency distribution of fibre cross-sectional area from fast muscles of goldfish (*Carassius auratus* L.) acclimated to either aerated water (a), or anoxia (b)(n=6)

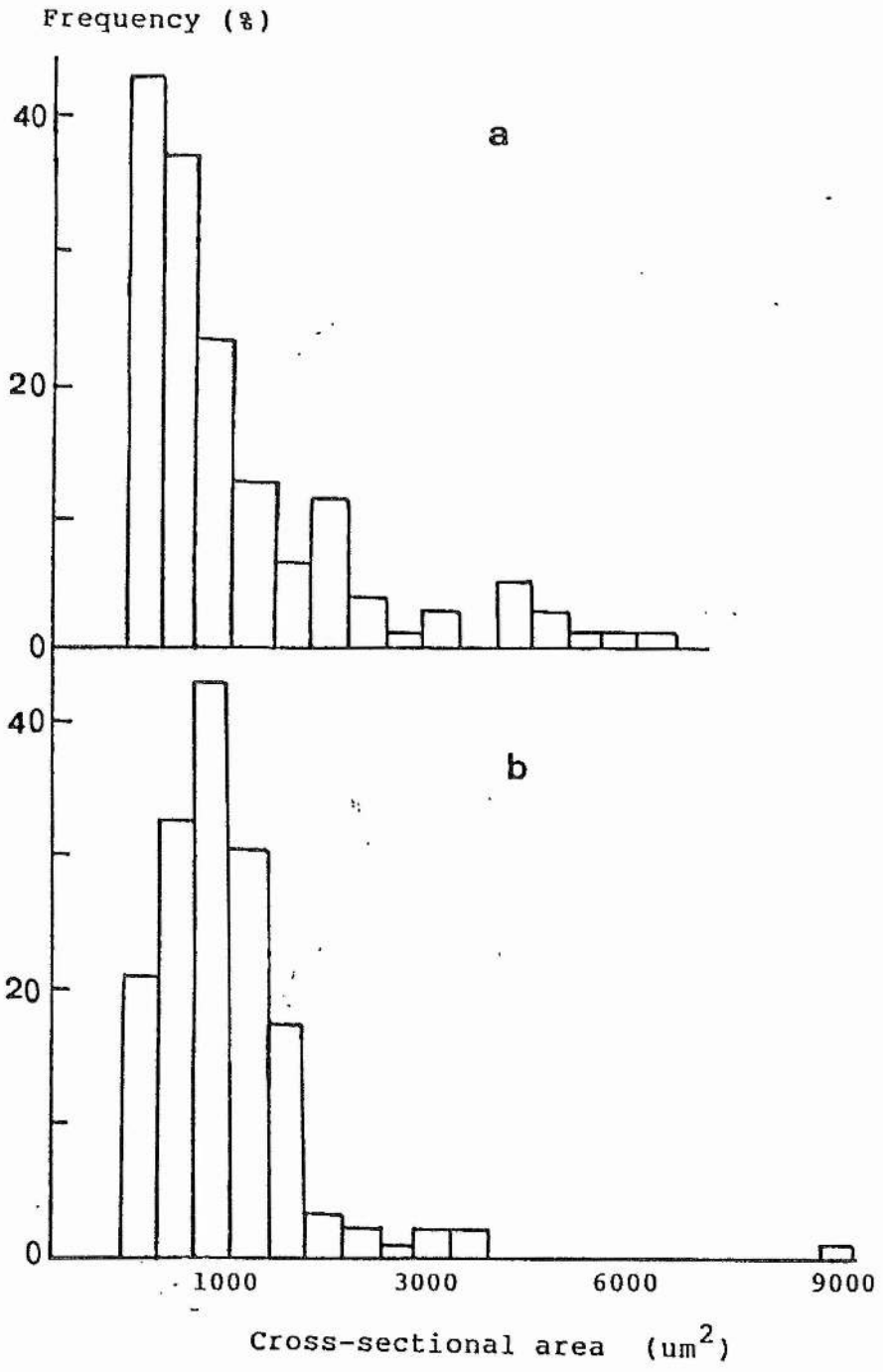

Frequency (%)
40
20
0
a
40
20
0
b
1000
3000
6000
9000
Cross-sectional area (um2)

FIG.6.1.

FIGURE 6.2. Histogram showing frequency distribution of fibre cross-sectional area from slow muscles of goldfish (*Carassius auratus* L.) acclimated to either aerated water (a), or anoxia (b)(n=6)

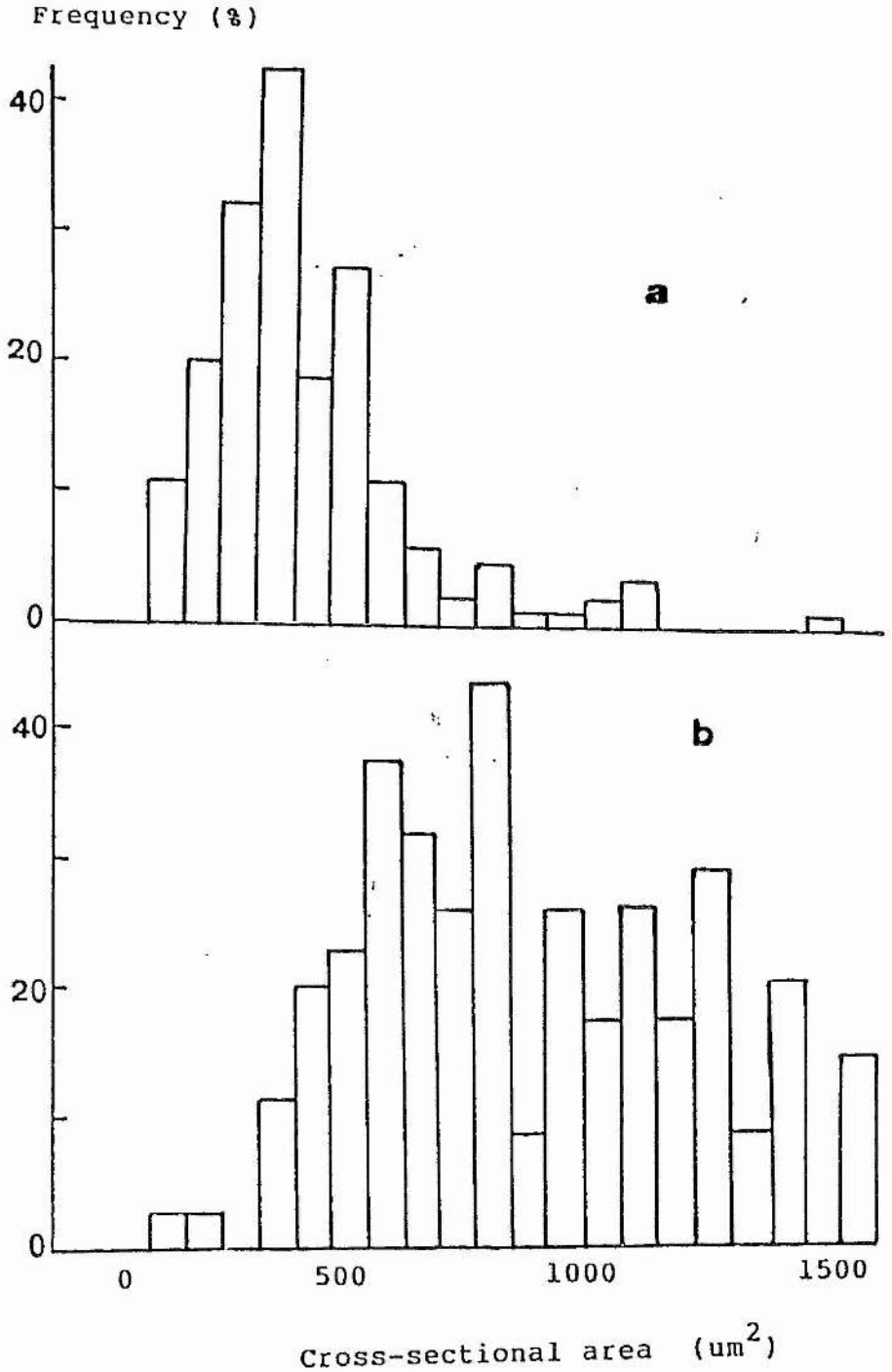

Frequency (%)
40
20
0
a
40
20
0
b
0
500
1000
1500
Cross-sectional area (um2)

FIG. 6.2.

FIGURE 6.3. Frequency distribution of the number of capillaries per fibre for slow muscles from goldfish (Carassius auratus L.), following acclimation to either aerated water (a), or anoxia (b).

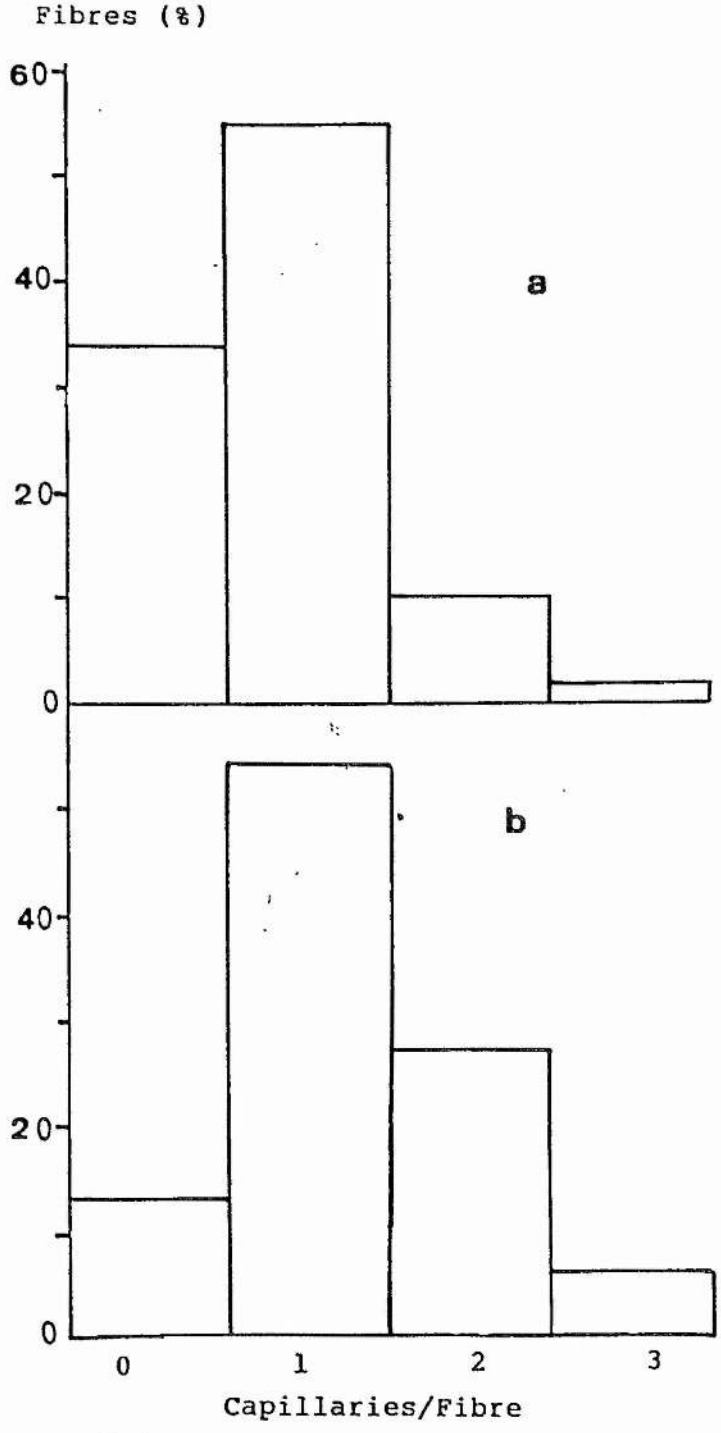


FIG. 6.3.

FIGURE 6.4. Frequency distribution of the number of capillaries per fibre for fast muscles from goldfish (*Carassius auratus* L.) following acclimation to either aerated water (a), or anoxia (b).

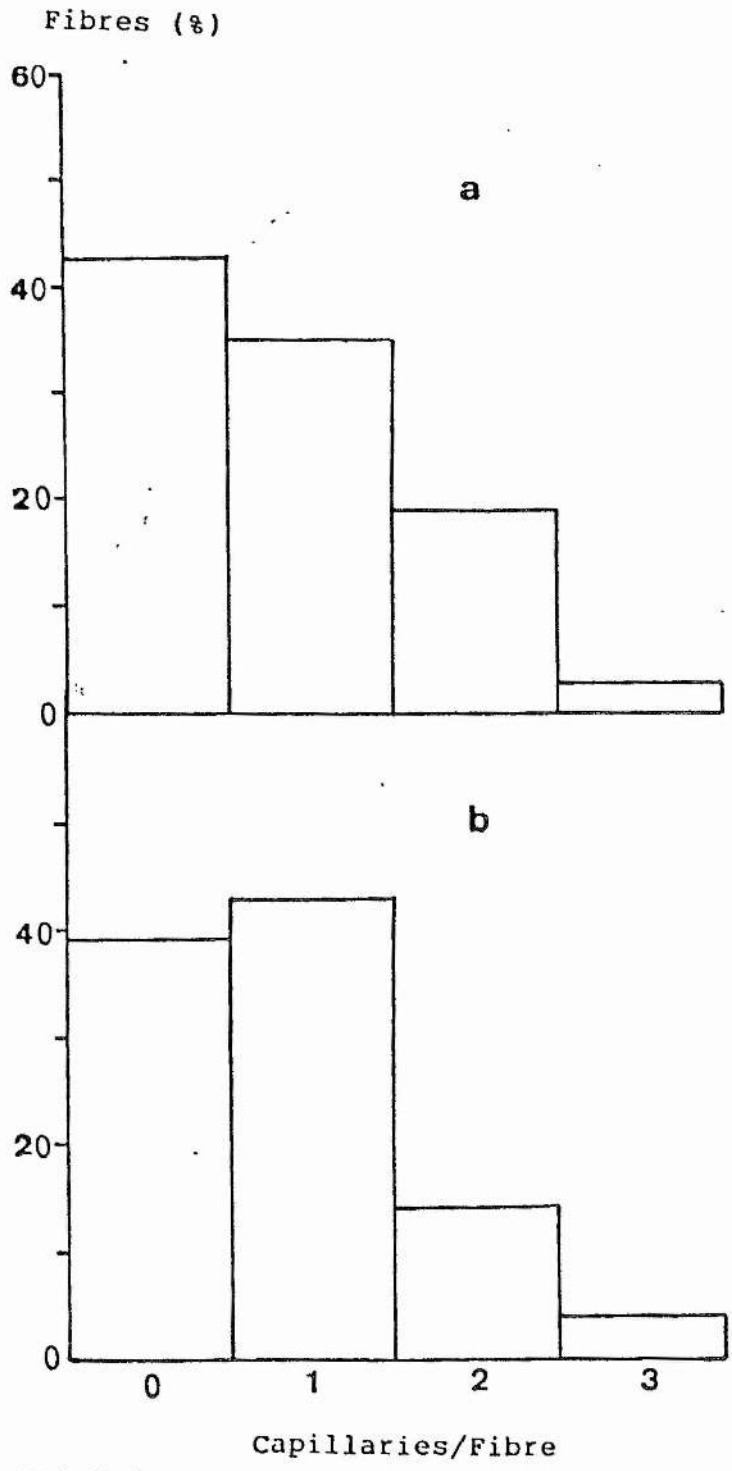


FIG.6.4.

FIGURE 6.5.

Relationship between mitochondrial volume density, $V_v(c,f)$ and the number of capillaries per mm^2 of muscle fibre cross-sectional area NA(c,f) for fish slow fibres of various species.

References:

(1,2). This paper (3,4). Johnston & Bernard (1984). (5,6). Johnston & Bernard (1983). (7,8). Johnston & Bernard (1982a).

(9). Salamonski & Johnston (1982).

(10). Beardall & Johnston (1983).

(11). Johnston (1983).

(12). Salamonski & Johnston (1983).

(13). Egginton & Johnston (1983).

(14,15). Totland,Kryvi & Bone (1981).

(16). Kryvi,Flood & Gulyaer (1980).

(17). Totland et.al. (1981).

(18). Egginton & Jonston (1982).

(19). Totland et. al. (1981).

(20). Fitch & Johnston (1983).

(21). Fitch et. al. (1982).

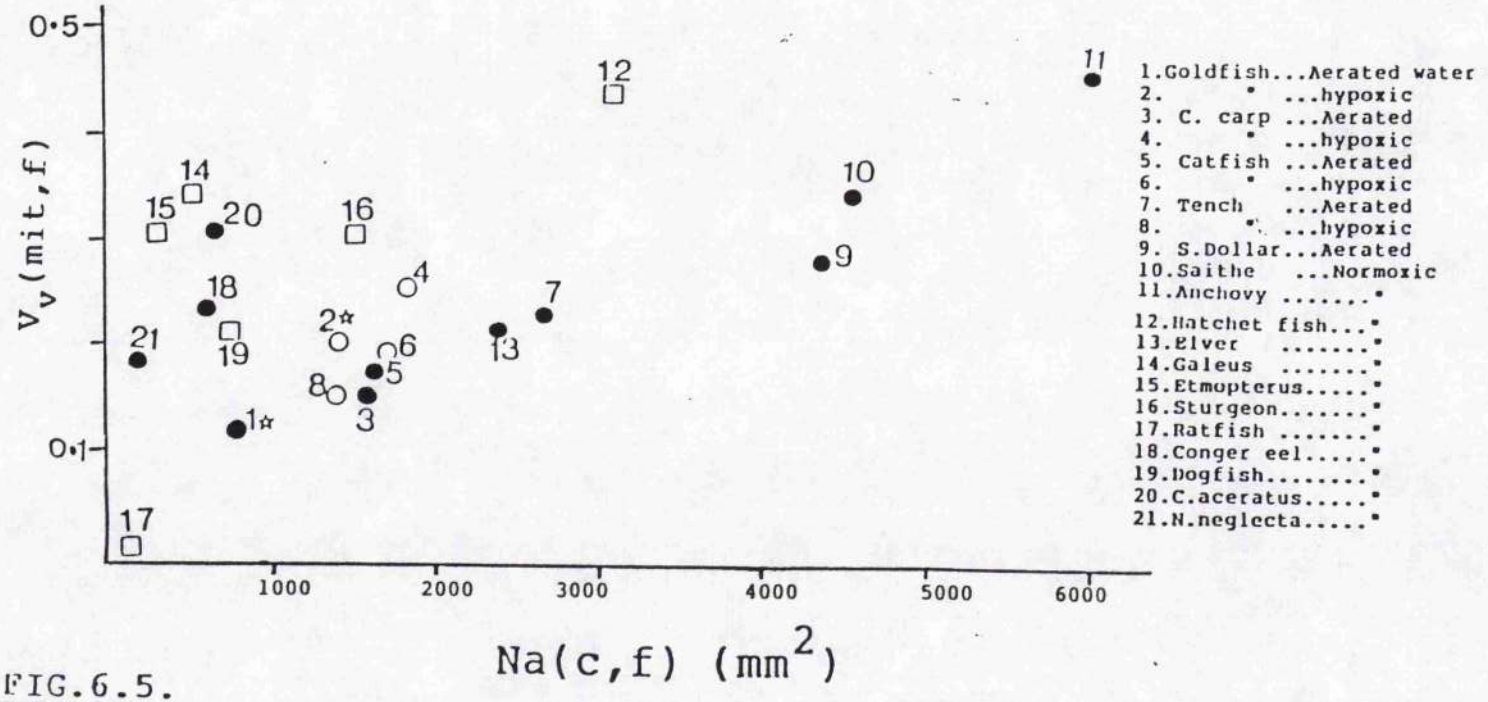

0·5
0·1
V_v(mit,f)
1000
2000
3000
4000
5000
6000
Na(c,f) (mm^2)
1.Goldfish...Aerated water
2. " ...hypoxic
3. C. carp ...Aerated
4. " ...hypoxic
5. Catfish ...Aerated
6. " ...hypoxic
7. Tench ...Aerated
8. " ...hypoxic
9. S.Dollar...Aerated
10.Saithe ...Normoxic
11.Anchovy .....
12.Hatchet fish...
13.Elver .....
14.Galeus .....
15.Etmopterus.....
16.Sturgeon.....
17.Ratfish .....
18.Conger eel.....
19.Dogfish.....
20.C.aceratus.....
21.N.neglecta.....

FIG.6.5.

PLATE 6.1.

Low power electron micrographs showing the distribution of subsarcolemmal mitochondria in slow fibre cross-sectional area of goldfish (Carassius auratus L.) in either (A), aerated water; or (B), hypoxic water. Degeneration of myofibrils in the fibre centre (see arrows) may be due to the effects of periodic hypoxia exposure. Note location of capillaries (C) at fibre junctions; (L) lipid droplets. Scale bars: A,B (4 μm). Magnification: A,B (2958 x).

PLATE 6.1.

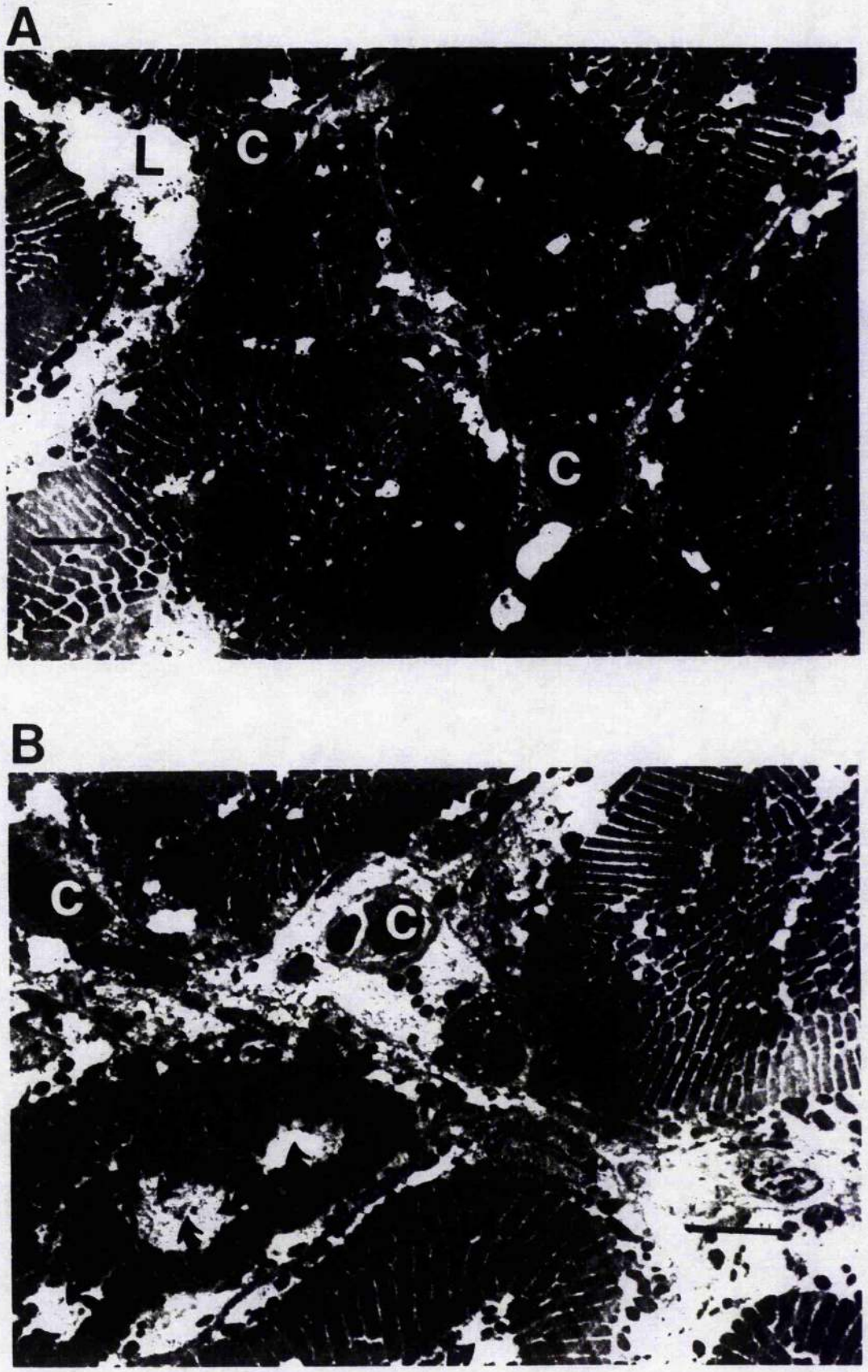
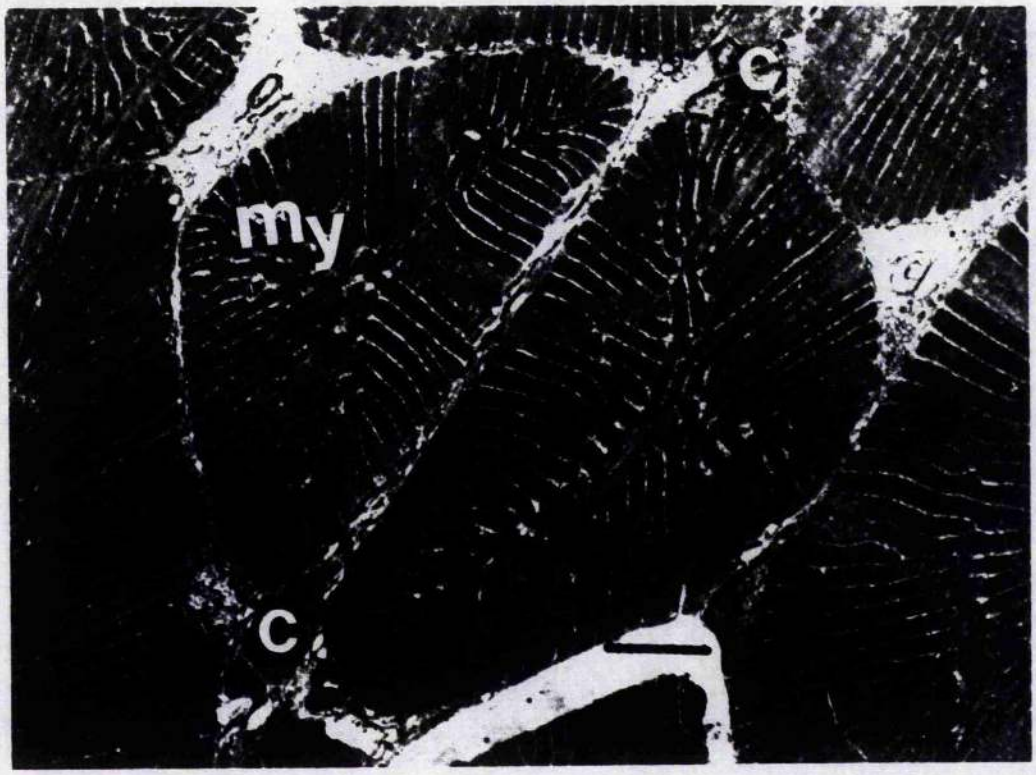


PLATE 6.2. Transverse micrographs showing fast fibre cross-sectional areas of goldfish (Carassius auratus L.) acclimated to either (A), aerated water; (B), hypoxic water. Scale bars: A, (4 μm); B, (0.3 μm). Magnification: A, (2958 x); B, (32,000 x).

PLATE 6.2

A



B

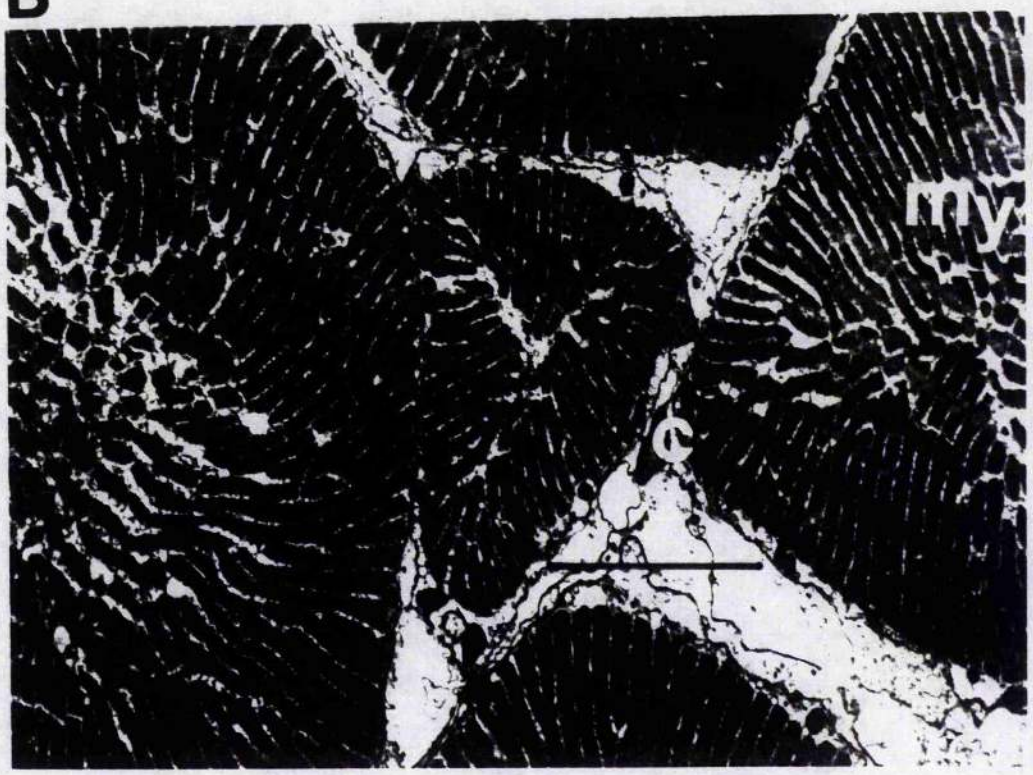
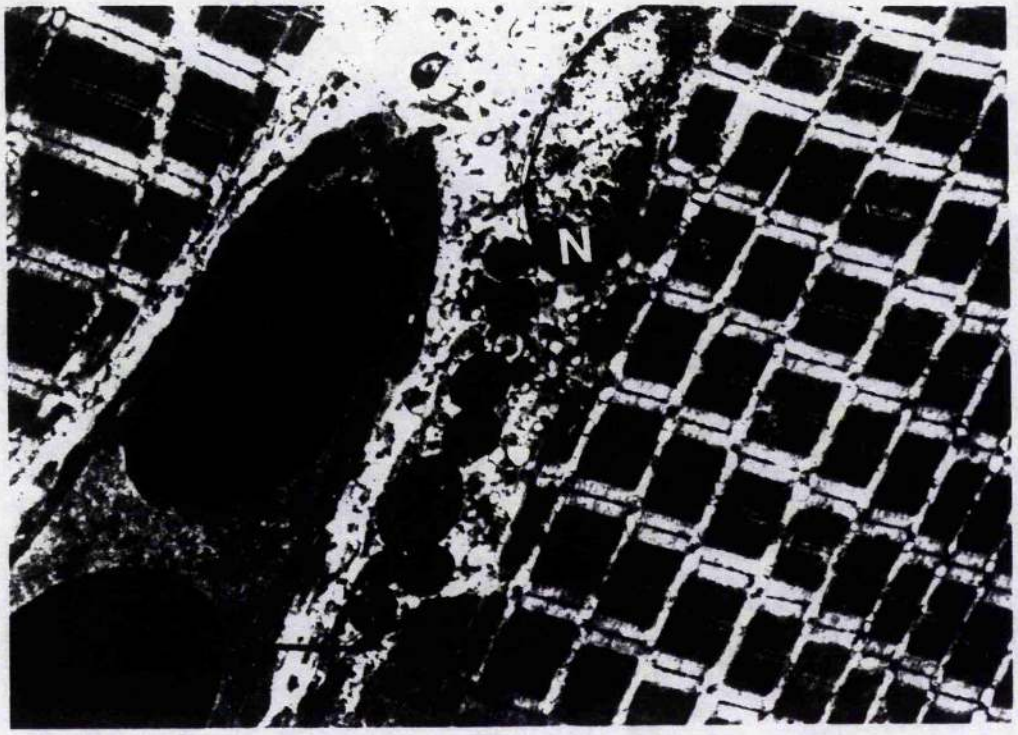


PLATE 6.3. Longitudinal section micrographs of slow fibres from goldfish (Carassius auratus L.) showing the distribution of mitochondria in (A), low power; (B), high power; note: N, nucleus; Mit, mitochondria with well- defined cristae formation. Scale bars: A, 4 µm; B,1 µm. Magnification: A, 2958x; B, 32,500 x.

PLATE 6.3

A



B

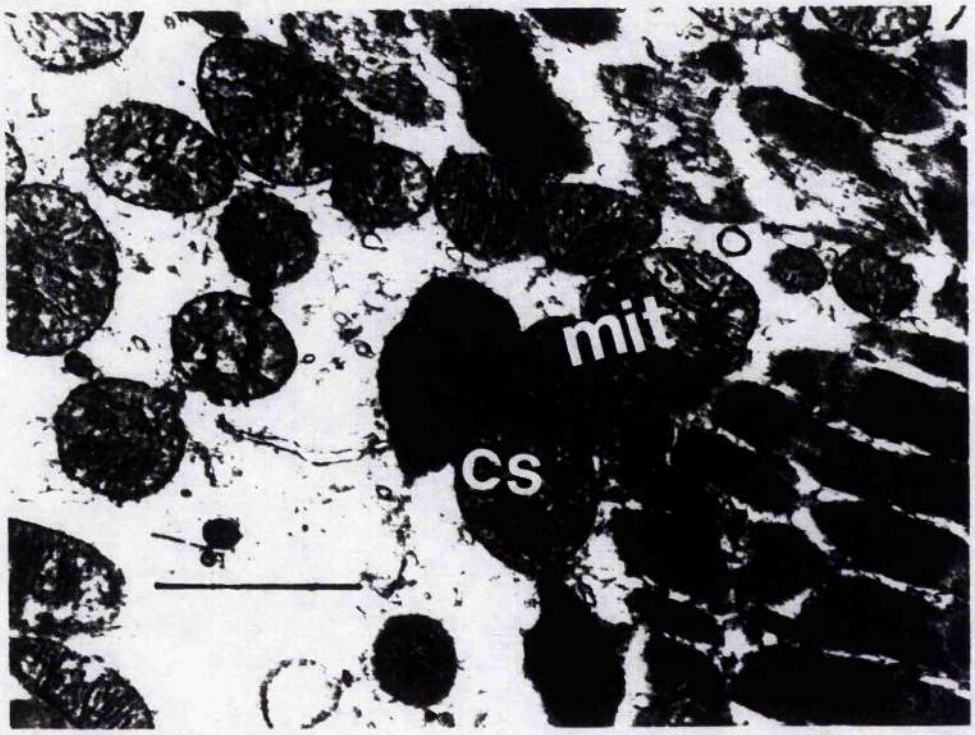


PLATE 6.4.

High power electron micrographs of mitochondria from goldfish (Carassius auratus L.) acclimated to (A), aerated water ; (B), hypoxic water showing well defined cristae (CS) in both. Scale bars: A, 0.5 µm; B, 0.4 µm. Magnification A, (58,500 x); B, (74,100 x).

PLATE 6.4.

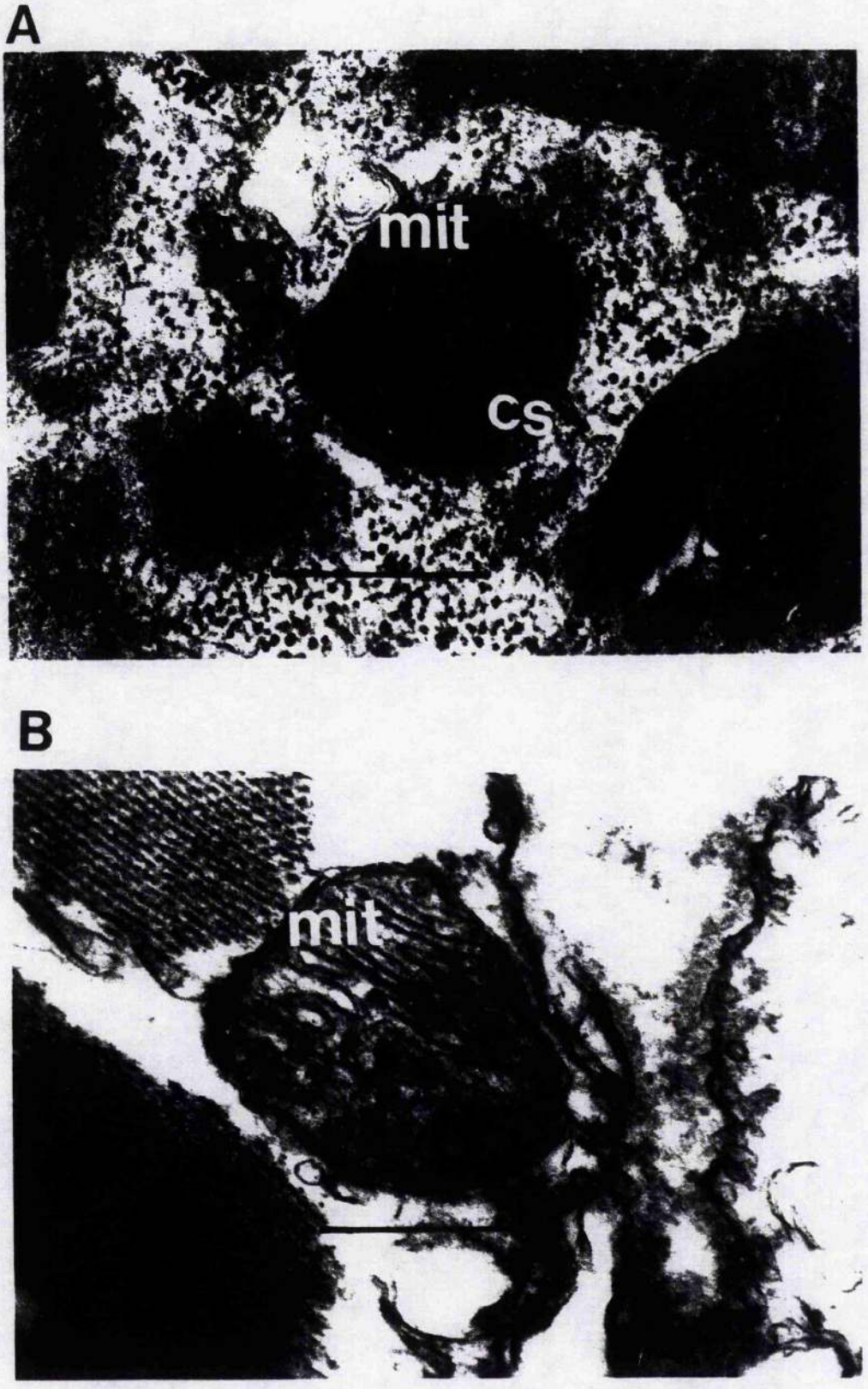
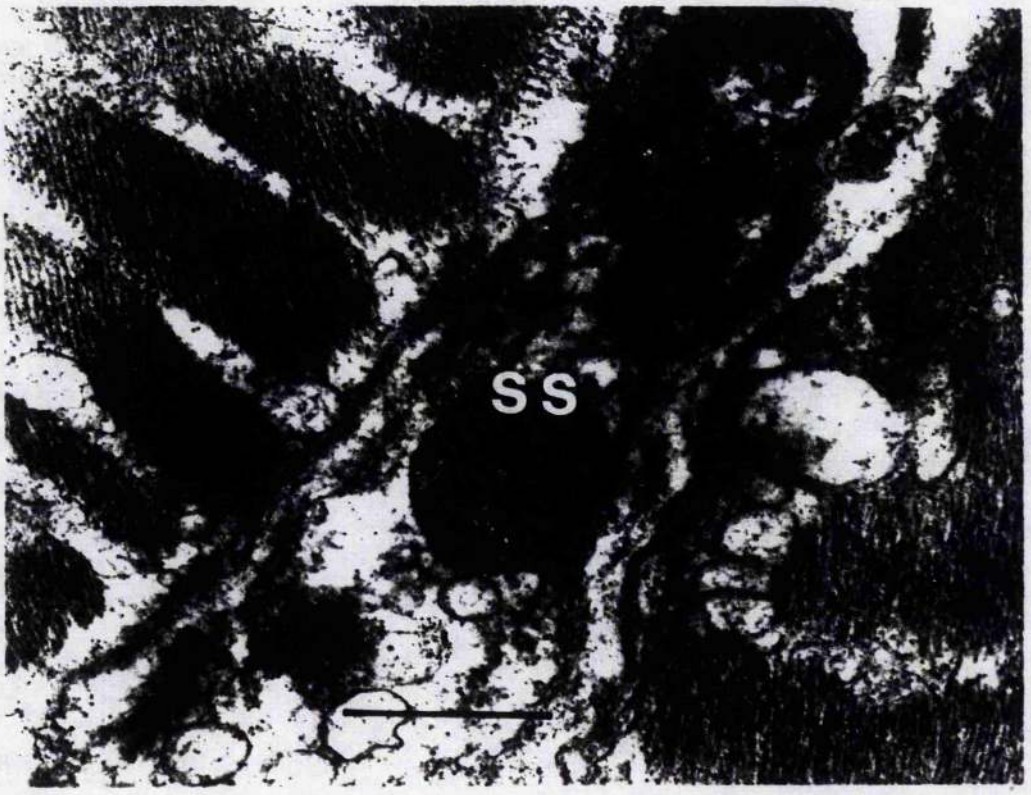


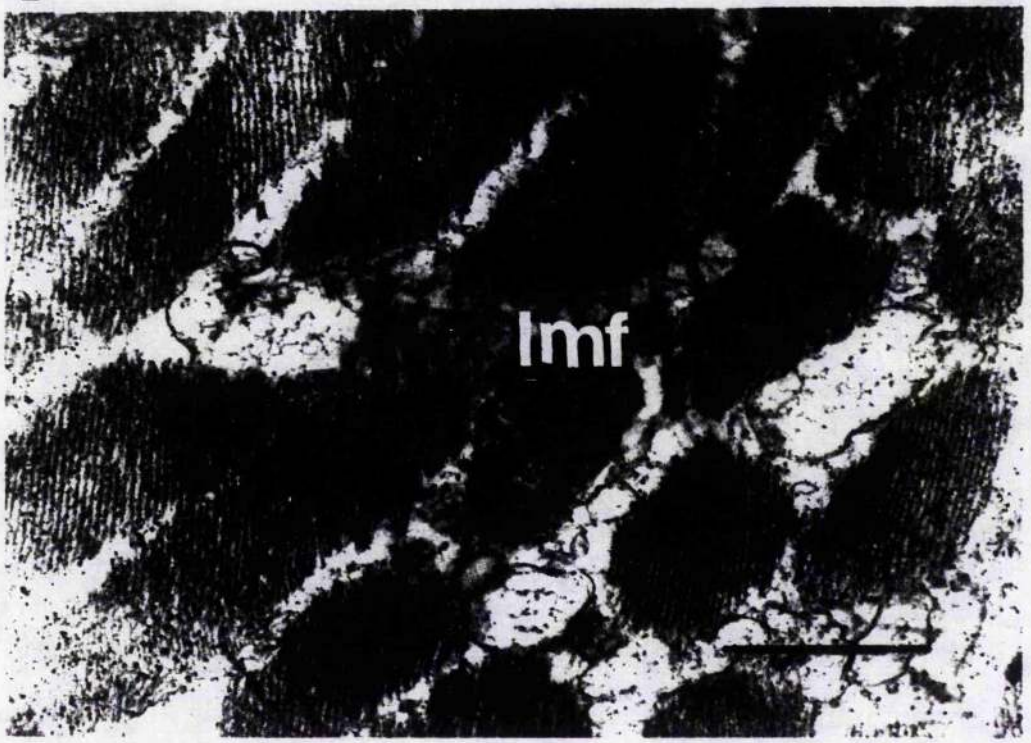
PLATE 6.5. High power electron micrographs of mitochondria from goldfish (Carassius auratus L.) showing (A), subsarcolemmal mitochondria, (SS), with more distinct cristae formation than (B), intermyofibrillar mitochondria (Imf). Scale bars: A, B, (1 μm). Magnification A, B, (32,500 x).

PLATE 6.5.

A



B



CHAPTER 7.

GENERAL DISCUSSION.

Fish undergo seasonal changes in temperature and so can tolerate a range of temperatures, but their muscle performance and hence swimming ability show optima, each species having a preferred performance temperature. Some species of fish become sluggish in winter, but others are able to move their optima as the seasonal temperature changes, thus enabling performance at any time of the year. For example, many freshwater fish are found in environments in which temperature ranges from 0-4^{o}C in winter and 25-30^{o}C in summer. Thus mummichog (Fundulus heteroclitus), a small fish is usually exposed to rapid temperature changes between 15^{o}C and 30^{o}C during summer tidal cycles. Sidell et.al., (1983) have shown that the contractile apparatus (Myofibrillar ATPase) is relatively temperature independent. The two kinds of response, i.e., torpid, relatively inactive or improvements in swimming performance with low temperature acclimation, are not mutually exclusive and sometimes occur in the same species depending on the temperature. For example, largemouth bass (Micropterus salmoides), become torpid below 7^{o}C, yet maintain a similar level of spontaneous activity over the range 7^{o}C to 30^{o}C (Lemons & Crawshaw, 1985).

Temperature acclimation studies show that fishes are able to function with a variable body temperature (Chapter 2). The adaptations which occur at the cellular level to offset the effects of temperature changes are very complex and are poorly

understood. Passive effects parallel the time course of thermal change and are due to changes in the free energy of ions and molecules. Other metabolic changes reflect an attempt to maintain celluluar homeostasis despite the direct or passive effects of temperature change. The time course of these changes ranges from seconds to several weeks. The plasticity of the muscle in adapting to different work loads and physiological conditions has been reviewed (Holloszy & Booth, 1976). Poikilothermic species show a complete or partial compensation in metabolic rate following acclimation to different environmental temperatures (Hazel & Prosser, 1974). In general the strategies adopted by ectotherms to compensate for changes in temperature fall into two categories: quantitative changes in enzyme concentration (Wilson et.al., 1974; Sidell, 1977) and/or a qualitative change in isozyme synthesized or the modification of existing protein. For example, changes in membrane composition (Hazel & Prosser, 1972; Van den Thillart, 1978), contractile properties (Johnston et.al., 1975; Johnston et. al., 1978) and metabolism (Hochachka & Hayes, 1962) have all been recorded following temperature acclimation. It would appear that cellular responses to stress result in the turnover of its component parts; the more rapid the turnover is, the faster the response, whether neuronal or hormonal (Chapter 3).

The present studies show the responses to periodic hypoxia vary among the species (Chapters 4,5) following acclimation. For example, in plaice (Pleuronectes platessa), increase in lactate concentrations resulting from periodic exposure to acute hypoxia were accompanied by parallel increases in the activities of lactate dehydrogenase (Chapter 4). The absence of alcohol dehydrogenase activity, and the consequent lack of ethanol

pathway utilization, suggests that ATP production during hypoxia is dependent only on anaerobic glycolysis. An excess production of lactate is likely to disturb the redox balance, resulting in metabolic acidosis. This may contribute to the failure of plaice to survive anoxia. In contrast, in goldfish, acclimation to periodic anoxia exposure resulted in the increase in mitochondrial volume density in slow and fast muscle fibres (Chapter 6) which is correlated with ethanol production (Chapter 5) and thus confirms ethanol pathway utilization. This is supported by induction of alcohol dehydrogenase activity in the skeletal muscles (Chapter 5). The conversion of lactate to ethanol (Shoubridge, 1982) suggests excessive lactate accumulation is prevented and glycolysis is allowed to continue without acidosis. This constitutes the basis for survival of goldfish during prolonged anoxia.

Ultrastructural findings:

Ulrastructural studies (Chapter 6) show that in goldfish (Carassius auratus L.) following acclimation to periodic anoxia exposure, proliferation of mitochondrial volume density was accompanied by a reduction of oxygen/and or metabolites diffusion pathlength between mitochondria and the S.R. of the cytosol. Similar results have been found in other teleosts (crucian carp, Johnston & Bernard., 1984; striped bass, Egginton & Sidell., 1989). Interestingly, changes in mitochondrial volume density would appear to depend not only on the type and severity of environmental stress but also on the physiological state of the organism. For example, following acclimation to hypoxia

exposure mitochondrial volume density V_v(mit,f) showed a reduction in tench, Tinca tinca (Johnston & Bernard, 1982), and no change in catfish, Clarias mossambica (Johnston & Bernard 1983). In contrast, V_v(mit,f) for slow myotomal muscle fibres are 28% for 5°C and 14% for 25°C - acclimated fish (Johnston, 1982a). Increases in mitochondrial volume density and surface density would enhance the rate of ATP provision. Support for this comes from the increases in associated electron transport enzymes following cold acclimation (Chapter 2). However the present studies on goldfish show that mitochondrial proliferation during anoxia is correlated with ethanol pathway utilization (Chapter 5).

Acclimation to periodic anoxia exposure resulted an increase in capillary volume density NA(c,f) in the slow fibre of goldfish (Chapter 5). Interestingly, anoxia had no effect on the capillary supply in carp (Johnston & Bernard, 1984). Hudlicka et. al., (1988), have indicated increase in capillary density NA(c,f), may represent increased blood flow to effect faster removal of metabolite such as lactate and does not necessarily indicate increased aerobic capacity ,since other intracellular factors also come into play.

Survival time during anoxia is temperature dependent, ranging from 16 h at 20° C to several weeks at 0° C (Van den Thillart, 1977; Walker & Johansen, 1977).

How can these laboratory studies be related to the natural environment.

Magnuson et.al., (1983) suggested, in seasonally anoxic water, survival might be due to some alternative oxygen sources like bubbles under ice. However, it would appear the Cyprinid

species have the capability for true anaerobiosis (Blazka, 1958). For example, Crucian carp has been found to survive prolonged anoxia in snow-capped lakes for 6 months (Blazka, 1958., Holopainen & Hyvarinen, 1985). Most species stop feeding and must rely on energy reserves, (e.g. fat or carbohydrate deposits) collected prior to wintering. However, in goldfish and crucian carp cold acclimation is associated with an increase in myoglobin, mitochondria and the component enzymes (Johnston & Lucking, 1978; Sidell, 1980; Jones & Sidell, 1982), and enzymes of fatty acid oxidation (Johnston et. al., 1985), resulting in increased provision of ATP. (Johnston et. al., 1985). In goldfish, anoxia under cold acclimation results in the induction of alcohol dehydrogenase activty which converts lactate to ethanol especially in the red skeletal muscle (Chapter 5, Mourik et.al., 1982) thus enabling anaerobic metabolism to proceed without redox balance disturbance (Hochachka, 1980). Anoxia acclimation in the cold is also known to increase glycogen stores. For example, prolonged anoxia survival in carp was matched by a parallel increase in glycogen (Holopainen & Hyrarinen, 1985), and a reduction in the activity of phosphorylase activity (Hyrarinen & Holopainen, 1986). This is consistent with the results obtained on goldfish (Rahman & Storey, 1988), which showed the anoxic forms of the glycolytic enzymes, PK and PFK have reduced activities compared to the aerobic enzymes.

Suggestions for future work:

Studies of hypoxia or temperature should involve interaction of environmental variables in order to mimic the

natural environment e.g. (i) intermittent hypoxia (ii) periodic hypoxia at varying temperatures, (iii) periodic hypoxia and anoxia). These procedures mimic the natural fluctuating conditions and predict results that are similar. For example, in the studies of Kleckner & Sidell (1985), a comparison of maximal enzyme activities in tissues from 5°C- and 25°C-acclimated chain pickerel to those from winter- and summer-acclimatized fish, showed the general metabolic response was similar between the laboratory acclimated and the natural acclimatized groups. However, activities were generally higher for laboratory held than wild fish. This may be due to changes in the pattern of swimming or differences in food availability.

Continued efforts should be made to identify the biochemical pathways and their relative energetic importance during rest, hypoxia and recovery.

Enzyme kinetic studies on control and anoxic fish.

Differences in the metabolism of aerobic and anoxic goldfish have been attributed to the reduced kinetic activities of the anoxic enzymes compared to the aerobic forms. For example, the in vitro treatment of both the aerobic forms of PK and PFK from the liver of goldfish (Carassius auratus L.) with cAMP dependent protein kinase altered the kinetic properties (e.g. reduced Vmax, increased substrate for PEP and increased K_a for F16P for PK; and a decrease in F26P for PFK) consistent with reduced PK or PFK activity which mimicked the effect of anoxia on the enzymes. Interestingly, alkaline phosphatase treatment of the anoxic enzyme forms had the opposite effect (Rahman & Storey,

1988). Sprang et al. (1980) have also shown, the conversion of phosphorylase b to phos a is concomitant with a significant reduction in the allosteric constant for phosphorylase a relative to phos b.

Studies on hypoxia should include the brain and heart, since as noted by Shoubridge and Hochachka, (1983), glycogen reserves in these tissues are depleted early in anoxia, while muscle glycogen is retained to fuel burst swimming or as needed.

Simultaneous direct and indirect calorimetry:

Van den Thillart et. al., (1976) had shown that lactate level of whole goldfish reaches a stable concentration after approximately 6 h anoxia. Similar results have been obtained (Hochachka and Shoubridge, 1980 for goldfish; see also chapter 5; and Johnston & Bernard, 1982, for carp). If for one of the endproducts, the animal fails to reach a steady state , this unknown production can be determined by simultaneous direct and indirect calorimetry. These tests together with biochemical determinations of metabolites show that during normoxia and anoxia the same substrates are used by starving goldfish, but the endproducts are different (Waversveld & Van den Thillart, 1989). During normoxia oxidation is complete - yielding CO_2, H_2O and ammonia while during anoxia oxidation is incomplete with ethanol and CO_2 as end-products. Increased fat content was also observed. This is supported by increased fat content following anoxia in Crucian carp (Blazka, 1958). This led to the suggestion that lipid metabolism serves as a sink for reducing equivalents in the slow spinning anaerobic TCA cycle (Van den

Thillart & Van Waarde, 1985) in two ways: (i) by chain elongation, (ii) by saturation of double bonds.

Nuclear Magnetic Resonance (^{31}P NMR) spectroscopy:

Recent advances in the application of phosphorus nuclear magnetic resonance (^{31}P NMR) to living muscle (Dawson, Gadian & Wilkie, 1977) have now made it possible to monitor all the substances quantitatively. This should be utilized in control and anoxic fish simultaneously directly or indirectly and to relate changes in them to concurrent changes in the mechanical performance of muscles. The substances most directly involved are ATP, ADP, Pi, H^+ and phosphocreatine. However, the disadvantage of this method is that results are obtained from a mixture of different systems (or muscle fibres) of varying energy capacities.

In conclusion, in studies of energy metabolism of animals in relation to environmental hypoxia, no single method is considered ideal, hence a combination of a variety of techniques is advisable, such as the calorimetry, respirometry, NMR, metabolite determination, enzyme analysis and electron microscopy.

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"Let me tell you the secret that has led me to my goal. My only strength lies in my ténacity".

Pasteur.