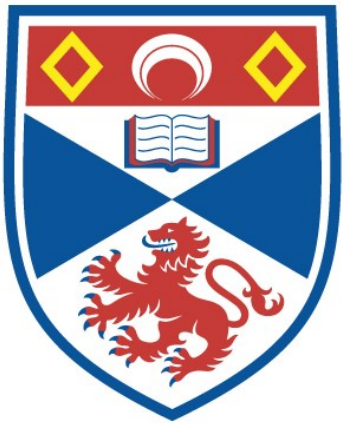
THE EFFECTS OF TEMPERATURE ACCLIMATION ON THE STRUCTURE AND FUNCTION OF MYOTOMAL MUSCLES FROM THE CARP (CYPRIMUS CARPIO, L.)

John Richard Fleming

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



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The effects of temperature acclimation on the structure and function of myotomal muscles from the carp (*Cyprinus carpio* L.)

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

by

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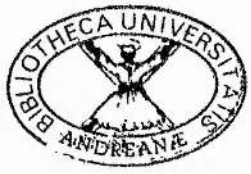

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DECLARATION

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. Leonard, University of St. Andrews, under the direction of Professor Ian A. Johnston.

CERTIFICATE

I hereby certify that John Fleming 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.

SUMMARY

Chapter One

Introduction

Research in the structure, biochemistry, electrophysiology and mechanical properties of fish muscle is reviewed. A variety of mechanisms underlying temperature acclimation and adaptation is described.

Chapter Two

The effects of temperature acclimation on muscle relaxation in the carp (Cyprinus carpio L.)

1. Common carp were acclimated to either 7 or 21 °C for a minimum of one month, and the twitch contraction kinetics of a nerve-muscle preparation investigated.
2. A significant compensation for the acute effects of a temperature change was achieved in twitch kinetics by acclimation to 8 °C. An acute drop in temperature from 20 to 8 °C in 20 °C-acclimated preparations led to approximately 2 and 3 fold increases in the half times for activation and relaxation.
3. At 8 °C, values for $t_{0.5a}$ and $t_{0.5r}$ were only around 50%

lower in 8 °C-acclimated preparations relative to those at 20 °C from 20 °C-acclimated fish, indicating a partial capacity adaptation in rates of both twitch activation and relaxation.

4. A quantitative ultrastructural study was performed on the sarcoplasmic reticulum (SR) of fast and slow myotomal muscles.

5. During processing for electron microscopy a greater degree of shrinkage was observed in fibres from warm- than cold-acclimated fish.

6. Significantly more SR was revealed using osmium ferricyanide stain than with Reynolds lead citrate alone.

7. The only difference observed in SR membranes following temperature acclimation was a higher surface density of SR and terminal cisternae in slow fibres from warm- than cold-acclimated fish.

8. The Ca^{2+}-ATPase activity of SR-enriched microsomes prepared from fast myotomal muscle was 60% higher at 8 °C in cold- than warm-acclimated fish. This suggests that changes in the kinetics and/or density of Ca^{2+} pumps contribute to the observed capacity adaptation in relaxation rate with temperature acclimation.

9. Other possible mechanisms for enhancing muscle relaxation rate in cold-acclimation are briefly discussed.

Chapter Three

The effects of temperature acclimation on the pCa-tension relationship of slow myotomal muscle fibres from the carp

1. Common carp (Cyprinus carpio L.) were acclimated to either 7 °C or 23 °C for at least one month.
2. The pCa-tension relationship of skinned slow fibres isolated from the anterior myotomes was investigated at pH 7.0, 7.6 and at 0 °C and 15 °C.
3. Sigmoid pCa-tension curves were obtained with 'n' (i.e. Hill coefficient) values between 2 and 3.
4. Temperature acclimation did not alter the sensitivity of the contractile apparatus to calcium under any of the conditions of pH or temperature investigated.
5. An increase in temperature from 0 °C to 15 °C at pH 7.6, but not at pH 7, shifted the pCa-tension relationship towards higher pCa values.
6. Lowering pH from pH 7.6 to pH 7.0 resulted in a rightward shift of the pCa-tension relationship at both 0 °C and 15 °C.
7. The results are discussed in the context of providing a mechanism to compensate for the effects of low temperature on the contraction and relaxation of muscle following cold-acclimation.
8. It was concluded that a shift in the pCa-tension relationship following cold-acclimation does not provide a

mechanism to explain a more rapid rate of relaxation in muscle fibres from cold- than warm-acclimated carp.

Chapter Four

The role of thyroid hormones in temperature acclimation

1. Common carp (Cyprinus carpio L.) were acclimated to either 8 °C or 22 °C for at least one month.
2. One group of cold-acclimated fish were made hypothyroid by treatment with methimazole (80mg/100ml 0.9% NaCl) 0.5µg/g body weight once every two days.
3. The level of T3 and T4 in the plasma of carp was determined using a radioimmunoassay technique.
4. Frozen sections of m. hyohyoideus muscles were cut and stained for the activity of succinic dehydrogenase and alkali-stable myosin ATPase to determine the percentage cross-sectional area of different fibre types.
5. m. hyohyoideus muscles from cold-acclimated fish had a higher proportion of slow oxidative fibres and fast oxidative glycolytic fibres than similar muscles from warm-acclimated carp.
6. The ultrastructure of slow myotomal muscle was investigated by electron microscopy. The volume fraction of mitochondria was higher in fibres from cold- than warm-acclimated fish ($P<0.01$). The volume fraction of

myofibrils decreased in the series cold-acclimated > warm-acclimated > cold-acclimated, hypothyroid ($P<0.01$). The mean cross-sectional area of individual myofibrils decreased in the series, warm-acclimated > cold-acclimated > cold-acclimated hypothyroid.

7. Hypothyroidism did not affect the total volume fraction of mitochondria in cold-acclimated fish. The volume fraction of mitochondria in the intermyofibrillar zone was higher in cold-acclimated euthyroid than hypothyroid fish ($P<0.001$).

8. Neither temperature acclimation nor hypothyroidism affected the volume or surface density of cristae within mitochondria.

9. The effects of temperature acclimation and hypothyroidism on the performance, composition and ultrastructure of muscle are compared and the involvement of thyroid hormones in temperature acclimation is discussed.

Chapter Five

General Discussion

The results presented in this report are discussed in the context of providing mechanisms that may compensate for the effects of low temperature on the rate of diffusion of small molecules through the cytosol.

CHAPTER 1

INTRODUCTION

The structure and function of fish skeletal muscle

In terrestrial locomotion the power required for motion increases linearly with increasing speed. In aquatic locomotion each increment in swimming speed requires an increase in power proportional to swimming velocity cubed (Webb, 1975). To meet this demand for power fish require a disproportionately larger mass of muscle for every additional increment in swimming speed. The bouyancy of muscle in water reduces the need for weight economy and allows fish to carry large masses of muscle specifically for fast swimming speeds. Depending on the species, muscle may constitute between 40 and 75% of total body mass of which 90% may be fast contracting, white fibres, used during burst swimming (Greer-Walker and Pull, 1975; Bone, 1978a). Unlike most other vertebrates where fibre number is fixed at birth, fibre number in fish continues to increase throughout life (Greer-Walker, 1970).

The orientation of muscle fibres in myotomes

In fish, red and white muscle fibres are arranged in myotomes. There are two myotomes per vertebrae (Nursall,

1959). Each myotome consists of muscle fibres that are inserted, via tendons, at both ends into sheaths of flexible connective tissue called myosepta or myocommata. The orientation of fibres within the myotome varies both along the body and with distance from the vertebral column. This complex orientation of fibres is thought to enable similar degrees of sarcomere shortening at different stages in the contraction cycle optimising the overlap between the thick and thin filaments at all depths within the myotome (Alexander, 1969).

In general, slow muscle fibres form a thin sheet lying just underneath the lateral line, running from head to tail parallel to the long axis of the fish (Greene and Greene, 1913; Johnston, Patterson, Ward and Goldspink, 1974; Johnston and Moon, 1980a, b; Johnston, Davidson and Goldspink, 1977). However in skipjack tuna (Katsuwonus pelmis), red muscle shows a more complex arrangement. In addition to forming a wedge along the lateral line, the red muscle also extends deep into the white muscle and is associated with a counter-current heat exchange portal system. These fibres differ markedly from more superficial red fibres by having ten times as many mitochondria (with more complex cristae) and a more highly developed capillary bed (Bone, 1978b).

Beneath the superficial myotomes of slow muscle the myotomes of fast muscle fibres are arranged in a complex W-shape to form a series of overlapping dorso-ventral cones

(Wainwright, 1983). Successive myotomes form a series of helices with their axis running parallel to the long axis of the body. The development of this W-shape is dependent on the evolutionary advancement of the fish, being best formed in teleosts and least in the chondricthyes. The cross-sectional area of the muscle mass decreases cranio-caudally (Alexander, 1969).

The myotomal muscles contract in waves which originate from the head region and run backwards to end at the tail fin. It is thought that the thicker head muscles provide the propulsive force to the tail fin while the thin tail muscles stiffen the caudal peduncle. The complex forces produced by this movement can be resolved into two components, caudo-cranial and ventro-dorsal. The former propels the fish forwards and the latter provides bouyancy (Videler, 1977; Wardle, 1985).

Types of muscle fibre

Originally muscle from the electric ray (Torpedo) was divided, on the basis of colour, into two classes, red and white (Lorenzini, 1678). High concentrations of myoglobin, (50-350 nmol mg^{-1} wet weight ; Giovane, Greco, Maresca and Tota, 1981; Kryvi, Flatmark and Totland, 1981), give red muscle its characteristic colour. Muscle fibres from some antarctic fishes in the family Channichthyidae, such as the benthic icefish (Chaenocephalus aceratus) are exceptional as

they lack haemoglobin and the myoglobin gene is only expressed to a limited degree (Feller and Hamoir, 1981). The muscles in these fish are highly aerobic and are either yellow or white depending on the concentration of cytochrome present (Rudd, 1954; Walesby, Nicol and Johnston, 1982).

By using biochemical, histochemical, mechanical, ultrastructural and physiological criteria red and white fibres have been sub-divided into a number of other fibre types. For example, Bone (1966) has distinguished four fibre types from superficial red to deep white in dogfish. Two fibre types have been reported in brook trout, Salvelinus fontinalis (Johnston and Moon, 1980b), and mackerel, Scomber scombrus (Bone, 1978b) while in other teleosts such as the carp, Cyprinus carpio L. three fibre types have been identified (Johnston et al., 1977).

Slow muscle

Recently, histochemical techniques and investigations into the mechanics of muscle contraction have shown that red muscle has low myofibrillar ATPase activity (Johnston et al., 1974) and a low V_{max}, typically 30-50% of that found for fast fibres (Johnston and Brill, 1984).

Following stimulation red fibres take considerably more time to reach peak twitch tension than white fibres. For example, red and white opercular fibres from the perch, Perca fluviatilis took 92ms and 14ms respectively to reach

peak twitch tension at 20°C (Akster et al., 1985). Muscle fibres with these characteristics are now termed slow muscle.

Maximum force production by fish slow fibres at 10°C is in the range 50-100 kN m^{-2} (Altringham and Johnston, 1982; Bone et al., 1986).

In addition, slow muscle has large lipid and glycogen stores (Johnston and Walesby, 1980; Johnston, 1982; Love, 1980), high activities of tricarboxylic acid cycle enzymes (Johnston et al., 1977) and an extensive capillary bed confering a high aerobic capacity. In teleost slow fibres the major lipid fuel for contraction is the oxidation of fatty acids (Bilinski, 1974) while ketone bodies are the main lipid fuel in elasmobranchs (Zamitt and Newsholme, 1979). In tuna, both fast and slow muscle fibres have a significant capacity for aerobic glucose metabolism (Guppy, Hubert and Hochachka, 1979).

The proportion of slow to fast muscle is generally higher in active pelagic species such as the Anchovy Engraulis encrasicholus, (29%) while demersal, deep-sea and those species which use their fins as a primary means of locomotion can have proportions of slow muscle as low as 0.5% (Greer-Walker and Pull, 1975).

Fast muscle

White fibres have a high myosin ATPase activity (Bàràny, 1967; Bone, 1978a; Love, 1980; Johnston, 1981a) enabling a fast maximal velocity of contraction (V_{max}) and a high power output. For skinned fast fibres from cod (Gadus morhua) and dogfish (Scyliorhinus canicula) V_{max} was around 1-3 muscle lengths s^{-1} ($L_o s^{-1}$) at 8°C (Altringham and Johnston, 1982).

Recent research showed that V_{max} is higher for intact fibres. For example fast myotomal fibres from Myoxocephalus scorpius have V_{max} values of around 4-6 $L_o s^{-1}$ at 3°C (Altringham and Johnston, 1988a). Maximum isometric tension (P_o) for skinned fast fibres at 12 °C is around 180 KN m^{-2} (Bone et al., 1986). Fibres that have these histochemical and mechanical properties are termed fast fibres. Generally, fast fibres have larger diameters than slow fibres. However there is a continuous distribution of fibre diameter depending upon the age of a fibre and the size of fish (Magnuson, 1973). Exceptionally large fast fibres occur in some antarctic species. These fibres may have diameters in excess of 500μm (Davison and Macdonald, 1985).

The capillary density is lower (3-110mm^{-2}) in fast than slow fibres but varies with the habit of the fish. Capillary density is generally higher in pelagic than sedentary, demersal species (Mosse, 1979). A well developed capillary bed is of less importance for fast muscle since fuel for contraction is mainly derived through anaerobic

pathways. Low aerobic capacity is further reflected by a very low but variable volume density of mitochondria (0.5-8%).

Ultrastructure of muscle fibres

Transverse sections of muscle fibres reveal small, polygonal myofibrils (1μm^2) that become more ribbon-like on the periphery of the myofibrillar mass (Nag, 1972).

Longitudinal sections show that each myofibril consists of two sets of interdigitating filaments which appear as bands. These bands are called A (anisotropic) and I (isotropic). The A-band is composed of "thick" myosin filaments which are about 1.7 µm long in fish (Akster et al., 1985). The I-band is made up of "thin", actin filaments which eminate from the Z-line and extend into the thick filaments. Thin filaments are usually around 2 µm long (e.g. sharks, Kryvi, 1977). The region between two Z-lines is called a sarcomere. This is the basic contractile unit of muscle and its length depends on the degree of overlap between the thick and thin filaments. In contrast to other vertebrates, fast and slow fibres from fish have a prominent M-line midway between the two Z-lines (Patterson and Goldspink, 1972; Johnston, 1981a). Thick filaments are joined together at the M-line.

Electrical stimulation of a muscle fibre depolarises the

sarcolemma. The transmission of this depolarisation throughout the myofibrillar mass, occurs *via* a three dimensional network of tubules called the T-system. Tubules are visible at the level of the Z-line and form diads and triads with the terminal cisternae of the sarcoplasmic reticulum (SR) (Nag, 1972).

In fish, fast and slow fibres have similar surface and volume densities of SR per unit myofibrillar volume. (Patterson and Goldspink, 1972; Johnston, 1980, 1980a). For example, volume densities of SR in slow fibres are 6% for the eel (*Anguilla anguilla*) (Egginton and Johnston, 1982) 2.7% for anchovy (*Engraulis encrasicolus* L.) (Johnston, 1982a) and 5.1% for trout (*Salmo gairdneri*) (Nag, 1972).

Comparison of the ultrastructure of slow and fast fibres

Transverse sections of slow muscle fibres reveal a volume density of myofibrils varying between 50 and 65%. This is around 30% lower than that found in fast fibres (Bone, 1978a; Johnston, 1981a). Longitudinal sections of fish slow and fast muscle reveal straight Z-lines and prominent M-lines. Z-lines are thicker in slow than fast fibres (Patterson and Goldspink, 1972; Bone, 1978a).

Slow muscle fibres contain a volume density of mitochondria in the range 15-46%, among the highest found for any striated muscle (Johnston, 1981a). In contrast, the

volume density of mitochondria in fast muscle is usually less than 8%.

Slow muscle from more advanced teleosts shows a good correlation between the volume density of mitochondria and the volume density of capillaries (Johnston and Bernard, 1982; Egginton and Johnston, 1983; Egginton, 1986). Capillary density for slow fibres varies between 118 mm^{-2} for Chimera monstrosa (Totland, Kryvi, Bone and Flood, 1981) to 6000 mm^{-2} for the anchovy (Johnston, 1982). Average cross-sectional area of capillaries in fish slow muscle is in the range 15-30 μm^2 (Johnston and Bernard, 1982). Notothenoid fishes from the antarctic are exceptional as average capillary cross-sectional area can be 2-3 times greater than for other teleosts (Fitch and Johnston, 1983).

Intermediate muscle fibres

Histochemical techniques have identified another fibre type in the myotome of a number of species of fish (Johnston et al., 1974; Patterson et al., 1974). In the mirror carp (Cyprinus carpio L.) these fibres comprise around 10% of the myotomal bulk making them slightly more numerous than red fibres (Davison et al., 1976). These fibres are called fast oxidative glycolytic (FOG) and possess an ATPase activity, aerobic capacity, lipid content, volume fraction of mitochondria and capillary density intermediate between slow

and fast muscle fibres (Johnston and Maitland, 1980; Johnston et al., 1977). FOG fibres have high concentrations of glycogen and utilise both oxidative and glycolytic pathways for energy. In addition, FOG fibres have unique myosin heavy chains (Scapolo and Rowlerson, 1987) and the light chains of fast fibres (Johnston et al., 1977).

Tonic fibres

Fibres with similar structural, electrophysiological, and contractile properties to vertebrate tonic fibres are uncommon in fish. However, in the dogfish, Scyliorhinus canicula a few superficial large diameter fibres form an interupted single layer external to the zone of red fibres. These fibres have a number of characteristics not shared by other types of muscle fibre. For example, although they have large diameters and contain few mitochondria, they have low activity for myofibrillar ATPase and are multiply innervated (Bone, Johnston, Pulsford and Ryan, 1986). Moreover they form only a small proportion (< 0.5%) of the total number of fibres in the myotome.

At the ultrastructural level, superficial fibres are distinguishable by the absence of M-lines in longitudinal section and by the poor alignment of myofilament bundles across the fibre compared to fast and slow fibres. The volume density of the SR is intermediate between that found

in fast and slow fibres (Bone et al., 1986).

Maximum Ca^{2+}-activated force for superficial fibres from the dogfish was 49 kN m^{-2} compared with 70 kN m^{-2} and 180 k Nm^{-2} for slow and fast fibres respectively. At 12°C unloaded contraction velocity was 0.58 $L_o s^{-1}$ compared with 1.53 $L_o s^{-1}$ for slow and 4.5 $L_o s^{-1}$ for fast fibres. Superficial fibres may have a role in maintaining body tone and/or attitude when the dogfish is resting on the seabed (Bone et al., 1986).

In addition, the myotomes of some freshwater fish such as the stickleback, Gasterosteus aculeatus L. contain a small number of fibres that have small diameters, long sarcomere lengths, thick Z-lines and stain weakly for succinic dehydrogenase activity compared with other fibre types (Kilarski and Kozlowska, 1983). These fibres may posses a number of characteristics in common with superficial fibres from the dogfish but more work is required to determine their physiological properties.

Muscle fibre recruitment

In less advanced fish groups (elasmobranchs, dipnoans, primitive teleosts) there is a complete anatomical and functional divison between fast and slow motor systems (Bone, 1966). For example in dogfish and Pacific herring, slow sustainable swimming speeds are supported by the activity of slow fibres alone. Burst swimming involves the

recruitment of fast glycolytic (FG) fibres which fatigue rapidly in 1-2 minutes (Bone, 1966; Bone et al., 1978b).

Fast muscle in advanced teleosts is unusual among vertebrates in having multiple innervation. Recruitment of these fibres occurs over a lower, broader range of swimming speeds compared to focally innervated fast fibres. For example in saithe (Johnston and Moon, 1980a), brook trout (Johnston and Moon, 1980b), rainbow trout (Hudson, 1973) and common carp (Johnston et al., 1977) the threshold for the recruitment of fast fibres is around 0.8-1.9 body lengths per second for fish 40 cm in length at 15 °C. In teleosts, electromyographical studies have shown the order of recruitment to be red >intermediate >white (Hudson, 1969; Johnston et al., 1977; Johnston and Moon, 1980a). For example in carp and coalfish, the e.m.g. activity recorded from fast muscle fibres during low sustainable swimming is similar to that found in slow muscle (Johnston and Moon, 1980a). Higher amplitude spike potentials are seen during high speed and burst swimming. The recruitment of fast fibres also depends on their depth within the myotome, with superficial fibres being recruited before deeper fibres (Johnston and Moon, 1980b). FG fibres are recruited at high burst swimming speeds and since they rely on anaerobic metabolism, fatigue rapidly limiting burst activity. Fatigue is probably due to the accumulation of lactic acid.

Other fast fibres (Fg) found in some antarctic species of fish such as Chaenocephalus acaeratus and Notothenia

rossii), are characterised by having low activities of both aerobic and anaerobic enzymes and are thought to rely on the the hydrolysis of phosphocreatine for their energy supply (Johnston and Harrison, 1985; Johnston and Dunn, 1987). The supply of phosphocreatine is very limited and they fatigue rapidly.

FOG fibres are less susceptible to fatigue than either FG or Fg fibres and are recruited over a range of swim speeds from low sustainable speeds to high burst speeds (Johnston and Moon, 1980a; Rome, Loughna and Goldspink, 1984).

Innervation

A distributed, multiple innervation pattern has been found for teleost red myotomal muscles in all taxonomic groups examined (Bone and Ono, 1982). However the pattern of innervation in white myotomal muscles is more variable.

All non-teleost fishes examined so far possess terminally innervated fast muscle fibres. Primitive teleosts (e.g. Elopomorpha and Clupeomorpha with the exception of denticeps which has distributed innervation) possess terminally innervated fast fibres while a trend towards distributed innervation occurs in the Neoteleostei. The primitive teleost Brevoortia tyrannus, a clupeid, is unusual since each fast fibre has dual innervation

(Gillerman cited by Ono, 1983). Stomiiformes possess a rather different distributed pattern which may be a transitional stage from terminal to a distributed pattern in teleosts (Bone and Ono, 1982).

The innervation of slow muscle

Slow fibres are multiply innervated with small diameter myelinated axons, forming "en-grappe" type terminals along each fibre (Bone, 1966, 1970). Tonic fibres are multiply innervated and are activated by junction potentials (JPs) only. Early reports found that slow myotomal muscles in teleosts were activated by JPs (Barets, 1961), while Stanfield (1972) suggested that homologous fibres in elasmobranchs could be capable of generating action potentials (APs). Recent studies showed that respiratory and pectoral muscles of teleosts are composed of slow twitch fibres (Granzier et al., 1982; Akster et al., 1985; Johnston, 1987). Slow myotomal muscle fibres from Myoxocephalus scorpius are activated by JPs and overshooting APs (Altringham and Johnston, 1988b).

The innervation of fast fibres

There are two distinctive types of innervation in fast

fibres. Elasmobranchs, dipnoans, agnathans, chondrosteans and holosteans possess focally innervated fast fibres, with en-grappe type terminations at one end of the fibre. For example, in dogfish, Scyliorhinus canicula L. each fibre is innervated by two large diameter axons that fuse to form one end-plate (Bone, 1964, 1972). These fast fibres show a propagating action potential that overshoots zero potential (Hagiwara and Takahashi, 1967).

On the other hand most teleost fast fibres are multi-terminally innervated. Each fibre receives numerous nerve terminals from axons that run down the myosepta and then spread across the surface of the myotome (Bone, 1964). In cod Gadus morhua, each fast fibre may receive up to 23 motor terminations, depending on where in the myotome the fibre is situated. Superficial fast fibres have a higher density of end plates than deeper fibres (Altringham and Johnston, 1981). The number of end plates per fast fibre is also related to the position of the fish taxonimically (Bone, 1964, 1970).

With the exception of the work done by Barets (1961) and Hudson (1969), most early studies on the electrophysiology of fish muscles were confined to fin and opercular muscles. Hudson (1969) studied the multiply innervated fast muscle of the teleost fish, Myoxocephalus scorpius and found that stimulation of spinal nerves produced either local junction potentials leading to a slow graded contraction or a propagated action potential resulting in a fast twitch.

These studies suggested that polyneuronally innervated fast fibres were capable of responding to stimulation with JPs/local contractions at slow swimming speeds, and APs/twitches at higher swimming speeds. In recent studies, fast fibres have responded to nerve stimulation with APs only (Westerfield et al., 1986; Altringham and Johnston, 1988a, b).

The resting membrane potential of fast fibres from either pectoral fin muscle or myotomal muscle are similar at between -80 mV and -85 mV (Westerfield et al., 1986; Altringham and Johnston, 1988a, b). This is determined by potassium conductance in all fish except elasmobranchs where the concentration of chloride ions inside and outside the cell determines the resting membrane potential (Luttgau, 1963; Hagiwara and Takahashi, 1967). APs that overshoot zero voltage have been reported as rare (Yamamoto, 1972) or frequent (Hagiwara and Takahashi, 1967; Hidaka and Toida, 1969). In contrast, APs were absent in fast and slow fibres from the dorsal fin of the pacific sand dab. Contraction of these fibres resulted from JPs only (Gilly and Aladjem, 1987).

The response of muscle fibres to nerve stimulation may be altered by the physiological condition of the fish. For example, in experiments on gravid female sculpin Myoxocephalus scorpius, Altringham and Johnston (1988a) found that resting membrane potential was low, APs were slow and undershooting, JPs were small (5-15mV) and the

recruitment of several axons was required to trigger an AP. The composition of the Ringer solution used during electrophysiological experiments may also alter the response of fibres to stimulation (Hudson, 1969).

Times to peak twitch tension for slow fibres are about 2-3 fold longer than for fast fibres. For example, at 20 °C, fast and slow muscle fibres from the m. hyohoideus muscle of Cyprinus carpio L. had times to peak twitch tension of 32 ms and 59 ms respectively (Granzier et al., 1983).

On stimulating different fibre types repetitively, fatigue occurs at different rates. Fast fibres fatigue more rapidly than slow fibres. Fast and slow fibres have similar fusion frequencies of around 20-60 Hz at temperatures between 0 and 17 °C (Takeuchi, 1959; Altringham and Johnston, 1987, 1988a, b).

The function of polyneuronal innervation

Altringham and Johnston (1988a) studied the electrophysiological properties of focally (Anguilla anguilla) and polyneuronally (Myoxocephalus scorpius) innervated fast muscle and concluded that polyneuronal innervation did not confer any unique electrophysiological properties compared with focal innervation. Nevertheless there seems to have been strong selection pressure for

polyneuronal innervation as several groups of fish seem to have evolved it independently (Bone and Ono, 1982). Reasons for this are unclear but one suggestion is that the sequential activation of spinal nerves, whose myotomal fields overlap, may lead to the multiple stimulation of muscle fibres and thus maximise power output over a range of swimming speeds (Altringham and Johnston, 1988b). Evidence to support this hypothesis was provided by Josephson (1985). Working on insect fight muscle he showed that multiple stimuli during an oscillatory work cycle increased power output above that generated from single twitch contractions.

Other advantages of polyneuronal innervation may include an increase in fine motor control. If individual muscle fibres can respond to both JPs and APs, a graded contractile response may be possible (Gilly and Aladjem, 1987; Altringham and Johnston, 1988b). On the other hand, polyneuronal innervation may ensure the synchronisation of muscle firing. Such a pattern of firing may be useful during an escape response (Hudson, 1969; Ono, 1983).

INTRODUCTION PART II

Temperature acclimation

The body temperature of most fishes varies directly with the temperature of their surroundings. For example, the mean excess body temperature of Tilapia mossambica was 0.27°C at an ambient temperature of 22 °C (Stevens and Fry, 1970). However certain groups of fishes such as tuna and lammnid sharks appear to be warm bodied. In such fish the temperature of eye, brain and muscle can be 2-12 °C higher than the ambient water temperature. Elevated body temperatures are achieved by use of a counter current heat exchange system (Stevens and Fry, 1971).

Despite the profound effect of temperature on the rate of biochemical reactions, groups of fishes have evolved to exploit a wide range of thermal niches. Environmental temperature can range from -1.9 ° C for antarctic fish to over 40 °C for fish inhabiting geothermal hot springs. In some species the annual variation in body temperature is less than 1 °C while in others temperature can change by 20-30 °C over a few hours.

Within the cell an intricate chemical network of feedback loops activate or inhibit key regulatory enzymes to co-ordinate responses to varied metabolic demands. Changes in metabolism may occur almost instantaneously, over several

days or even months. For example, glycolytic flux in skeletal muscle may increase up to 100-fold following the onset of burst activity (Newsholme and Start, 1973). On the other hand, acclimatory responses such as changes in mitochondrial density may take several weeks to complete (Precht, 1958; Johnston and Maitland, 1980).

Resistance adaptations modify upper and/ or lower lethal limits and therefore delineate the temperature range over which normal function is maintained. Capacity adaptations modify rate processes and compensate for the effects of temperature over the normal thermal range of the fish (Precht, 1958).

Oxygen consumption and cellular metabolism

An estimate of total energy production can be obtained from measurements of oxygen consumption provided there is no net contribution from anaerobic metabolism. Standard or basal metabolism is represented by the oxygen uptake of animals in the post-absorbtive state in the absence of activity. Measurements of steady state oxygen consumption at maximum sustainable swimming speeds (i.e. speeds that can be sustained for periods between 30 and 60 minutes (Brett, 1972)) are more useful as they provide an estimate of the aerobic capacity and aerobic scope for activity (Beamish, 1970; Duthie and Houlihan, 1982).

When a fish experiences an acute drop in temperature, oxygen consumption declines immediately due to the direct effects of temperature on the reactions involved. After a period of time the animal begins to acclimate and oxygen consumption rises to a value characteristic of the cold-acclimated state. When fishes are subjected to a persistent change in environmental temperature adaptive changes are initiated that allow the fish to maintain a similar metabolic rate across a range of body temperatures.

Adaption to a given situation usually consists of a genetic and a non-genetic component. Genetic adaptation is the basis for evolution. Individuals with favourable characteristics in a given environment, survive and reproduce better than less-fit individuals, and if these characteristics have a genetic basis they gradually become fixed within the population after several generations.

Non-genetic adaptations, where fish respond to seasonal fluctuations in environmental conditions during a lifetime are termed acclimatizations. The ability to acclimate is passed to the next generation rather than the adaptions themselves. Acclimatization involves changes in the phenotype, the limits of which are set by the genotype. These adaptations are induced by many interacting stimuli such as temperature, photoperiod, oxygen tension and the availability of food. This complexity makes experimental study rather difficult and it is usual to vary one factor only in an otherwise controlled laboratory environment.

Physiological responses to a well-defined stimulus under laboratory conditions are called acclimations (Prosser, 1973). There are often major differences between fish acclimated to low temperatures in the laboratory and under winter conditions in the field.

Several patterns of temperature acclimation have been observed. These responses are usually classified according to schemes devised either by Precht (1958) or Precht et al. (1973). Precht (1958) has categorised capacity adaptation into five subdivisions: 1) overcompensation 2) perfect compensation 3) partial compensation 4) no compensation and 5) inverse compensation (Fig. 1). These responses are based upon the comparison of the rate of any particular process immediately after transfer to a new temperature with the rate measured at the new temperature after a period of acclimation. Based on the oxygen consumption of whole animals type 2 and type 3 are the most common responses to thermal stress (Meuvis and Heuts, 1957, carp; Precht, 1951, eel; Roberts, 1964 sunfish). Most stenothermal species of fish have lost the ability to acclimate to changes in temperature and therefore show a type 4 response. Several examples of type 5 (inverse) acclimation have been reported particularly for amphibians and reptiles which do not maintain normal levels of activity in the cold (Holzman and McManus, 1973; Patterson and Davies, 1978). Inverse compensation may be of adaptive value as metabolic reserves may be conserved during periods of cold-induced torpor.

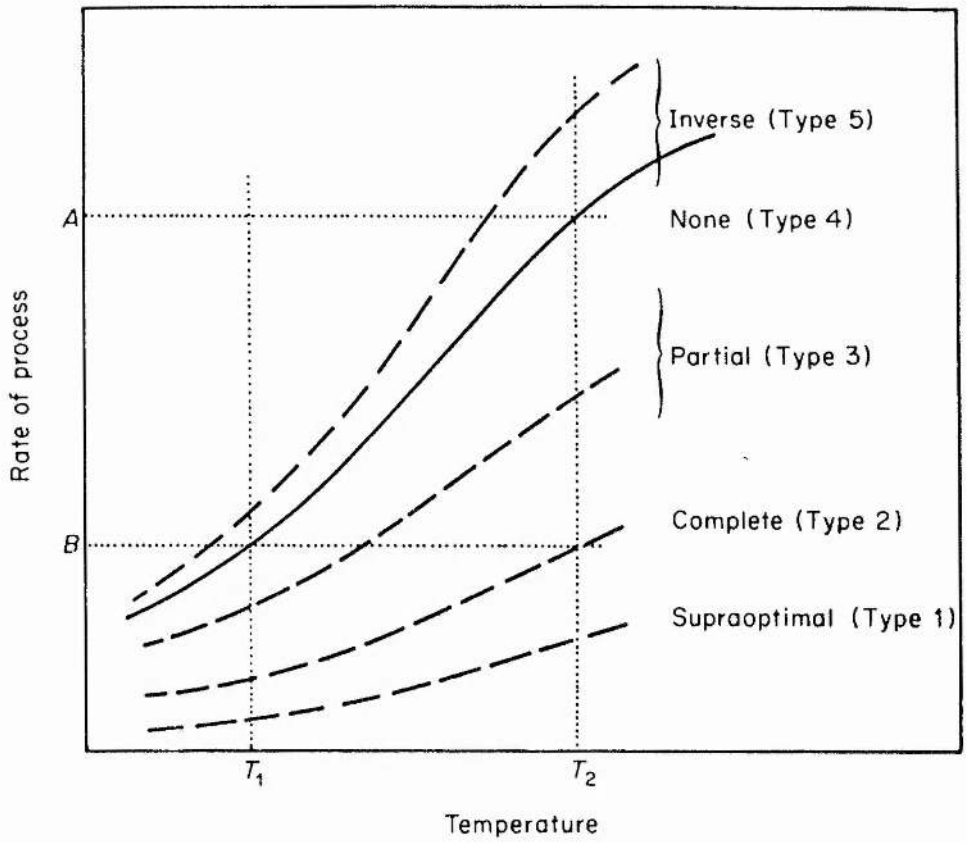


Fig 1. Schematic diagram to illustrate the terminology of Precht (1958) concerning the compensation of rate processes following transfer of fish from T_1 to T_2. The solid line represents the increase in rate upon transfer to the higher temperature. After a period of acclimation at T_2 the rate may become altered to give new rate-temperature curves which lie above or below the original depending upon the type of compensation. The broken lines represent the rate-temperature curves after acclimation for each of the categories. (After Precht, 1958).

Thus some fish species such as the eel (Walsh et al., 1983) and the striped bass (Crawshaw, 1984), cease feeding altogether and can remain buried in the mud for the whole winter. Inverse compensation is usually accompanied by a high temperature coefficient which permits a rapid transition from torpidity to activity in response to an increase in temperature. Starvation can cause digestive enzymes in the carp to exhibit either no or inverse compensation compared to partial compensation in well fed fish (Mews, 1957). In addition, starvation can also reduce the ability of fish to compensate for changes in the environment by reducing protein synthesis to a low level (Heap, Watt and Goldspink, 1986).

Acclimated largemouth bass, Micropterus salmoides become torpid below 7 °C, yet maintain a similar level of spontaneous activity between 7-30 °C (Lemons and Crawshaw, 1985). Feeding in this species is inhibited at higher temperatures (10 °C) than are required to supress locomotion, suggesting that not all physiological systems respond in the same manner to a given change in body temperature (Lemons and Crawshaw, 1985).

Due to the effect of increasing temperature on the solubility of oxygen, a fish exposed to an increase in temperature is faced with a higher oxygen demand in an environment that is relatively hypoxic. Homeostatic responses by the cardiovascular and respiratory systems can ensure a constant oxygen supply to tissues even though the

oxygen content within the environment may vary widely. In largemouth bass (Micropterus salmoides), aerobic scope increased between 15 and 30 °C but was lower at 35 than 30°C (Beamish, 1970). It was suggested that the temperature at which aerobic scope starts to decline marks the point at which energy demands for ventilation can no longer be met (Jones, 1971).

In some freshwater teleosts increased oxygen demand at higher temperatures is met by increasing the oxygen-carrying capacity of the blood. In carp (Houston and Dewilde, 1968), rainbow trout (Houston and Dewilde, 1969) and brook trout (Dewilde and Houston, 1967) there is a direct correlation between the composition of the blood (i.e. red cell number, packed cell volume and haemoglobin content) and acclimation temperature.

The relationship between adaption temperature, basal oxygen consumption and active oxygen consumption is complicated by interspecific variation in activity patterns since activity has a greater effect on total energy production than changes in acclimation temperature (Brett, 1964).

Adaptations in whole animal oxygen consumption are reflected by comparable compensations in tissue oxygen consumption following a period of acclimation to the cold. For example, Jones and Sidell (1982) found an overcompensation in the rate of oxygen consumption for skeletal muscle from striped bass (Morone saxatilis). The

rate of oxygen consumption in cold-acclimated striped bass was greater than that of warm-acclimated bass even when measured at their respective acclimation temperatures. However not all tissues reflect the acclimation pattern of the whole animal. For example brain tissue from rainbow trout showed complete compensation of oxygen consumption while gill tissue showed no compensation following temperature acclimation (Evans, Purdie and Hickman, 1962).

Spontaneous activity and locomotory performance

Physiological compensation in fish permits similar levels of locomotory performance and activity over a range of body temperatures. A higher rate of spontaneous locomotor activity was reported for 6 °C- than for 18 °C-acclimated Atlantic salmon when measured at an intermediate temperature (Peterson and Anderson, 1969). Many fish show optimal locomotory performance at the temperature to which they have been acclimated (Fry, 1967). For example, green sunfish (Roots and Prosser, 1962) and juvenile coho salmon (Griffiths and Alderdice, 1972) exhibited a positive correlation between acclimation temperature and maximum swimming speed.

Recent reports have suggested mechanisms to explain the maintenance of swimming speed at low temperatures. Electromyography in carp has shown that mechanical power

output of oxidative fibres is reduced at low temperature and thus glycolytic fibres must be employed at lower swim speeds resulting in fatigue (Loughna, Rome and Goldspink, 1983). Following acclimation to 8 °C, 15 °C and 26 °C carp were swum at 10 °C and 20 °C. At 10 °C, the swimming speed at which white muscle was initially recruited was significantly higher in fish acclimated to low temperatures. Thus temperature acclimation of muscle allows fish to maintain higher sustainable swimming speeds at low temperatures (Loughna et al., 1983; Rome et al., 1984, 1985).

The effects of temperature acclimation on the mechanical properties of muscle fibres

Acclimatory mechanisms which may allow the maintenance of sustained activity at low temperature include increases in the relative proportion of aerobic to anaerobic fibre types in cold-acclimated fish (goldfish, Johnston and Lucking, 1978; Striped bass, Jones and Sidell, 1982). In addition, Johnston, Sidell and Driedzic (1985) found that when measured at 7 °C, contraction velocity was about 2-fold higher and maximal force was about 70% higher in slow fibres from 7 °C- compared with 23 °C-acclimated carp. Furthermore, pectoral fin adductor muscles from cold-acclimated goldfish showed a higher rate of tension generation and relaxation at low temperature compared to

similar muscles from warm-acclimated fish (Heap, Watt and Goldspink, 1987). Penney and Goldspink (1980) reported a higher surface density of the SR in cold- than warm-acclimated goldfish. This could promote more rapid relaxation in the cold by reducing the diffusion distance of calcium out of the contractile apparatus.

The molecular mechanisms underlying changes in contractile properties following temperature acclimation are unknown. It is likely that both qualitative and quantitative changes in myofibrillar proteins are involved. Actomyosins that have been stripped of their tropomyosin-troponin complex have similar actomyosin ATPase activities at all temperatures regardless of the acclimation temperature of the fish from which they were prepared (Johnston, 1979). Higher activities of myofibrillar ATPase have been reported for cold- than warm-acclimated fish for a number of eurythermal species (Johnston, Davison and Goldspink, 1975; Sidell, 1980; Penney and Goldspink, 1981; Heap, Watt and Goldspink, 1985). For example, the activity of myofibrillar ATPase from fast muscle of 1 °C-acclimated goldfish was 2.8-fold higher than for 26 °C-acclimated fish when assayed at a temperature of 1°C (Johnston, Davidson and Goldspink, 1975).

Altringham and Johnston (1985a) determined the activity of myofibrillar ATPase in skinned muscle fibres from carp under force generating conditions by measuring ADP production using high performance liquid chromatography.

When measured at 7 °C, ATP turnover per myosin head was 50% lower and the amount of power (force.time) obtained per ATP hydrolysed was 50% higher in fibres from cold- than warm-acclimated fish.

Molecular mechanisms that underlie metabolic compensation to temperature acclimation

Biochemical studies of fish tissues showed that the maximal activities of some enzymes involved in pathways of aerobic energy metabolism increased following cold-acclimation (Sidell, 1980; Shaklee et al., 1977; Johnston, Sidell and Driedzic, 1985; Jones and Sidell, 1982; Johnston and Wokoma, 1986) while those enzymes involved in glycolysis (eg lactate dehydrogenase) found in the cytoplasmic compartment showed no compensation (Shaklee et al., 1977; Sidell, 1980; Johnston et al., 1985). This may result from an increase in the volume density of mitochondria in muscles from cold- compared to warm-acclimated fish (Johnston and Maitland, 1980) and by higher activities of aerobic enzymes in mitochondria from cold- than warm-acclimated fish. Wodtke (1981) found similar amounts of cytochrome c oxidase per mg mitochondrial protein and similar K_m values for cytochrome c from warm- and cold-acclimated carp. However the molar activities of cytochrome c intercalated in 10 °C-acclimated mitochondrial membrane were higher than for cytochrome c in

mitochondrial membrane from 30 °C-acclimated fish. Wodtke (1981) concluded that during thermal acclimation the molar activity of cytochrome c was controlled by the fluidity of the mitochondrial membrane (Wodtke, 1980).

Adaptive value of adjusting enzyme concentration

A wide range of environmental stimuli can elicit changes in the concentration of cellular proteins. Temperature is one of the most important and has profound effects on the level and type of metabolism in fish. For example cold-acclimation results in an increase in the concentration of both cytochrome oxidase (Wilson, 1973) and cytochrome c (Sidell, 1977, 1980) in the skeletal muscle of fish.

At least two mechanisms are available for changing the concentration of active enzymes within the cell: (1) readjustment of the rate of synthesis and breakdown of the enzyme and (2) enzymatic interconversion between existing pools of active and inactive forms, frequently by reversible phosphorylation of the enzyme. Responses to thermal acclimation are often accompanied by changes in the level of protein synthesis and in the activity of enzymes involved in protein synthesis such as aminoacyltransferase (Haselkorn and Rothman-Denes, 1973).

The exposure of green sunfish to lower temperature resulted in a rapid decrease in the rate of synthesis of

skeletal muscle cytochrome c, but the rate constant for the degradation of this protein was reduced even more. Consequently the concentration of cytochrome c increased during cold- and decreased during warm-acclimation. The differential changes in the rates of synthesis and degradation enable the organism to achieve an increase in the concentration of an enzyme while actually reducing the rate of enzyme synthesis. Therefore a change in the concentration of cytochrome c results from a passive effect of temperature on the rate constants for synthesis and degradation.

Enzyme-modulator interactions

The lowered environmental/ cell temperature encountered during cold-acclimation decreases the rate of diffusion of oxygen and metabolites within the cell because of both the direct effect of temperature on molecular kinetic energy and the resultant increase in the viscosity of the cytosol (Sidell and Hazel, 1987; Egginton and Sidell, 1989). As a result the diffusion coefficient for oxygen in frog skeletal muscle decreases more than 40% between 23 °C and 0 °C (Mahler, 1978). Muscle from cold-acclimated fish has a higher oxygen capacitance and higher concentrations of cellular lipid than muscle from warm-acclimated fish (Sidell, 1980). Egginton and Sidell (1989) suggested that

these factors may compensate for the effects of low temperature on the diffusion of oxygen through the cytosol. In contrast, Sidell (1980) reported an increase in the concentration of cellular myoglobin in slow muscle of the goldfish following cold-acclimation. This result is unexpected since increased concentrations of myoglobin in muscle from warm-acclimated fish would alleviate a reduction in the dissolved oxygen content in the cell at higher temperatures. More recently, Johnston et al. (1985) found higher concentrations of myoglobin in slow muscle from warm- than cold-acclimated common carp.

The effect of temperature on interactions between enzymes and various modulating metabolites can modify catalytic function. For example, lactate dehydrogenase isozymes present in fast skeletal muscle of the mudsucker exibit substrate (pyruvate) inhibition at temperatures below 25 °C. Therefore at high ambient temperatures, when oxygen availability is low and activity high, pyruvate is efficiently converted to lactate. Conversely at low temperatures, when the availability of oxygen is increased, pyruvate is channelled through the Krebs cycle (Somero, 1973). If the effectiveness of two metabolic pathways to compete for a common substrate varies with temperature, a change in temperature can shift the balance of that competition. As a result one pathway may exhibit a Q_{10} of approximately one whereas the Q_{10} of the other pathway may be considerably greater than one. In this way temperature

affects both the flow of carbon through a pathway and the contribution of various pathways to overall metabolism.

Measurement of the activities of enzymes *in vitro* following temperature acclimation may provide information on the relative capacities of metabolic pathways *in vivo* (Hazel and Prosser, 1974). Newsholme and Paul (1983) suggested that the activities of non-equilibrium enzymes can be used to provide a quantitative index of the flux through a pathway. For example, the activity of hexokinase and carnitine palmitoyltransferase may provide quantitative measures of glucose and fatty acid oxidation, respectively (Newsholme and Paul, 1983). Kacser and Burns (1979) suggested that the activities of equilibrium enzymes may also exert a degree of control over total flux.

The rate of carbon flow through glycolytic pathways is increased in the cold (Hochachka, 1967; Ekberg, 1962). For example glucose-6-phosphate, a branch point metabolite, can either be stored as glycogen or catabolised *via* the hexose monophosphate shunt and glycolysis. Furthermore Hochachka (1968) showed that higher temperature increased the rate of glycogen synthesis and the activity of the hexose monophosphate shunt relative to the rate of glycolysis.

The catalytic activity and regulatory ability of enzymes is dependent on proper ligand (substrate, cofactor and allosteric modulator) binding to enzyme molecules. Temperature changes may affect enzyme-metabolite interactions in at least two ways 1) bonds that stabilise

enzyme-metabolite complexes are weak and can be directly influenced by the thermal energy of the environment; complex formation may be hindered or facilitated by the resultant change in enzyme structure. 2) temperature may alter the affinity of an enzyme for its substrate due to changes in the geometry of the binding site. Substrate binding necessitates that the enzyme is able to form highly stable complexes with its substrate in addition to having a binding capacity such that the enzyme does not become saturated with substrate. Reserve capacity for activity confers greater scope for metabolic regulation, allows the system to accomodate increased demands, perhaps resulting from an increase in locomotory activity and also reduces the build up of highly reactive intermediates (Atkinson, 1969).

At sub-saturating concentrations of substrate, velocities are dependent on the ability of an enzyme to bind substrate (approximated by apparent K_m values) and therefore the effect of temperature on K_m can control the rate of reaction at physiological substrate concentrations. Enzymes with temperature-dependent K_m's are found in both poilkilotherms and homeotherms but the sensitivity of K_m to a change in temperature may be higher in poilkilotherms, permitting a greater degree of positive thermal modulation. For example, the K_m for isocitrate of pig heart isocitrate dehydrogenase increased 2.4 fold from 20-50 °C whereas the K_m for this enzyme in trout liver increased 3.4-fold from 1-20 °C (Moon and Hochachka, 1972).

Acid base balance

Low molecular weight constituents of the cell may have a major influence on the *in situ* structural and functional properties of enzyme and contractile proteins. Hydrogen ions are the most important of these constituents and may affect the catalytic ability of enzymes as well as effecting the assembly and function of other proteins.

The normal blood pH of fish varies inversely with temperature with a change in pH/°C value of around -0.018. The change in pH/°C for neutral water between 3 °C and 37 °C is -0.017, therefore the difference between the pH of blood and that of neutral water remains constant suggesting that fish regulate a constant degree of alkalinity in relation to the neutral point of water (Reeves, 1977).

The regulation of pH in this way is thought to conserve the fractional disociation state of imidazole groups over a range of temperature. This in turn maintains key properties of proteins that are dependent on the degree of protonation or deprotonation of histidine residues (Somero, 1981).

However changes in the intracellular pH of muscle with temperature are often less than required by the alphastat hypothesis (Walsh and Moon, 1982; Mutungi and Johnston, 1988). Consequently enzymes that exhibit pH dependencies within the normal physiological range of pH are subject to pH regulation. For example, the small increase in intracellular pH accompanying cold-acclimation was

sufficient to cause a marked increase in the activity of phosphofructokinase in goldfish and may explain the enhanced glycolytic flux characteristic of many cold-acclimated fish (Freed, 1971). Many other enzymes may be activated by changes in pH due to temperature acclimation (reviewed by Hazel and Prosser, 1974).

In fast skeletal muscle of fishes the amount of buffering capacity increases with rising potential for anaerobic glycolysis (Castellini and Somero, 1981). Adaptive differences in the buffering capacity of white muscle would seem to ensure optimal pH for muscle function despite the build up of acidic end-products during burst swimming. A variety of substances are used to absorb protons generated during metabolism including the bicarbonate system and phosphates. However the family of histidine-containing buffers including free histidine, dipeptides (e.g. carnosine) and protein-bound histidyl residues are the most important cell buffers in tissues having a high buffering capacity (Burton, 1978).

CHAPTER 2

The effects of temperature acclimation on muscle relaxation in the carp, a mechanical, biochemical and ultrastructural study

Introduction

Muscle contractility is modified by temperature acclimation in some freshwater fish (family Cyprinidae) (Johnston and Dunn, 1987). For example, the Mg^{2+}-Ca^{2+} myofibrillar ATPase activity of myofibrils at 1 °C was found to be 3-times higher in goldfish acclimated to 1 °C than 26 °C (Johnston, Davidson and Goldspink, 1975). The myofibrillar ATPase from cold-acclimated goldfish was also much more susceptible to heat denaturation. Changes in ATPase activity with temperature acclimation require several weeks for completion and probably involve the synthesis of new proteins (Heap, Watt and Goldspink, 1985). Studies with skinned muscle fibres from common carp have shown that cold acclimation increases force production, contraction velocity (Johnston, Sidell and Driedzic, 1985), and the economy of isometric contraction (Altringham and Johnston, 1985a). Recently, Heap *et al.*, (1987) studied preparations from the pectoral fin muscles of the goldfish, acclimated to either 10 °C or 28 °C for two months. At 5 °C the half times for both

activation and relaxation of twitch tension were two times faster in cold- than warm-acclimated fish.

What mechanisms underlie capacity adaptation in relaxation rate ? Penney and Goldspink (1980) reported that cold-acclimation in goldfish resulted in a remodelling of sarcoplasmic reticulum (SR) and myofibrils. They suggested that the surface density of SR, and the reduction in myofibril diameter found in fast fibres from cold- relative to warm-acclimated fish, may provide at least part of the explanation.

However, preliminary experiments indicated that during preparation for electron microscopy muscle fibres from warm-acclimated fish exhibited a greater degree of shrinkage. Furthermore, an osmium ferricyanide staining method (Peachey, Waugh and Sommer, 1974) revealed significantly more sarcoplasmic reticulum membrane than the Reynolds (1963) lead citrate stain used by Penney and Goldspink (1980). These results prompted a re-investigation of their original findings using the common carp. The present study was also extended to include slow muscle. In order to obtain further insights into the effects of temperature on muscle relaxation rates, the Ca^{2+}-ATPase activity of isolated SR vesicles and the twitch contraction kinetics of a myotomal nerve-muscle preparation were also investigated.

Materials and methods

Fish

Common carp (*Cyprinus carpio* L.) were obtained from Humberside Fisheries, Driffield, England. For electron microscopy experiments, 14 fish (142g ± 5g body weight, 17.4cm ± 0.5cm standard length, mean ± s.e.) were maintained at either 7 °C (± 0.5 °C) or 23 °C (± 1 °C) under an 8h light : 16h dark photoperiod in filtered recirculated freshwater for at least one month. A second population was used for the mechanical and biochemical studies, maintained under a similar regime at 8 and 20 °C (10 fish, 691g ±36g body weight, 31.0cm ± 0.6cm standard length, mean ± s.e.). All fish were fed commercial fish pellets *ad lib*. Fish were killed by a blow to the head followed by transection of the spinal cord.

Mechanics

Twitch contraction parameters of fast fibres were recorded from a nerve-muscle preparation of abdominal myotomes. The abdominal cavity of the fish was opened along the ventral midline, and a rectangular block of tissue removed. This included the 4 myotomes either side of the insertion of the pectoral fin, and the full depth of the abdominal cavity. No slow fibres are located in this region. The preparation

was pinned out in a temperature controlled bath, and immersed in ringer solution of the following composition: NaCl, 132 mM; Na pyruvate, 10 mM; KCl, 2.6 mM; Na HCO_3, 18.5 mM; Na H_2PO_4 , 3.2mM; $MgCl_2$, 1mM; $CaCl_2$, 2.7 mM; pH 7.4 at 8 °C (Hudson, 1969). The central spinal nerve was drawn into a suction electrode, and single stimuli yeilding a maximum force response were given through the spinal nerve. Muscle force was recorded by resting a strain gauge (AE801, AME, Horten, Norway) on the surface of the myotome immediately rostral to the spinal nerve. This method gave highly reproducible measurements of contraction kinetics in the intact myotome, independent of the positioning of the strain gauge. However, the system could not be calibrated for force generation by the muscle, since although the strain gauge was arranged to record in a plane parallel to the longitudinal axis of the fibres, some of the fibres in adjacent myotomes also contracted. Nevertheless, it was usual to see an increase in the amplitude of the force records with increasing temperature. Experiments were performed at both 8 °C and 20 °C over at least 2 temperature change cycles, and the times taken to reach half-maximal tension ($t_{o.5a}$) and half-relaxation ($t_{o.5r}$) were measured. The results were fully reversible, and the data from each preparation were averaged.

Electron microscopy

Tissue preparation

Small bundles of fast and slow muscle fibres were dissected from dorsal myotomes at a point adjacent to the insertion of the first dorsal fin ray. Tissue was processed by one of two methods. In the first, fibre bundles were pinned to cork strips at their *in vivo* resting length and fixed overnight in 2% gluteraldehyde, 0.15M phosphate buffer at pH 7.2 (at 4°C). The outer fibre layers were removed, washed in buffer and post-fixed in 1% osmium tetraoxide, 0.15M phosphate buffer, pH 7.2 for 1 hour. Fibre bundles were rinsed in distilled water, stained in uranyl acetate and lead citrate, dehydrated in a series of alcohols up to absolute, cleared in propylene oxide and embedded in araldite CY 212 resin.

A second method was used in an attempt to improve the contrast and resolution of SR membranes. Fibre bundles were fixed in several changes of 2% gluteraldehyde, 0.15M cacodylate buffer, 10 mM $CaCl_2$, pH 7.2 (at 4 °C) overnight. Following further dissection the tissue was washed in cacodylate buffer and post-fixed for 2h in 2% osmium tetraoxide, 0.15M cacodylate buffer, 10mM $CaCl_2$, 0.8% potassium ferricyanide, at pH 7.2 (Peachey, *et al.*, 1974). The tissue was washed in fresh cacodylate buffer and

distilled water and stained en bloc for 30 min in a saturated aqueous solution of uranyl acetate to improve contrast. Fibres were subsequently dehydrated in acetone and vacuum embedded in araldite resin. This method of processing was found to reduce lipid leaching.

Ultra-thin sections (50-100 nm) were cut from blocks prepared by both methods on a RIECHERT OMU2 ultramicrotome and mounted on 150 mesh copper grids coated with 3% pyroxyline in amyl nitrate. Sections were double stained with aqueous, saturated uranyl acetate (30 min) and Reynolds (1963) lead citrate (5 min) and examined with a PHILIPS 301 electron microscope at 60KV.

Five fish were taken from each acclimation group and five blocks made of each fibre type per fish, giving a total of 50 blocks per acclimation temperature (25 fast, 25 slow). Plates were analysed from all the fish in each group.

Quantitative analysis

Volume and surface densities of SR were estimated from plates (19-25000x) of longitudinal sections (enlarged three times using a photographic enlarger) and quantified using digital planimetry (Walesby and Johnston, 1980). This may over estimate absolute values of SR (Akster, Granzier and ter Keurs, 1985), but a systematic error will not affect

comparisons between acclimation groups or fibre types. Maximum measurement error was $\pm$3%, estimated by repeated tracing of the same SR profiles (5 times 5 plates). The volume and surface density of total SR and terminal cisternae were calculated per unit myofibrillar volume using stereology software (Imagan system, Kompira, Salsburgh, Scotland). The mean length of 10 A-filaments was measured for each plate. Surface density of SR was negatively correlated with measured A-band length (least squares regression, $P<0.01$). This suggests that A-band length provides a good measure of shrinkage. A-band length (Table 1) was therefore used to correct for shrinkage between acclimation groups, assuming a reference A-band length of 1.5 µm (Page and Huxley, 1963; ter Keurs, Luff and Luff, 1984). No assumptions were made about differential shrinkage between organelles, due to the lack of satisfactory methods.

Sarcoplasmic reticulum calcium ATPase activity

Samples of fast myotomal muscle (20g) were minced with scissors and homogenised for three X 20s in three volumes of 0.3M sucrose, 10 mM imidazole, pH 7.3 (at 0 °C) using a polytron blender (Kinematica Gmbh, Switzerland) at 3/4 full speed. Myofibrils and fragments of muscle fibre were

removed by centrifugation at 2,500g for 30 min. The supernatant was centrifuged at 15,000g for 30 min to pellet the mitochondria. Microsomes enriched with SR were obtained by centrifuging at 95,000g for 1.5h. Protein concentration was measured using a modification of the Lowry method (Maddy and Spooner, 1970).

Ca^{2+}-ATPase activity was measured in an assay medium containing: 40 mM Tris-HCl, 25 mM KCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.3 mM EGTA (free Ca^{2+} concentration approx. 50μM), 0.2-0.4mg ml^{-1} SR protein, pH 7.2 at 10 °C (McArdle and Johnston, 1981). Assay temperature was controlled to within ± 0.1 °C in a water bath. The reaction was initiated by the addition of ATP to the pre-incubated medium (final concentration 2 mM), and terminated by the addition of an equal volume of 10% trichloroacetic acid. Denatured protein was removed by centrifugation and inorganic phosphate (Pi) measured in the supernatant by the method of Rockstein and Herron (1951).

Statistical analysis

A normal probability plot showed that the volume and surface density data was normally distributed, permitting the use of an unpaired students t-test (Two-tailed), to compare acclimation groups and fibre types. Log transformation of the data, or the use of a non parametric Mann-Whitney U-test

did not alter the level of statistical significance. $P<0.05$ was used as the level of statistical significance.

Results

Mechanics

Representative twitches are shown in Fig. 1. Acclimation to 8 °C led to significantly lower values of both $t_{o.5a}$ and $t_{o.5r}$ relative to 20 °C-acclimated fish at both temperatures (Fig. 2). An acute drop in temperature from 20 to 8 °C in 20°C-acclimated preparations led to approximately 2 and 3 fold increases in $t_{o.5a}$ and $t_{o.5r}$ respectively. A partial but very major compensation for the acute effects of temperature was achieved in both $t_{o.5a}$ and $t_{o.5r}$ by acclimation to 8 °C. At 8 °C, values were only around 50% greater in 8 °C-acclimated preparations relative to those at 20 °C from 20 °C animals.

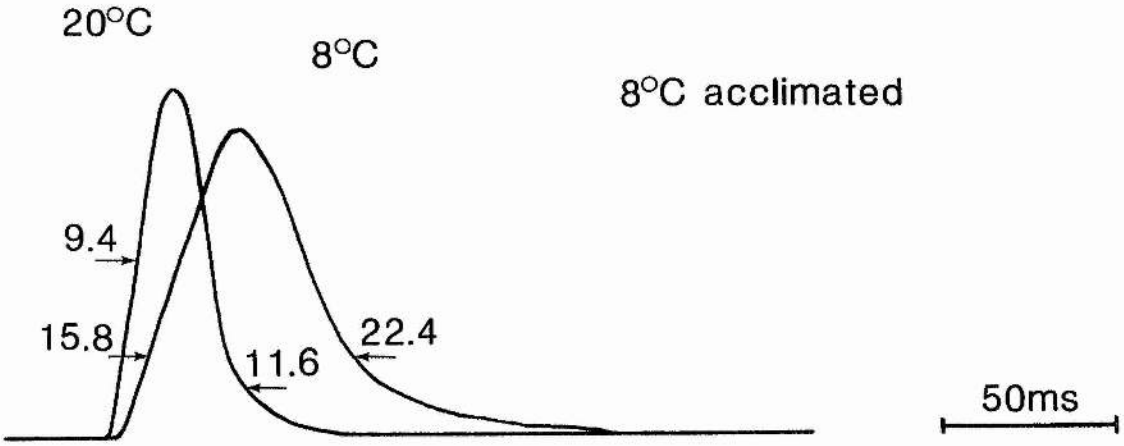
Electron microscopy

Methodology

In order to determine whether the degree of shrinkage during processing for electron microscopy varied between

Fig. 1

Isometric twitch contraction times recorded from a nerve-fast muscle preparation of carp (Cyprinus carpio L.) abdominal myotomes. The force is uncalibrated for reasons given in the methods. Fish were acclimated for several months to 8 °C (upper records) and 20 °C (lower records), and experiments performed at both temperatures for each population. The figures are the times for half activation ($t_{o.5a}$) and relaxation ($t_{o.5r}$) in ms.


20°C
8°C
8°C acclimated
9.4
15.8
11.6
22.4
50ms

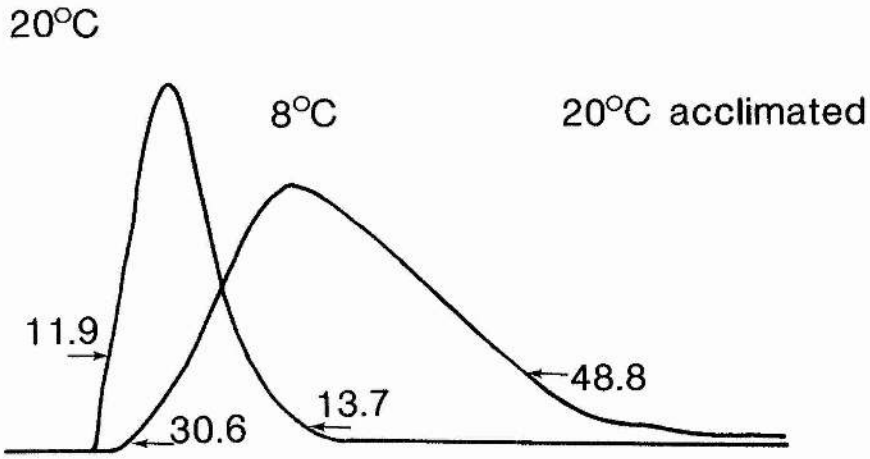
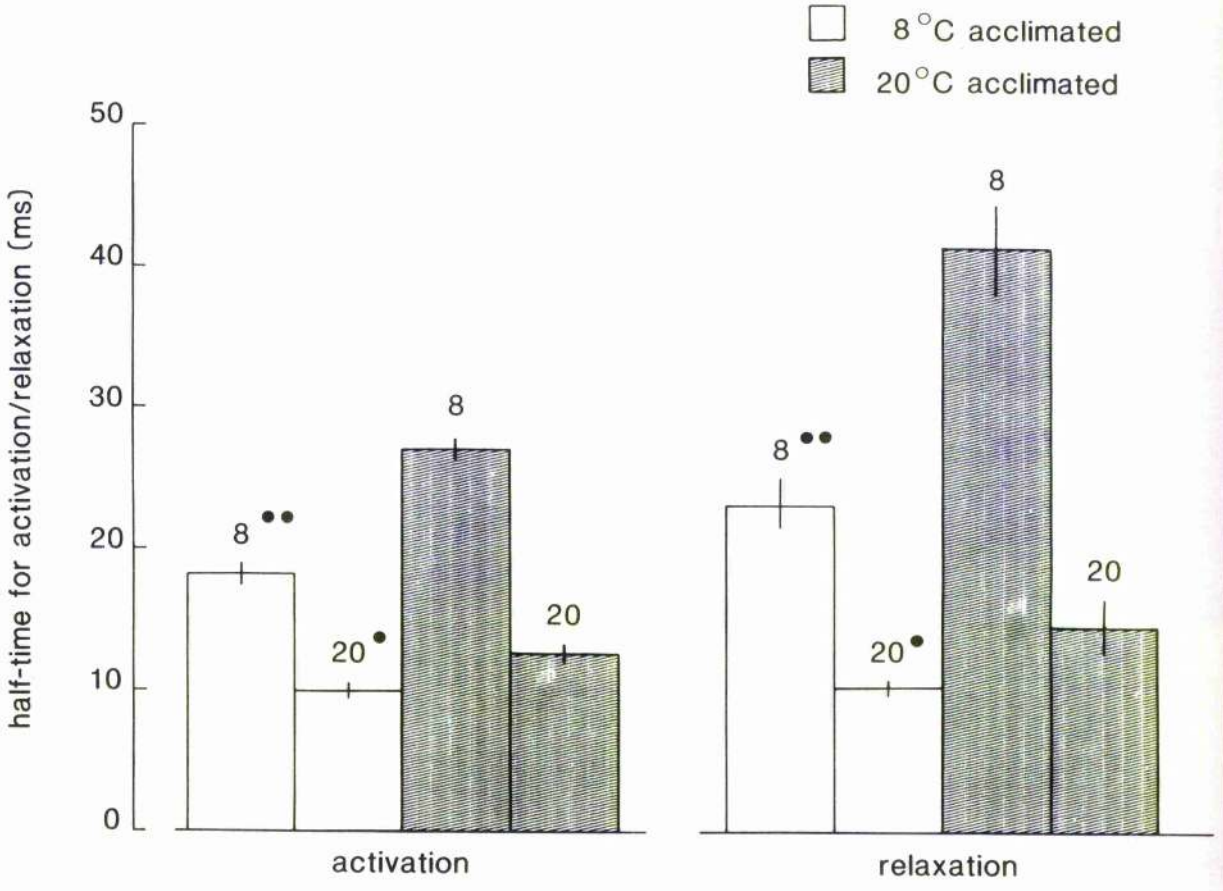

20°C
8°C
20°C acclimated
11.9
30.6
13.7
48.8

Fig. 2.

Histogram of half-times of activation ($t_{0.5a}$) and relaxation ($t_{0.5r}$) of fast fibre twitches in a nerve-muscle preparation, at 2 temperatures. The figures above each bar denote experimental temperatures of 8 °C and 20 °C. Data are means $\pm$ s.e. (n = 6 fish). Filled circles denote degrees of statistical significance (• $P<0.05$; ••, $P<0.01$) in the differences between acclimation groups at 8 or 20 °C.


8 °C acclimated
20 °C acclimated
half-time for activation/relaxation (ms)
50
40
30
20
10
0
8
20
8
20
activation
8
20
8
20
relaxation

acclimatory groups, A-band length was measured in fast muscle fibres. A-band length was 1.4 ± 0.02 μm (mean ± s.e.) in 7 °C- and 1.27 ± 0.04 μm in 23 °C-acclimated fish, indicating a greater degree of shrinkage in in warm-acclimated fish ($P<0.01$). The volume density of SR was significantly higher in 7 °C- than in 23 °C-acclimated fish ($P<0.001$, Table 1). However, there was no significant difference in the surface density of SR between acclimation groups either before or after correction for shrinkage.

Osmium ferricyanide was found to improve the definition, resolution and contrast of all cell membranes (Fig. 3). This resulted in an increased amount of SR visualised relative to fibres stained with uranyl acetate and lead citrate alone (Table 2). For example, the total volume density of SR from the fast muscle of warm-acclimated fish was nearly two-fold higher for fibres stained with osmium ferricyanide ($P<0.001$). The osmium ferricyanide staining technique was therefore used for all subsequent quantitative studies (all results after Table 1).

Comparison of fast and slow muscle fibres

The ultrastructure of the T-system and SR in carp fast and slow fibres is typical of that described for teleosts (Kilarski, 1967; Franzini-Armstrong, 1973; Akster *et al.*,

Table 1.

The effects of shrinkage during processing for electron microscopy, on the ultrastructure of sarcoplasmic reticulum from fast fibres of 7 ^{o}C- and 23 ^{o}C-acclimated common carp.

	correction for shrinkage	23 ^{o}C-acclimated (36)	7 ^{o}C-acclimated (24)
A-band length (μm)	not applicable	1.27±0.04	1.40±0.02**
Volume Density SR (%)	no	2.8±0.2	3.9±0.2***
Surface density SR (μm^{-1})	no	3.1±0.1	2.9±0.2^{ns}
	yes	2.6±0.1	2.7±0.2^{ns}

Sections were stained with uranyl acetate and lead citrate. The number of plates analysed is shown in brackets.

** = statistically significantly different at P<0.01;

*** = P<0.001; ns = not significant at P<0.05 level. All values are mean ± s.e.

Table 2.

Comparison of methods for staining the sarcoplasmic reticulum of fast fibres from carp skeletal muscle. Note that the results were corrected for shrinkage during tissue processing.

	23 °C-acclimated fish		7 °C-acclimated fish	
	uranyl acetate/ lead citrate (36)	osmium ferricyanide (39)	uranyl acetate/ lead citrate (24)	osmium ferricyanide (27)
Volume density SR (%)	2.8±0.2	5.4±0.3***	3.9±0.2	5.1±0.3**
Surface density SR (μm^{-1})	2.6±0.1	3.7±0.2***	2.7±0.2	3.3±0.2*
Volume density terminal cisternae (%)	1.4±0.1	4.4±0.3***	2.8±0.3	3.9±0.4*
Surface density terminal cisternae (μm^{-1})	1.0±0.07	2.3±0.2***	1.1±0.08	1.9±0.1**

The number of plates analysed from 7 fish in each acclimation group is shown in brackets. * = statistically significantly different at $P<0.05$; ** = $P<0.01$; *** = $P<0.001$. All values are mean ± s.e.

1985). Small tubules constituting the T-system run between facing terminal cisternae at the level of the Z-line forming diads and triads ("Z-type" organisation of Porter and Palade, 1957) (Fig. 4). The surface and volume densities of SR and terminal cisternae were significantly higher in fast than slow muscle fibres (Figs. 4, 5, Table 3, $P<0.05$). The presence of distinct M-lines and H-bands in fast and slow muscles provides evidence that super-contraction did not occur even though 10 mM $CaCl_2$ was added to the fixative as a component of the osmium ferricyanide stain. Glycogen deposits were more numerous in warm- than cold-acclimated fish in both fibre types, occuring as small granules and droplets distributed throughout the myofibrils and intermyofibrillar space (Fig. 4).

Effects of acclimation temperature

No significant differences between the two acclimation groups were noted in the surface or volume densities of the SR or terminal cisternae of fast fibres (Table 3).

The surface density of SR in slow muscle was higher in fibres from warm-acclimated fish (Table 3). This indicates that the SR vesicles become more spherical with acclimation to low temperature. The surface densities of terminal cisternae and SR in slow fibres were 32% and 15% higher

Fig. 3.

Electron micrographs of fast muscle fibers stained with (left) lead citrate (right) osmium ferricyanide (see text for details). Note the greatly improved staining of the cell membranes with osmium ferricyanide. Abbreviations: A, A-band; I, I-band; M, M-line; SR, sarcoplasmic reticulum; T, T-tubules; TC, terminal cisternae; Z, Z-line. Scale bar = 1μm.

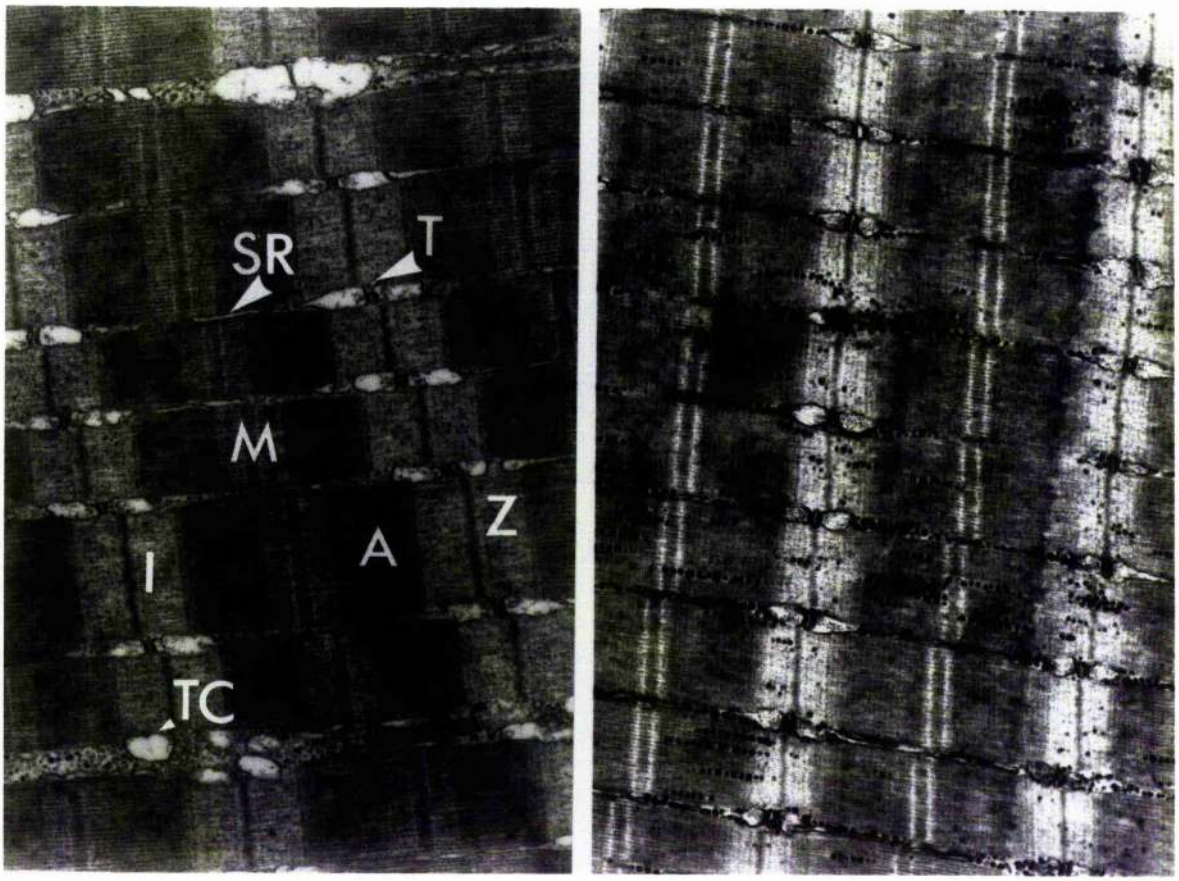

SR
T
M
Z
A
I
TC

Fig. 4.

Electron micrographs (longitudinal sections) of fast muscle fibers stained with osmium ferricyanide from (left) warm-acclimated and (right) cold-acclimated carp. Scale bar = 1μm.

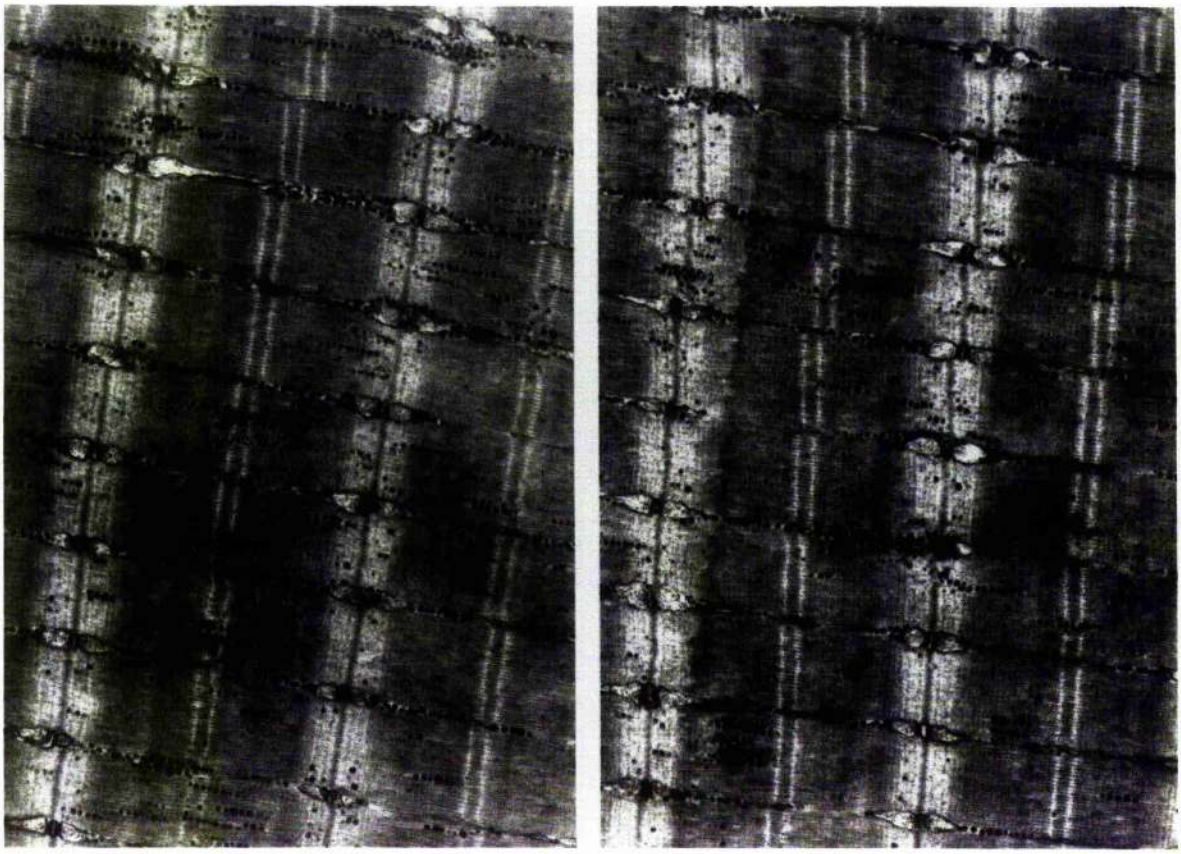


Fig. 5.

Electron micrographs (longitudinal sections) of slow muscle fibers stained with osmium ferricyanide from (left) warm-acclimated and (right) cold-acclimated carp. Abbreviation: Mt, mitochondria. Scale bar = 1μm.

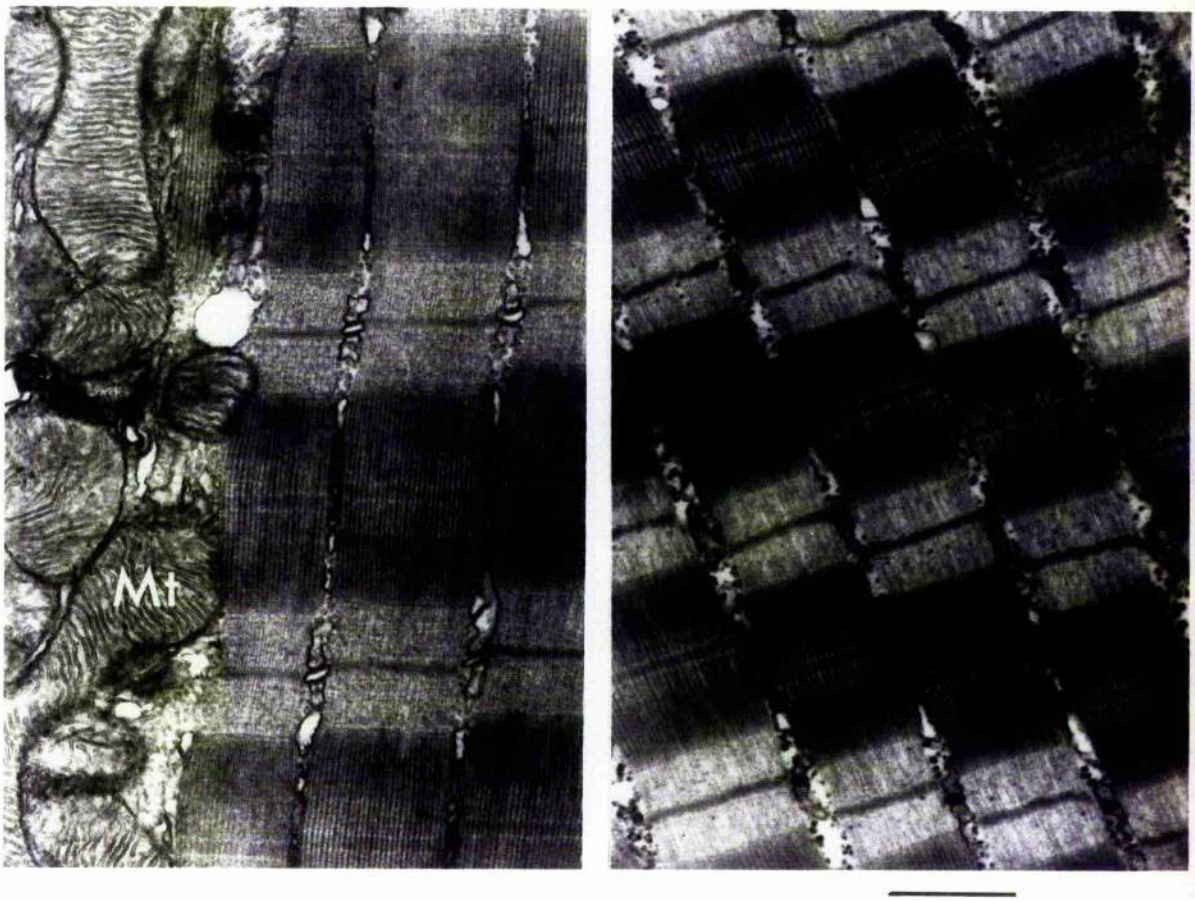

Mt

Table 3.
The effect of acclimation temperature on the sarcoplasmic reticulum of fast and slow muscle fibres of the carp.

Fibre type	Parameter	component	23 ^{o}C-acclimated fish	7 ^{o}C-acclimated fish
Fast	Volume density (%)	total S.R.	5.4±0.3	5.1±0.3^{ns}
		terminal cisternae	4.4±0.3	3.9±0.4^{ns}
	Surface density (μm^{-1})	total S.R.	3.7±0.2	3.3±0.2^{ns}
		terminal cisternae	2.2±0.1	1.9±0.1^{ns}
Slow	Volume density (%)	total S.R.	3.7±0.1	4.0±0.2^{ns}
		terminal cisternae	1.7±0.1	1.8±0.1^{ns}
	Surface density (μm^{-1})	total S.R.	3.0±0.2	2.6±0.1*
		terminal cisternae	1.2±0.1	0.9±0.05*

Number of micrographs analysed for each group: fast and slow fibres from 23^{o}C-acclimated fish, 39 and 26 respectively, fast and slow fibres from 7 ^{o}C-acclimated fish, 36 and 29 respectively. Preparations were stained with Osmium ferricyanide, and the results corrected for shrinkage during tissue preparation. * = statistically significantly different at $P<0.05$; ns = not significant at $P<0.05$ level. All values are mean ± s.e.

Table 4.

Ca^{2+}-activated ATPase activity of fast muscle fibres of 8 °C- and 20 °C-acclimated carp.

	Ca^{2+}-activated ATPase activity	
Assay temperature	8 °C-acclimated fish	20 °C-acclimated fish
8 °C	93±8	58±5**
20 °C	206±27	161±11

Values represent mean ± s.e. for preparations from 5 fish. Activity is in nmoles ATP cleaved mg S.R. protein^{-1} min^{-1}. ** = statistically significant at $P<0.01$ level.

respectively in warm- relative to cold-acclimated fish ($P<0.05$).

Sarcoplasmic reticulum calcium ATPase activity

Microsomal Ca^{2+}-ATPase activity was 60% higher at 8 °C for 8 °C- than 20 °C-acclimated carp ($P<0.01$) (Table 4). ATPase activities for the two acclimation groups measured at 20 °C were more similar (Table 4).

Discussion

Mechanics

The acute effects of decreasing temperature on twitch contraction time were largely compensated for by acclimation to low temperature. Changes in twitch contraction time will greatly influence locomotor performance, since it is a major determinant of tailbeat frequency and swimming speed (Wardle, 1975). A similar acclimatory response has been reported for the pectoral fin muscles of the goldfish by Heap et al., (1987), who found that at 10 °C the half-relaxation time was two times faster in 10 °C- relative to 28 °C-acclimated fish. Several mechanisms could be

responsible for the increased relaxation rate following cold-acclimation, the present study investigated two of these: possible changes in the surface density and Ca^{2+}-ATPase activity of the SR.

Effects of shrinkage and staining technique on quantifying the SR

One of the problems associated with quantitative ultrastructural studies is the effect of shrinkage during tissue preparation. The extent of shrinkage is dependent on a large number of variables including buffer type, osmolarity, fixative and the method of dehydration. In addition, some plastics used for embedding (e.g. methacrylate) also cause shrinkage (see Lee, 1984 for review). The degree of shrinkage is positively correlated with tissue water content, which is known to change with acclimation (Heap, Watt and Goldspink, 1986). In the present study the A-band was found to shrink to a greater extent in warm- than cold-acclimated fish (Table 1). Sub-cellular organelles have also been shown to shrink to different extents during processing e.g. osmium fixation causes muscle I and A filaments to shrink by 10% and 6% respectively, while gluteraldehyde causes shrinkage of 2-3% in I-band length but has little effect on A-band length (Page and Huxley, 1963). Acetone is preferable to ethanol

as a dehydrating agent since it causes less shortening of muscle filaments (Page and Huxley, 1963). The estimate of shrinkage based on A-band length provides a general measure of shrinkage between acclimation groups, and is not necessarily representative of all organelles. No methods are available for estimating differential shrinkage between organelles.

Another factor causing systematic error in the quantification of SR membranes is the staining technique employed (Table 2). Using uranyl acetate/lead citrate stain, surface and volume densities of SR in fast muscles of the carp are similar to those reported for other species. For example, Nag (1972) reported the surface density of total SR in fast fibres from axial muscle in Salmo gairdneri to be 2.4 μm^{-1}. Similarly the total SR surface density observed in oblique sections of fast fibres of the axial muscles of Carrasius auratus was 2.5 μm^{-1} (Penney and Goldspink, 1980). Comparable values have been found for Perca fluviatilis L. (Akster et al., 1985), Tinca tinca (Johnston and Bernard, 1982) and Anguilla anguilla (Egginton and Johnston, 1982). The addition of osmium ferricyanide to the uranyl acetate/lead citrate stain revealed significantly higher volume and surface densities of SR particularly in the case of terminal cisternae (Table 2). This may be due to changes in the specific surface of the SR between acclimation groups (Table 2). The mode of action of osmium ferricyanide is unclear but it is thought that OsO_4, in the

presence of ferricyanide, forms labile, cyano-bridged osmium complexes in which osmium is arrested in its VIII, VII or VI oxidation states rather than its IV state. White, Mazurkeiwicz and Barrnet (1979) proposed that metal-containing proteins present within the SR membrane (e.g. calsequestrin), chelate these complexes resulting in the deposition of large amounts of electron dense material making the SR easier to visualise.

Comparison of SR in fast and slow muscles

Half times for relaxation during isometric twitches are generally 3 times shorter for teleost fast than slow fibres (Johnston, 1987; Altringham and Johnston, 1988b). A number of mechanisms have been proposed to explain the more rapid relaxation rate observed in fast muscles. Terminal cisternae are particularly important in excitation-contraction coupling and are generally more developed in fast-contracting muscle types (Winegrad, 1970; Franzini-Armstrong, 1973; and Table 3). The total surface area of the SR membrane is responsible for calcium uptake during relaxation (Winegrad, 1970) and therefore one might expect a more extensive SR in fast muscles as found in the present study. A number of reports have correlated faster rates of calcium uptake (and therefore relaxation) in fast fibres with a higher surface density of SR compared to slow

fibres (e.g. Fawcett and Revel, 1961; Franzini-Armstrong, 1973) but the relationship between the surface density of the SR and relaxation rate is not universal. For example, differences in half-relaxation time found between pink and red fibres from the hyohyoideus muscle from carp were not associated with a corresponding change in SR surface area (Akster, 1985; see also Akster et al., 1985). McArdle and Johnston (1981) found a higher calcium uptake rate in fast relative to slow muscle in the rainbow trout. Similar results have been reported for mammalian fast and slow fibres (Sreter, 1969; Briggs, Poland and Solaro, 1977; Kim, Witzmann and Fitts, 1981).

Other mechanisms that may also augment faster rates of relaxation include a higher rate of calcium uptake resulting from changes in the density and/or kinetics of the calcium ATPase (McArdle and Johnston, 1981; Zubrzycka-Gaarn, Korezak, Osinska and Sarzala, 1982).

Effects of temperature acclimation

The only effect of acclimation on muscle ultrastructure found in the present study was an increase in the suface density of the SR and terminal cisternae in slow fibres from warm-acclimated fish (Table 3). Adaption of relaxation rate in the cold has not therefore been achieved by producing more SR. These results are at variance with the study on

another cyprinid, the goldfish (Carassius auratus L.) reported by Penney and Goldspink (1980), which found that the surface density of the SR increased in fast fibres of cold-acclimated fish. However, Penney and Goldspink did not correct for the effects of shrinkage, and used a uranyl acetate/lead citrate stain which reveals less SR (see Table 2).

There are a number of alternative mechanisms which could account for the higher rates of muscle relaxation in cold-acclimated fish. These include a faster sequestration of calcium by the SR, resulting from an increase in the number of calcium pumps or changes in their kinetics (Bennet, McGill and Warren, 1980; Martonosi, 1963; but see Cossins, Christiansen and Prosser, 1978). Penney and Goldspink (1980) found no difference in calcium uptake by isolated SR fragments from fast muscles of warm- and cold-acclimated goldfish. However, only temperatures above 13 °C were investigated, where differences in relaxation rate with temperature acclimation are small (Heap et al., 1987). In the present study the Ca^{2+}-ATPase activity of SR-enriched microsomes at 8 °C was found to be 60% higher for 8 °C- than 20 °C-acclimated carp (Table 4), suggesting that changes in the number or properties of calcium pumps are indeed important.

Other mechanisms which might enhance relaxation rate, and are worthy of further study, include changes in the concentration of soluble Ca^{2+} binding proteins

(parvalbumins) and modification of the pCa-force relationship of muscle.

A significant increase in the rate of twitch activation was observed in fibres from cold- relative to warm-acclimated fish. Although this cannot be explained by gross changes in the surface density of the terminal cisternae, it may be due to more subtle changes at the terminal cisternae/t-tubule junction (Block, Imagawa, Campbell and Franzini-Armstrong, 1988).

CHAPTER 3

The effects of temperature acclimation on the pCa-tension relationship of slow fibres from carp

Introduction

In vertebrate skeletal muscle the interaction between myosin and actin is regulated by the binding of Ca^{2+} to low-affinity sites on the troponin c (TnC) subunit of troponin (Tn) (Potter and Gergely, 1975). Recent research suggests that additional factors, including the rate of cross-bridge cycling and the interaction between adjacent and remote tropomyosin molecules on the same thin filament may modify the apparent affinity of TnC for calcium ions (Bremel and Weber, 1972; Fuchs, 1977; Brandt et al., 1980, 1982; Grabarek et al., 1983; Moss et al., 1985).

Cooperative binding of calcium within the thin filament was suggested as a possible explanation for the steepness of the pCa-tension curve which otherwise could not be modelled on the basis of two low-affinity Ca^{2+}-binding sites (Brandt et al., 1980, 1982).

The relationship between isometric tension development and free calcium ions is much steeper in fast-twitch skeletal (Moss et al., 1986) than in either cardiac (Kerrick et al., 1980) or slow-twitch skeletal muscles (Kerrick et

al., 1976). Such a difference in steepness, in terms of current models of contractile activation (Hill, 1983), might also be explained on the basis of a greater degree of cooperativity of Ca^{2+}binding in fast-twitch muscle fibres.

Both end-to-end interactions between adjacent tropomyosin molecules (Wegner, 1979) and the interaction of regions of the thin filament adjacent to already activated troponin-tropomyosin complex are thought to enhance the sensitivity of TnC to Ca^{2+}.

In addition, the structure of TnC is likely to affect the sensitivity of the contractile apparatus to Ca^{2+}; Fast-twitch skeletal TnC has two low affinity Ca^{2+}-binding sites (Potter and Gergely, 1975), whereas cardiac TnC (Van Eerd and Takahashi, 1975; Leavis and Kraft, 1978) and slow-twitch skeletal TnC have only one (Wilkinson, 1980). The partial substitution of cardiac TnC for fast TnC in skinned psoas (fast) fibres isolated from the rabbit showed that the pCa-tension relationship is affected by the type of TnC present, the number of low affinity Ca^{2+}-binding sites located on TnC and their relative affinities for Ca^{2+} (Moss et al., 1986).

The troponin-tropomyosin complex may also affect the activity of actomyosin ATPase. Arrhenius plots of natural actomyosin ATPase from the rabbit showed a break in the slope at around 15-18 °C (Bendall, 1969). In contrast, linear Arrhenius plots were obtained for desensitised actomyosin from which the regulatory proteins had been

removed (Hartshorne et al., 1972; Fuchs et al., 1975). This provides evidence that the calcium regulatory proteins can modify the kinetic behaviour of actomyosin ATPase. Recent research showed that alternative isoforms of regulatory proteins were produced by carp following temperature acclimation (Crockford and Johnston, 1989). These proteins may modify the pCa-tension relationship of muscle fibres and provide a mechanism to offset the effects of low temperature on the rates of activation and/ or relaxation.

The aim of the present study was to investigate the effect of thermal acclimation on the pCa-tension relationship of slow myotomal muscle fibres from carp under conditions of pH and temperature likely to exist following acclimation to low and high temperatures.

Materials and methods

Fish

Common carp (Cyprinus carpio L.) (420g± 80g bodyweight, 30 cm ± 3 cm standard length mean ± S.D., 27 fish) were obtained from Humberside Fisheries (Driffield, England) and maintained at either 7 °C ± 0.5 °C or 23 °C ± 1 °C under a 12 hr light: 12 hr dark photoperiod in filtered, recirculated freshwater for at least one month. Both groups were fed ad lib on commercial fish pellets. Fish were killed by a blow to the head followed by transection of the

spinal cord.

Apparatus

The apparatus used to measure isometric tension consisted of a stainless steel hook attached to the silicon beam of an A.M.E. 801 strain gauge (A.M.E., Horton, Norway), average sensitivity 0.38 mN V^{-1}. This was ridgidly held in a perspex adaptor and mounted on a one way micromanipulator allowing the length of fibres to be adjusted. The output from the strain gauge was recorded using a chart recorder.

The solutions were contained in three adjoining 2ml perspex baths mounted on a two-way micromanipulator. This allowed solution changes to be effected in less than 3s. Solution temperature was controlled to within 0.3 °C by circulating a 20% methanol/ water mixture around the baths using a Julabo F20 circulator (Julabo Labortechnik GMBH, West Germany).

Solutions

All the chemicals used in solution formulation were Sigma grade reagents (Sigma, Poole, England). Variation in EGTA purity and the hydration of ATP was taken into account in order to maintain solution composition. Three sets of

solutions were used, relaxing solution, skinning solution and activating solution. Relaxing solution was buffered by PIPES (piperazine-N, N'-bis 2-ethanosulphonic acid) buffer which has a pKa of 6.8 at 25 °C and is useful as a buffer over the pH range 6.1 to 7.6. Relaxing solution (0 °C, pH 7.6) contained (mM l^{-1}) EGTA 10, ATP 7, CP 7.5, PIPES 15, $CaCl_2$ 0, $MgCl_2$ 8, KCl 92. These values were varied slightly to maintain similar free ion concentrations under different conditions of temperature, pH and pCa. Skinning solution contained 1% polyoxethylene-20-cetyl ether (Brij 58), a non-ionic detergent, in relaxing solution.

Maximal activating solution was made by the addition of $CaCl_2$ to relaxing solution. This solution was mixed with relaxing solution in various proportions to provide a series of activating solutions having intermediate pCa values (Ashley and Moisescu, 1977). Calcium levels in activating solutions were regulated by the use of a CaEGTA/ EGTA buffer system.

The various ionic species present in the solutions take part in complex equilibria, therefore free ion concentrations were calculated from apparent dissociation constants using an iterative computer programme (Nicol, 1985). This incorporated corrections for pH and temperature. Using this programme, the composition of activating solutions was adjusted to give a range of pCa's from 4.5 to 8, a pMg of 2.5, a pMgATP of 2.25 and an ionic strength of 180 mM.

A high molecular weight polymer polyvinylpyrolidone, (PVP, 5% wt/vol) was added to all solutions to maintain filament lattice spacing (Matsubara et al., 1984).

Isolation of fibres

Small strips of slow muscle were dissected from myotomes 1-4 (counting from the head) and small fibre bundles isolated on a cooled stage in a drop of relaxing solution under silicon oil (BDH MS550). Bundles consisting of between two and five slow fibres were transfered to the apparatus and wrapped around the two stainless steel hooks using fine jeweller's forceps. The bundle of fibres was then secured using plexi-glass acetone glue (Altringham and Johnston, 1982).

Methods

Fibres were skinned for 15 min. Sarcomere length was measured using laser diffraction and set to 2.3 μm. Sarcomere length was calculated using Bragg's equation for diffraction:

$$s = \frac{\lambda}{\sin\theta}$$

Where s=sarcomere length, λ =wavelength of the laser (0.6328 μm) and θ= angle subtended by the zero and first order

diffraction patterns. The length and diameter of each bundle of fibres was measured *in situ*. Fibre bundles were transfered to relaxing solution for 5 min prior to being maximally activated at the selected temperature and pH. Following activation the fibre bundles were allowed to relax completely in relaxing solution before re-activation using an activating solution with a different pCa. Sub-maximal tensions were related to maximum isometric tension to give relative tension, (P/P_o), values. Sub-maximal contractions were interspersed with contractions in maximal activating solution to correct for the reduction in maximal tension that followed repeated activation. Fibre bundles were discarded if maximum tension fell below 85% of the maximal isometric tension of the first activation.

The transformation of pCa-tension curves

Data was linearized according to the Hill equation:-

$$\log \frac{(P)}{P_o - P} = n \log (Ca^{2+}) + h$$

where n is the Hill coefficient (i.e. gradient of the above relation) and h is a constant for each fibre. The pCa values required to give half-maximal tensions (i.e. log $(P/(P_o-P) = 0)$ were used as a measure of calcium sensitivity (Kerrick *et al.*, 1976; Moore *et al.*, 1983). Students t-test was used to compare the sensitivity of fibre bundles taken

from warm- and cold-acclimated fish to Ca^{2+} under different conditions of pH and temperature. Data from warm- and cold-acclimated fish were pooled to investigate the effects of temperature and pH on the pCa-tension relationship.

Curves were fitted to experimental data (Fig. 2) using

$$\frac{P}{P_o} = (C/K)^n/(1+ (C/K)^n)$$

where n is the Hill coefficient, K is derived from the calcium concentration needed to generate half-maximum tension (i.e. -log K), C is the Ca^{2+} concentration and P/P_o is the tension relative to maximum. All parameters were obtained by linearising data for each fibre using the Hill equation (Brandt, Cox, Kawai and Robinson, 1982).

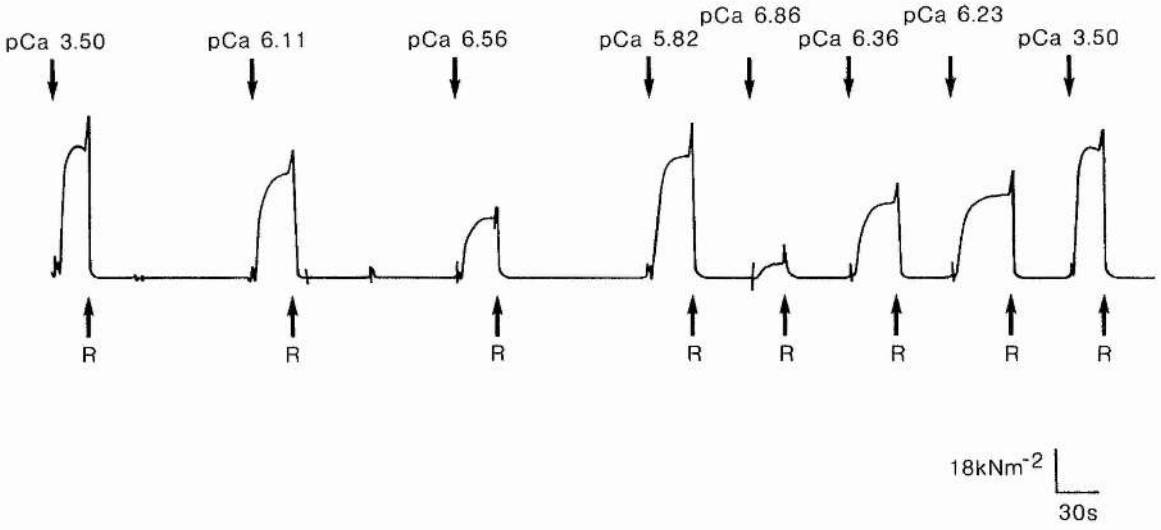
Results

A force-time trace demonstrating the experimental protocol used is shown in Fig 1. pCa-tension curves derived from such traces were sigmoid in shape with a threshold for tension generation around pCa 7.2 with maximum tension reached at pCa 5.3. Therefore tension was generated over 2 pCa units, equivalent to an 80-fold increase in the concentration of free Ca^{2+} from 0.063 µM to 5 µM. These values were dependent upon experimental pH and temperature (Fig. 2 A, B).

Half-maximal pCa values (Tables 1, 2 and 3) and 'n'

Fig.1

Isometric force records from a slow fibre at 0 °C and pH 7.0 illustrating the experimental protocol used. The transfer of the fibre into either activating solution or relaxing solution is indicated by pCa values and R respectively.


pCa 3.50
pCa 6.11
pCa 6.56
pCa 5.82
pCa 6.86
pCa 6.36
pCa 6.23
pCa 3.50
R
R
R
R
R
R
R
R
18kNm-2
30s

values were calculated from the Hill equation. 'n' values are thought to indicate the degree of cooperativity between Ca-binding sites on the troponin c molecule and cannot theoretically exceed the total number of binding sites available. 'n' values between 2 and 3 (Tables 4, 5), indicate a high level of cooperative binding of Ca^{2+} to troponin c.

The effects of acclimation on the pCa-tension curve of carp slow muscle

Temperature acclimation did not modify the sensitivity (as indicated by the pCa required to ellicit half-maximum tension) of slow fibres to Ca^{2+} under any of the conditions of pH or temperature investigated in this study (Table 1).

The effects of temperature and pH on the sensitivity of slow fibres to Ca^{2+}

Slow fibres from warm- and cold- acclimated fish were pooled to increase sample size for analysis of the effects of temperature and pH on the sensitivity of the contractile apparatus to Ca^{2+} (Tables 1, 2 and 3).

A temperature increase from 0 °C to 15 °C at pH 7.6 shifted the pCa-tension relationship to higher pCa values (ie. lower Ca^{2+} concentrations) and the pCa value required

Table 1
The concentration of calcium (μM) required to generate half maximum tension in slow fibres from warm- and cold-acclimated carp.

condition	warm-acclimated fish	cold-acclimated fish
0 °C, pH 7.0	6.11 ±0.08 (11)	6.16 ±0.05 ns (9)
0 °C, pH 7.6	6.27 ±0.06 (11)	6.18 ±0.07 ns (6)
15 °C, pH 7.0	6.04 ±0.05 (7)	6.00 ±0.04 ns (8)
15 °C, pH 7.6	6.53 ±0.11 (5)	6.56 ±0.04 ns (7)

Number of fibre bundles is shown in brackets. ns = not significant at $P<0.05$ level. All values are mean ± s.e.

Table 2
The effects of temperature at pH 7.0 and pH 7.6 on the sensitivity of the contractile apparatus to calcium.

	0 °C	15 °C
pH 7.0	6.13 ±0.05 (20)	6.02 ±0.03 ns (15)
pH 7.6	6.24 ±0.04 (17)	6.55 ±0.05 *** (12)

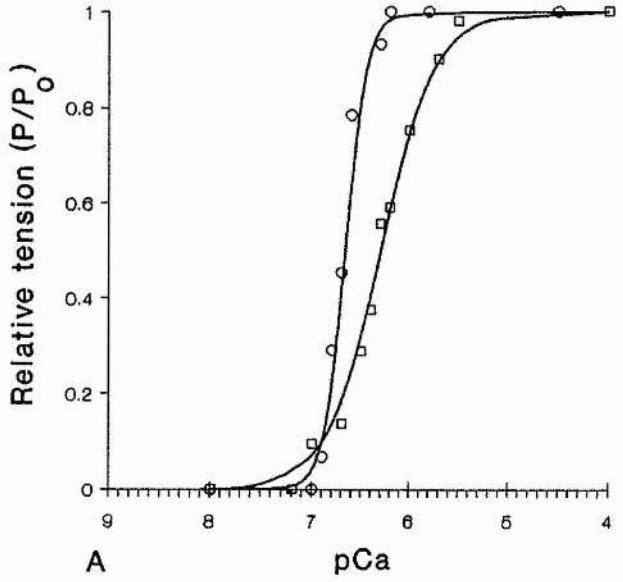
Number of fibre bundles is shown in brackets.
*** = P< 0.001; ns = not significantly different at P<0.05 level. All values are mean ± s.e.

Table 3
The effects of pH at 0 °C and 15 °C on the sensitivity of the contractile apparatus to calcium.

	pH 7.0	pH 7.6
0 °C	6.13 ±0.05 (20)	6.24 ±0.04 * (17)
15 °C	6.02 ±0.03 (15)	6.55 ±0.05 *** (12)

Number of fibre bundles is shown in brackets.
* = statistically significantly different at P<0.05 level;
*** = P<0.001; ns = not significant at P<0.05 level.
Values are mean ± s.e.

The effects of temperature and pH
on the pCa-tension relationship
of skinned slow fibres from the carp



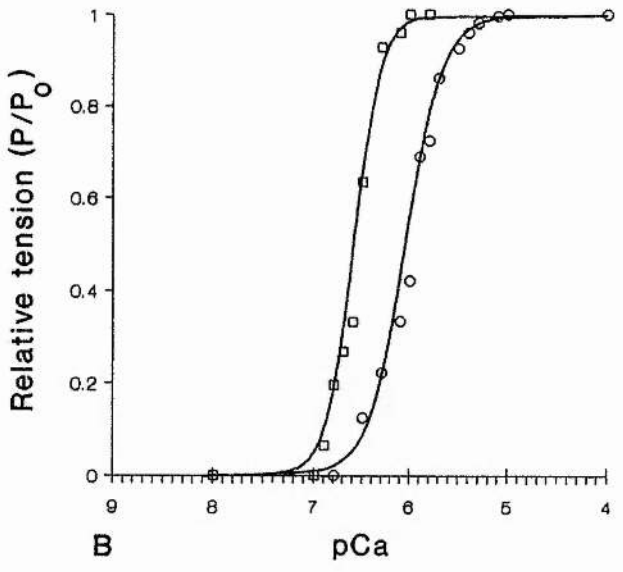


Fig 2

A The effects of temperature

o 15°C at pH 7.6

□ 0°C at pH 7.6

B The effects of pH

o pH 7.0 at 15°C

□ pH 7.6 at 15°C

Table 4
The effect of temperature at pH 7.0 and pH 7.6 on the Hill coefficient of slow fibres from the carp.

	0 °C	15 °C
pH 7.0	2.32 ±0.27 (20)	2.17 ±0.09 n.s. (15)
pH 7.6	1.68 ±0.07 (17)	2.83 ±0.21 *** (12)

Number of fibre bundles is shown in brackets.
*** = $P<0.001$; ns = not significant at $P<0.05$ level.
Values are mean ± s.e.

Table 5
The effect of pH at 0 °C and 15 °C on the Hill coefficient of slow fibres from the carp.

	pH 7.0	pH 7.6
0 °C	2.32 ±0.27 (20)	1.68 ±0.07 ns (17)
15 °C	2.17 ±0.09 (15)	2.83 ±0.21 * (12)

Number of fibre bundles is shown in brackets.
* = statistically significantly different at $P<0.05$ level;
ns = not significant at $P<0.05$ level.
Values are mean ± s.e.

to ellicit half-maximum tension increased from 6.24 to 6.55 ($P<0.001$, Tables 2 and 3, Fig. 2A). No significant difference was found between the pCa values required to develop half-maximum tension in fibres at 0 °C and 15 °C at pH 7 (Table 2).

A reduction in pH from 7.6 to 7.0 at both 0 °C and 15 °C shifted the pCa-tension relationship to lower pCa values (Table 3, Fig. 2B). For example, at 15 °C, the pCa required for the generation of half-maximum tension was lowered from 6.55 to 6.02 ($P<0.001$, Table 3).

The pCa's required for half-maximum tension increased in the series 15 °C, pH 7.0, (0.95 µM), < 0 °C, pH 7.0, (0.74 µm) < 0 °C, pH 7.6, (0.57 µM), < 15 °C, pH 7.6., (0.28 µM), (numbers in brackets = concentration of free Ca^{2+}, Table 2 and 3). Therefore lowering pH depresses the sensitivity of the contractile apparatus to calcium but raising the temperature at relatively alkaline pH reduces the concentration of Ca^{2+} required to reach half-maximum isometric tension (Tables 2 and 3).

<u>The effects of temperature and pH on the maximum isometric tension of slow muscle fibres from warm- and cold-acclimated carp</u>

For fibre bundles from both acclimation groups, a complete set of activation/ relaxation cycles at various pCa's,

Table 6
The effects of temperature and pH on the maximum isometric tension (P_o) of skinned slow fibres from the carp.

Acclimation temperature	pH	Experimental temperature 0 °C	15 °C
7 °C	7.0	53.6 ±4 (9)	69.3 ±6 * (8)
	7.6	54.2 ±6 (6)	71.9 ±6 * (7)
23 °C	7.0	40.8 ±4 (11)	56.3 ±6 * (7)
	7.6	39.3 ±4 (11)	58.1 ±6 * (5)

Tension values in KN m^{-2}. Number of fibre bundles is shown in brackets. * = statistically significantly different at $P<0.05$; ns = not statistically significant at $P<0.05$ level. All values mean ± s.e. The effect of pH on P_o was not statistically significant at $P<0.05$. See text for Q_{10} values.

(including up to four maximal activations), reduced maximum Ca^{2+}-activated force at both 0 °C and 15 °C by approximately 5-10% (Fig. 1). All fibres relaxed completely throughout the activation series without the development of residual tension.

The maximum isometric force (P_o) generated by skinned myotomal muscle fibres from the carp was dependent on acclimation temperature but not on experimental pH (Table 6). At 0°C, P_o was around 1.3-fold higher for slow fibres isolated from cold- than warm-acclimated carp ($P<0.05$, Table 6). At 15 °C P_o for slow fibres from warm- and cold-acclimated carp was more similar ($P>0.05$). Increasing the temperature from 0°C to 15 °C increased P_o for both acclimatory groups ($P<0.05$, Table 6). The $Q10_{(0-15°C)}$, for P_o was 1.20 and 1.25 for slow fibres from cold- and warm-acclimated fish respectively.

Discussion

The pCa-tension relationship

The pCa-tension relationship for slow fibres isolated from carp is similar to that described for frog, (Hellam and Podolsky, 1969) rabbit, (Kerrick et al., 1976; Metzger and Moss, 1987b) cod and dogfish, (Altringham and Johnston,

1982) and barnacle (Ashley and Moisescu, 1977). pCa-tension curves were sigmoidal indicating positive cooperativity between multiple Ca^{2+}-binding sites (Wyman, 1963). The data (Tables 4 and 5) suggests that at least three Ca^{2+} binding sites are involved in the activation of slow fibres from the carp. Since the overall steepness of the Hill plot may reflect cooperativity among Ca^{2+}-binding sites along the thin filament (Grabarek et al., 1983), as well as cross-bridge binding and Ca^{2+} binding (Bremel and Weber, 1972), the present results suggest that these factors may have more effect at higher temperatures and at alkaline pH than at low temperature and lower pH (Tables 4 and 5). In addition, a pCa-tension relationship for slow fibres with a higher cooperativity value than 1 may involve the interaction of separate TnC molecules, via neighbouring tropomyosins, (Grabarek et al., 1983), since TnC molecules from cardiac and slow muscle have only one low affinity Ca^{2+}-binding site (Wilkinson, 1980).

The number of Ca^{2+}-binding sites on each TnC molecule and the degree of cooperation between components of the troponin/ tropomyosin complex are also likely to effect the position and slope of the pCa-tension relationship. In general, fast fibres have higher n values and a higher degree of co-operativity between Ca^{2+}-binding sites than slow fibres (Kerrick et al., 1976; Altringham and Johnston, 1982; Metzger and Moss, 1987b).

The effects of temperature on the pCa-tension curve

Decreasing temperature from 15-0 °C resulted in a rightward shift in the pCa-tension relationship to higher Ca^{2+} concentrations at pH 7.6 but not at pH 7 (Table 3).

The binding of calcium to TnC has negative enthalpy (ΔH) (Potter, Hsu and Pownall, 1977; Godt and Lindley, 1982) and is therefore inhibited at higher temperature. If the force generated by muscle was directly related to the binding of calcium by TnC the pCa-tension curve would parallel the calcium binding curve of TnC and should shift to higher calcium concentrations following an increase in temperature. Orentlicher et al., (1977) showed that the pCa-tension relationship of skinned muscle fibres from crayfish shifted to the right (i.e. to higher free Ca^{2+} concentrations) following an increase in temperature from 5 °C to 20 °C. The pCa-tension relationship of skinned fast fibres (but not slow fibres) from the rat shifted in a similar direction following a temperature increase from 5 °C to 22 °C (Stephenson and Williams, 1981). Recently, Stephenson and Williams (1985) repeated their earlier work extending it to include fast and slow muscle fibres from the toad. In this latter report the calcium sensitivity of fast-twitch fibres from either the rat or the toad showed a greater temperature sensitivity compared with slow-twitch mammalian and amphibian muscle fibres.

In contrast, Ashley and Moisescu (1977) showed that skinned fibres from the barnacle had a similar pCa-tension relationship at both 4 °C and 20 °C.

Sigmoid pCa-tension curves show that the relationship between the binding of calcium to TnC and force generation is not a simple one. It is likely that kinetic steps in cross-bridge attachment/ detachment have varying degrees of dependence on temperature, perhaps shifting the pCa-tension curve to the left at higher temperatures as was found in this study (Fig. 2A).

The effects of pH on the pCa-tension relationship

Increasing pH from pH 7.0 to pH 7.6 shifted the pCa-tension relationship around 0.11 pCa units to the left at 0 °C and 0.53 pCa units to the left at 15 °C (Fig. 2B). A left-ward shift in the pCa-tension relationship following an increase in pH has been found in numerous studies (Ashley and Moisescu, 1977; Chen-Liu and Endo, 1973; Donaldson and Hermansen, 1978; Fabiato and Fabiato, 1978; Metzger and Moss, 1987b).

In addition, Metzger and Moss (1987b), found that a drop in pH increased the cooperative binding of Ca^{2+} to TnC in slow (soleus) fibres but decreased cooperativity in fast (vastus lateralis) fibres from the rabbit. In contrast, Donaldson and Hermansen, (1978) working on skinned fibres

isolated from various muscles from the rabbit found no correlation between the cooperative binding of calcium to TnC and pH.

The mechanism by which pH modulates force production is not known for certain and there are a number of contradictory reports. For example, hydrogen ions decrease (Blanchard, Pan and Solaro, 1984; Blanchard and Solaro, 1984; Stull and Buss, 1978) or leave unchanged (Fuchs, 1979) the affinity of the binding sites on TnC for calcium. Nevertheless, various mechanisms have been proposed to explain the effects of hydrogen ions on muscle contraction.

Katz and Hecht (1969) suggested that the depressed contractility of myocardial ischemia was caused by direct competition between H^+ and Ca^{2+} for receptor sites on TnC. Robertson et al., (1978) showed that H^+ compete with Ca^{2+} for binding at the Ca^{2+} specific (low affinity) sites on TnC but not at the Ca^{2+}-Mg^{2+} (high affinity) sites (Robertson and Kerrick, 1979).

More recently, Godt (1981) proposed an electrostatic model to explain the the effects of low pH on the sensitivity of muscle fibres to Ca^{2+}. He argued that a drop in pH would result in the electrostatic field surrounding the myofilaments becoming more electropositive, repelling cations, and thus reducing the sensitivity of the contractile apparatus to Ca^{2+}.

According to the cross-bridge hypothesis the force generated by muscle results from some form of chemical

bonding between myosin and actin followed by a conformational change in the cross-bridge-actin complex that shifts the thick and thin filaments relative to one another. Since this movement (about 1μm per half-sarcomere) is many times the length of a myosin molecule (0.15μm), it is natural to suppose that during shortening the cross-bridges break and re-attach further along the thin filament. These cycles of attachment, pulling, detachment and recovery enable mechanical work to be done. Energy for mechanical work is derived from the splitting of ATP.

A decrease in the sensitivity of the contractile apparatus to calcium may be due to a reduction in the number of cross-bridges as pH is lowered since both cycling (Guth et al., 1986, 1987) and rigor cross-bridges (Bremer and Weber, 1972) increase the binding of calcium by TnC in regulated thin filaments. However, fewer crossbridges would tend to reduce P_o which was not found in this report. Nevertheless a reduction in crossbridge cycling rate at low pH could account for a lowering in the calcium sensitivity of the contractile apparatus whilst maintaining P_o.

Edman and Mattiazzi (1981) showed that unloaded shortening velocity decreased with a drop in pH. This suggests that a lowering of pH may reduce the cycling rate of cross-bridges.

Shifts in pCa-tension curves may be caused by perturbations in the relationship between the cycling rate of crossbridges and the time that calcium ions are bound to

TnC during isometric contraction (Brandt et al., 1982). An increase in crossbridge cycling rate compared to the time calcium ions spend bound to TnC could result in an apparent decrease in calcium sensitivity due to a reduction in the cooperative activation of the thin filament by crossbridge attachments. This mechanism could explain the present results if the total number of attached crossbridges at any instant was sufficient to maintain P_o but an overall increase in the cycling rate of crossbridges is not consistent with the drop in V_{max} at lower pH reported by Edman and Mattiazzi (1981).

The effects of temperature acclimation on the pCa-tension relationship

In contrast to temperature and pH, thermal acclimation did not modify the sensitivity of the contractile apparatus to calcium ions under any combination of temperature and pH investigated in this study (Table 1).

Carp are similar to goldfish (Johnston, 1979) in that they produce alternative isoforms of regulatory proteins following cold-acclimation (Crockford and Johnston, 1989). A modification of the pCa-tension relationship resulting from a change in the regulatory proteins could provide a mechanism of adaption to low temperatures. A shift in the pCa-tension relationship to higher calcium concentrations

following cold-acclimation could have partially explained the higher rate of relaxation, at low temperature, of myotomal muscle fibres from cold- compared with warm-acclimated carp (chapter 2).

Comparison between the pCa's required to generate half-maximum tension at 15 °C, pH 7.0 and 0 °C, pH 7.6 (Table 2) indicates how the pCa-tension relationship for slow fibres would alter in response to a drop in temperature. The pCa-tension relationship shifts to the left following a drop in temperature from 15 °C-0 C and assuming a ΔpH /Δ°C of -0.0033 (Anguilla rostrata, Walsh and Moon, 1982) the intracellular pH of slow muscle will increase by 0.05 pH units from around pH 7.30 at 15 °C to about pH 7.35 at 0 °C. The pCa required to ellicit half-maximum tension will be shifted approximately 0.1 pCa units from pCa 6.28 at 15 °C, to pCa 6.18 at 0 °C. Thus at 15°C the calcium concentration required to reach half-maximum tension is only 80% that required at 0 °C. A leftward shift of this magnitude could increase the rate of contraction at high temperature. In contrast, the rate of relaxation may be higher in the cold if Ca^{2+} is removed from the contractile apparatus at a similar rate at both temperatures, since a relatively high concentration of Ca^{2+} would be required to generate an equivalent force.

The effects of temperature on P_o

Skinned slow muscle fibres from carp generate similar maximum tensions (P_o) as homologous fibres in other species (Altringham and Johnston, 1982; Mutungi, 1987).

P_o has a low thermal dependence over the physiological temperature range for a number of species including the carp (Table 6; Johnston et al., 1985) and frog (Godt and Lindley, 1982). Q_{10} $(0-15°C)$ values of 1.20 and 1.25 for slow fibres from warm- and cold-acclimated carp are similar to those reported by Johnston et al., (1985).

Ford, Huxley and Simmons, (1977) measured the stiffness of intact frog fibres and showed that increased tension with increasing temperature was due to a higher number of cross-bridges in the force generating state. Kuhn et al., (1979) suggested that increased tension at higher temperature could be due to an increase in the force generated per cross-bridge. It is likely that both mechanisms contribute to an increase in tension at higher temperatures.

The effects of pH on P_o

The maximum isometric tension of slow fibres is relatively insensitive to changes of pH within the normal physiological range in carp (Table 6), frog (Chen-Liu and Endo, 1973), cod

and terrapin (Mutungi, 1987). In addition, there is evidence to show that slow fibres (Donaldson, 1984; Metzger and Moss, 1987b) are more resistant to the depressant effects of low pH than fast fibres (Stracher, 1961; Watanabe and Gaffin, 1971; Fabiato and Fabiato, 1978; Metzger and Moss, 1987b). In general P_o is depressed outside the normal physiological range. It is unlikely that the pH range used in this study was wide enough to reduce P_o (Mutungi, 1987; Mutungi and Johnston, 1987; 1988).

CHAPTER 4

The role of thyroid hormones in temperature acclimation

Introduction

It is well established that exposure of a mammal to a cold environment stimulates thyroid metabolism and that this is related to the calorigenic action of thyroid hormone in this group of animals (Woods and Carlson, 1956). However reports on the effects of temperature on the thyroid gland of fish are contradictory. For example, Gorbman (1940) showed that the thyroid of goldfish responds to mammalian thyroid stimulating hormone (TSH) by increasing the synthesis of thyroid hormone. However Fortune (1956) reported that goldfish have an inactive thyroid.

Much of this confusion may be due to the many techniques used to assess thyroid activity. For example, histological and radioiodine-uptake measurements of thyroid activity on the same fish do not give comparable results (Drury and Eales, 1968).

The effect of temperature on thyroid activity varies considerably between different species of fish. Some species showed an inverse relationship between temperature and thyroid activity (Swift, 1959; Olivereau, 1955; Berg *et al.*, 1959) while other species of fish showed a direct

relationship (Barrington and Matty, 1954; Fortune, 1955; Berg et al., 1959). To confuse matters further species such as carp, tench, eel, chub and dogfish had similar levels of thyroid activity at either 5°C or 20°C. This suggests that the production of thyroid hormone is unaffected by temperature acclimation (Hoar and Eales, 1963; Olivereau, 1955). Nevertheless, serum from cold-acclimated carp and tench increased the oxygen consumption of muscles from both warm- and cold-acclimated fish (Precht, 1965a, b; Jankowsky, 1964). This suggests that chemical messengers in the blood of cold-acclimated fish can alter the metabolism of isolated tissues.

There are a number of similarities between the effects of temperature acclimation and the effects of hypo- and hyperthyroidism on the structure and function of muscle. Cold-acclimation results in an increase in the ratio of slow to fast fibres in goldfish (Johnston and Lucking, 1978) and striped bass (Sidell, 1980). Depletion of thyroid hormone results in a similar increase in the ratio of slow to fast fibres in the soleus and in the extensor digitorum longus muscle of the rat (Nwoye and Mommaerts, 1981; Nwoye, Mommaerts, Simpson, Seraydarian and Marusich, 1982; Nicol and Bruce, 1981).

A common finding is that cold-acclimation increases the capacity of muscle for aerobic metabolism (Hazel, 1972; Smit, van den Berg and Kijn-den Hartog, 1974; Van den Thillart and Modderkolk, 1978). The thyroid may have a role

in this process as an excess of thyroid hormones increases the activity of several mitochondrial enzymes including citrate synthase, cytochrome oxidase and alpha-glycerophosphate dehydrogenase, especially in slow muscles of the rat (Kubista, Kubistova and Pette, 1971; Hardeveld, van Rusche and Kassenaar, 1976; Winder and Holloszy, 1977; Janssen, Hardeveld and Kassenaar, 1979; Nicol and Johnston, 1981). Conversely, the depletion of thyroid hormones in mammals may reduce the activity of these enzymes in both slow and fast muscles (van Hardeveld and Kassennar, 1976; Baldwin, Hooker, Campbell and Lewis, 1978).

Myofibrillar ATPase isolated from the white muscle of cold-acclimated goldfish had a higher activity and reduced activation enthalpy at low temperatures compared with ATPase from warm-acclimated fish (Johnston et al., 1975; Johnston, 1979). Similar increases in the activity of myosin ATPase have been reported for soleus muscle from hyperthyroid rats (Ianuzzo, Patel Chen and O'Brien, 1980; Nwoye and Mommaerts, 1981). Conversely, the activity of ATPase was lower in cardiac, slow and fast muscles from hypothyroid compared with hyperthyroid rats (Baldwin et al., 1980; Nwoye et al., 1982; Nwoye and Mommaerts, 1981).

An increase in the rate of relaxation of myotomal muscle (chapter 2) and pectoral fin adductor muscles from cold- compared with warm-acclimated fish (Heap et al., 1987) may be explained by the increased activity of Ca^{2+}-pump protein in the SR of cold- compared with warm-acclimated fish

(chapter 2). Increased levels of thyroid hormones may be involved in this mechanism as hyperthyroidism increases the rate of Ca^{2+} uptake into the SR of rat muscle (Kim, Witzmann and Fitts, 1981, 1982; Nwoye et al, 1982).

Many acclimatory responses could be coordinated through an alteration in the level of thyroid hormones. This study investigated the effects of temperature acclimation and hypothyroidism on the ultrastructure of slow myotomal muscle from the carp.

Materials and methods

Fish

Common carp (Cyprinus carpio L.) (178.6g ± 5g bodyweight, 17.9 cm ± 2 cm standard length mean ± S.D. 15 fish) were obtained from Humberside Fisheries (Driffield, England) and maintained at either 8 °C (±0.5 °C) or 22 °C (±1 °C) under a 12 hr light: 12 hr dark photoperiod in filtered, recirculated freshwater for at least one month. Hypothyroidism was induced in one group of cold-acclimated fish using methimazole (0.5µg methimazole / g body weight approximately equivalent to 100µl of 80mg methimazole per 100ml 0.9% NaCl). Doses of methimazole were administered by intraperitoneal injection once every two days. Cold-acclimated controls were injected with 100µl 0.9% NaCl. All three groups were fed ad lib on commercial fish pellets. Fish were killed by a blow to the head

followed by transection of the spinal cord. Muscle from each fish was used for both electron microscopy and histochemical studies.

The collection of blood for the determination of T3 and T4 levels

Blood was removed from the ventral aorta in the caudal region of each fish using a heparinised syringe. Blood was then centrifuged at 13000 rpm (897g) for 5 min and the pellet discarded. Serum was stored at -150°C before being used to determine the levels of T3 and T4 within the blood.

The determination of the level of T3 and T4 in serum from temperature acclimated and hypothyroid carp

Serum was subjected to radioimmunoassay for T3 or T4 using antisera obtained from Steranti Research Ltd. The assay methods used were those recommended by the supplier of the antisera but separation of free and bound ligand in the T3 assay was accomplished using a second antibody technique (Sterasep, Steranti Research Ltd.). ^{125}I-T4 and ^{125}I-T3 were obtained from Amersham International plc. Buffer reagents and hormone standards were obtained from Sigma Chemicals Co Ltd. T4 was assayed on 50-µl aliquots of

serum. T3 was assayed on 10-µl aliquots (Youngson, McLay and Olsen, 1986). Each hormone determination was done in duplicate.

Tissue preparation

Bundles of slow fibres were taken from an area above the lateral line nerve directly below the insertion of the first dorsal fin ray and pinned to cork strips approximately at their resting length *in vivo* prior to fixation in 2% gluteraldehyde, 0.15M cacodylate buffer, 10 mM $CaCl_2$, pH 7.2 (at 4°C) overnight. Following further dissection the tissue was washed in cacodylate buffer and post-fixed for 2hr in 2% osmium tetraoxide, 0.15M cacodylate buffer, 10 mM $CaCl_2$, 0.8% potassium ferricyanide, at pH 7.2 (Peachey, Waugh and Sommer, 1974). The tissue was washed in fresh cacodylate buffer and distilled water and stained *en bloc* for 30 min in a saturated aqueous solution of uranyl acetate to improve contrast. Fibre bundles were rinsed in distilled water and dehydrated in a series of acetone solutions of increasing concentration up to absolute and vacuum embedded in araldite CY212 resin (Ciba-Geigy). This method of processing was found to reduce lipid leaching. Ultra-thin transverse sections, (50-100 nm thickness), were cut from blocks on a Reichert OMU2 ultramicrotome and mounted on pyroxyline, (3% in amyl nitrate), coated grids (150 mesh). Sections were

double stained with aqueous, saturated uranyl acetate (30 min) and Reynolds (1963) lead citrate (5 min) and examined with a Philips 301 electron microscope at 60KV.

Five fish were taken from warm-acclimated, cold-acclimated and cold-acclimated hypothyroid groups and five blocks made per fish, giving a total of 25 blocks for each group. Transverse sections were cut and analysed from all the fish in each group.

Quantitative analysis

Electron micrographs of whole muscle fibres (980-4300x), were enlarged three times using a photographic enlarger. The area of fibres and individual myofibrils was measured using digital planimetry (Walesby and Johnston, 1980). The perimeter and radius of individual myofibrils was calculated with a Hewlett Packard 68B computer in conjunction with stereology software (Kompira Ltd. Strathclyde, Scotland). The fraction of fibre volume occupied by myofibrils and mitochondria was estimated using a point counting method (Weibel, 1979). The total volume fraction of mitochondria was divided into two populations. Mitochondria surrounded by myofibrils were classified as intermyofibrillar while the remainder were counted as subsarcolemmal mitochondria.

Electron micrographs of mitochondria (mag 36-43000x) were enlarged 3 times using a photographic enlarger. The

volume fraction of cristae per unit volume of mitochondrion was estimated by the point counting method used previously to estimate the volume fraction of myofibrils (Weibel, 1979). The surface density of cristae and inner membrane per unit volume of mitochondrion (Svcr+mi) was estimated using the equation:-

$$Svcr+mi = (I_{cr+i}/P_{tot}).(M/d)$$

In which P_{tot} is the total point count within the mitochondrion and I_{cr+i} are the intersections of the cristae and inner mitochondrial membrane with both horizontal and vertical grid lines lying within the mitochondrion. M is the final magnification of the micrograph and d is the distance between parallel grid lines (Weibel, 1979).

Histochemistry of the m. hyohyoideus inferior muscle

One end of the m. hyohyoideus muscle of the carp inserts into the inner side of the epihyal while the other end inserts on the ventral raphe. The m. hyohyoideus consists of a bundle of red fibres, surrounded by white fibres at its ventral, lateral and caudal sides (Granzier, Wiersma, Akster and Osse, 1983). The m. hyohyoideus muscle is active in the contraction phase of the respiratory cycle (Ballintijn, 1969 a, b).

Tissue preparation

The m. hyohyoideus muscle was quickly dissected intact from the fish and mounted in O.C.T. embedding media (Labtek Co Ltd). The tissue was frozen by immersion in isopentane, cooled to its melting point with liquid nitrogen. Blocks were placed in a refrigerated cabinet at -25 °C for 1hr and then cryostat sections were cut at 8-12 μm thickness, and mounted in glycerol-gelatin on coverslips.

Histochemical methods

Succinic dehydrogenase (SDH)

Sections were stained for succinic dehydrogenase activity using the nitro-blue tetrazolium method of Nachlas, Tsou, DeSouza, Cheng and Seligman (1957).

Myofibrillar adenosine triphosphatase (Myofibrillar ATPase)

Myofibrillar ATPase activity was demonstrated using the method of Guth and Samaha (1970). Sections were preincubated for 2 min in a solution of 18 mM $CaCl_2$, 100 mM 2-amino-2-methyl-1-propanol buffer at between pH 10.2 and 10.3 for 2-3 min at 20 °C. Sections were incubated in the

media of Guth and Samaha (1970) at 20 °C for 20 min. Serial sections were used to compare the results for the activity of ATPase and SDH.

The measurement of the percentage cross-sectional area of muscle fibre types in the m. hyohyoideus muscle from the carp

Transverse sections of m. hyohyoideus muscles were magnified (x4) and traced on to paper to a final magnification of x50 using a microscope equiped with a side-arm. Preliminary experiments showed that the cross-sectional area of individual fast (FG), slow (SO) and fast oxidative glycolytic (FOG) fibres from m. hyohyoideus muscles of warm-cold- and cold-acclimated hypothyroid fish were not significantly different.

The percentage cross-sectional area of each fibre type was quantified using digital planimetry (Walesby and Johnston, 1980; Heap et al., 1987). The total cross-sectional area of each muscle was corrected for the presence of intramuscular spaces.

Statistical analysis

Data was compared using an unpaired students t-test

(Two-tailed). $P<0.05$ was the level of statistical significance.

Results

T4/T3 hormone radioimmunoassay

The concentration (ng/ml) of T4 in serum from warm-, cold- and cold-acclimated hypothyroid carp was 3.71 $\pm$ 1.4 (4), 2.82 $\pm$0.13 (4) and 2.76 $\pm$0.37 (6) respectively (mean $\pm$ s.e. number of fish in brackets). The level of T4 in serum from warm-, cold-, and cold-acclimated hypothyroid fish was not significantly different at $P<0.05$.

The concentration (ng/ml) of T3 in serum from warm- and cold-acclimated carp was 2.17 $\pm$ 0.38 (4), 1.10 $\pm$0.16 (5) respectively (mean $\pm$ s.e. number of fish in brackets). The level of T3 in serum was higher in warm- than cold-acclimated fish ($P<0.05$).

The concentration of T3 in samples from cold-acclimated hypothyroid fish was clearly lower than for cold-acclimated euthyroid fish since three out of six fish had levels of T3 below the detection limit of the assay technique (<0.6ng/ml). Levels of T3 in serum from the remaining hypothyroid fish were within one standard deviation of the mean for euthyroid cold-acclimated fish.

The effects of temperature acclimation on the percentage cross-sectional area of fibre types in the m. hyohyoideus muscle

The cross-sectional area of m. hyohyoideus muscle from warm-acclimated carp was composed of 37% fast glycolytic (FG), 62% slow oxidative (SO) and 1% fast oxidative glycolytic (FOG) fibres (Table 1, Fig. 1). The percentage cross-sectional area of oxidative fibre types increased folowing cold-acclimation. For example, the percentage cross-sectional area of FOG fibres was 6-fold higher in m. hyohyoideus muscles from cold- than warm-acclimated fish ($P<0.001$, Table 1).

In contrast, the percentage cross-sectional area of FG fibres was 14% lower in muscles from cold- than warm-acclimated fish ($P<0.05$, Table 1. Fig. 2).

The effects of hypothyroidism on the percentage cross-sectional area of fibre types in cold-acclimated carp

The percentage cross-sectional area of SO fibres in m. hyohyoideus muscles was 14% higher in euthyroid than hypothyroid fish ($P<0.05$, Table 1. Fig. 3). Conversely, the percentage cross-sectional area of FG fibres was 16% lower

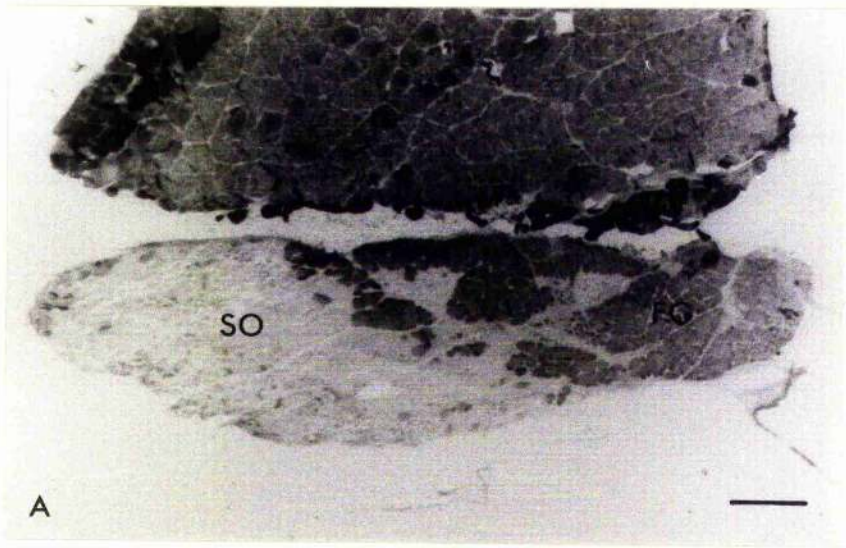
Table 1
The percentage cross-sectional area of fibre types in the m. hyohyoideus (inferior) muscle isolated from warm-, cold- and cold-acclimated hypothyroid carp.

Fibre Type	Warm-acclimated fish	Cold-acclimated fish	Cold-acclimated (hypothyroid) fish
Fast glycolytic	37.0 ±3.5	23.9 ±2	40.3 ±3.6
Fast oxidative glycolytic	1.1 ±0.3	6.4 ±1.9	4.5 ±0.51
Slow oxidative	61.9 ±3.7	69.7 ±3.2	55.2 ±1.5

Number of muscles for each group = 4. All values are mean ± s.e. Muscles from warm-acclimated fish had a higher percentage cross-sectional area of fast glycolytic and a lower percentage cross-sectional area of slow oxidative fibres than muscles from cold-acclimated fish $P<0.05$. Muscles from cold-acclimated fish had a higher percentage cross-sectional area of fast oxidative glycolytic fibres than muscles from warm-acclimated fish $P<0.001$.
The percentage cross-sectional area of fast glycolytic and slow oxidative fibres in muscles from cold- and cold-acclimated hypothyroid fish were significantly different $P<0.05$. The percentage cross-sectional area of fast oxidative glycolytic fibres in muscles from cold- and cold-acclimated hypothyroid fish were not statistically different at the $P<0.05$ level.

Fig. 1

Serial transverse sections of m. hyohyoideus muscle from warm-acclimated carp stained for ATPase activity (A) and Succinic dehydrogenase activity (B). Abbreviations for this figure and fig. 2 and 3; FG= fast glycolytic fibres; SO= slow oxidative fibres. Note the absence of fast oxidative (FOG) fibres. Scale bars =150 µm


SO
FG
A

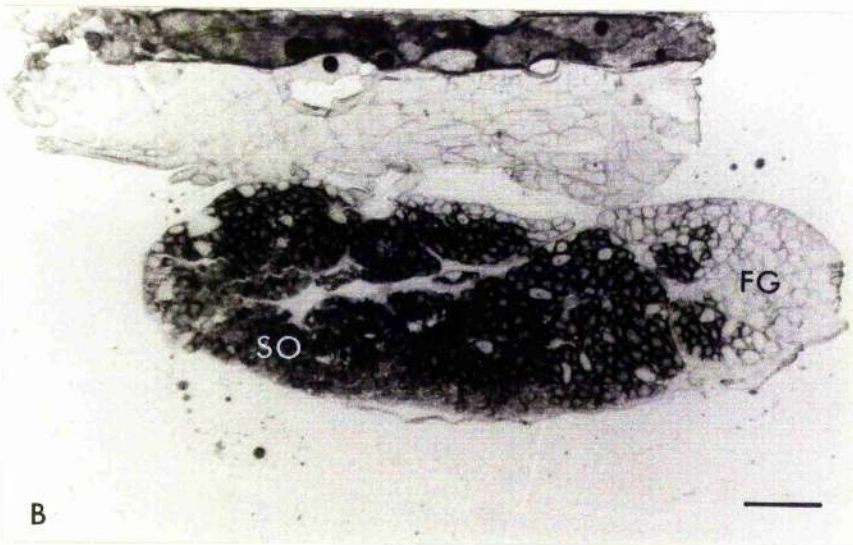

FG
SO
B

Fig. 2

Serial transverse sections of m. hyohyoideus muscle from cold-acclimated carp stained for ATPase activity (A) and Succinic dehydrogenase activity (B). Note the presence of FOG fibres. Scale bars =150 µm.


FOG
SO
FG
A

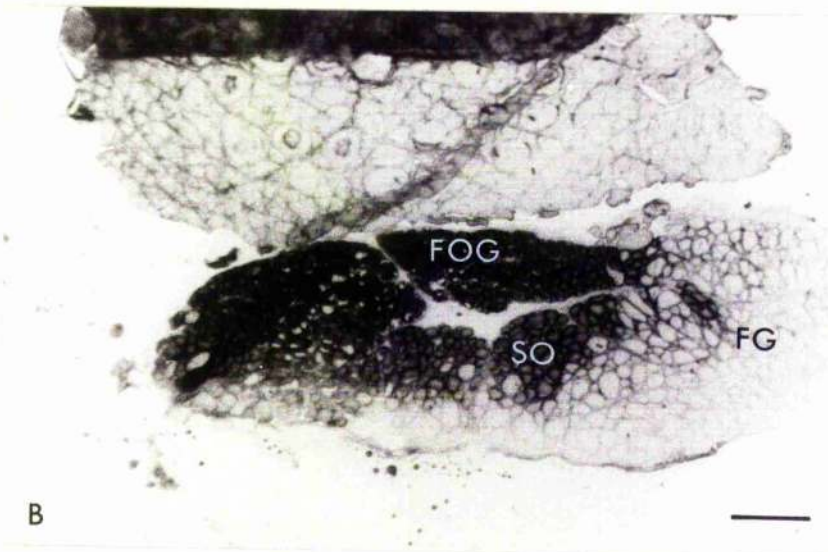
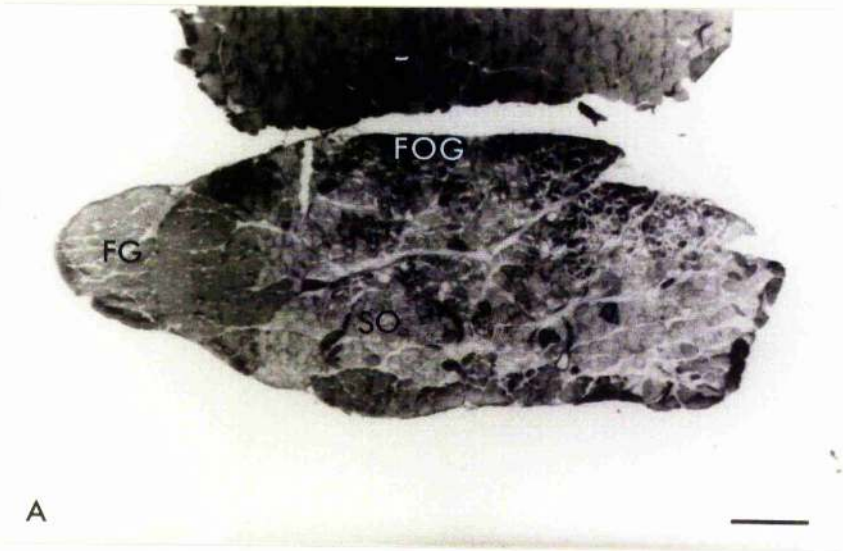
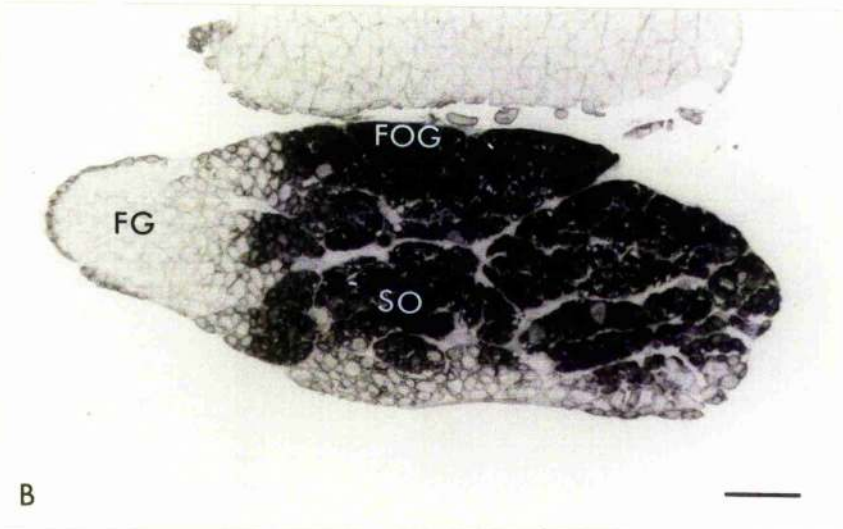

FOG
SO
FG
B

Fig. 3

Serial transverse sections of m. hyohyoideus muscle from cold-acclimated hypothyroid carp stained for ATPase activity (A) and Succinic dehydrogenase activity (B). Scale bars =300 μm



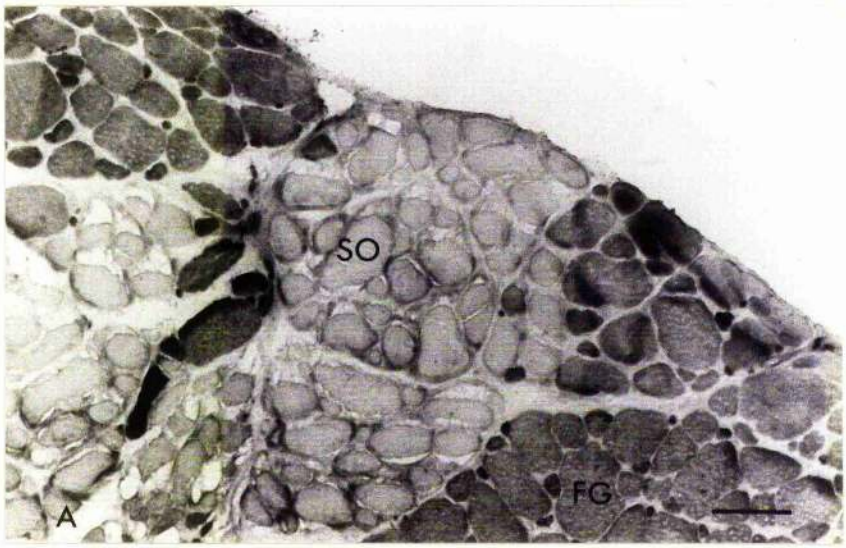
A



B

Fig. 4

High power serial transverse sections of m. hyohyoideus muscle from warm-acclimated carp stained for ATPase activity (A) and Succinic dehydrogenase activity (B). FG= fast glycolytic fibres; SO= slow oxidative fibres. Scale bars =60 μm


SO
FG
A

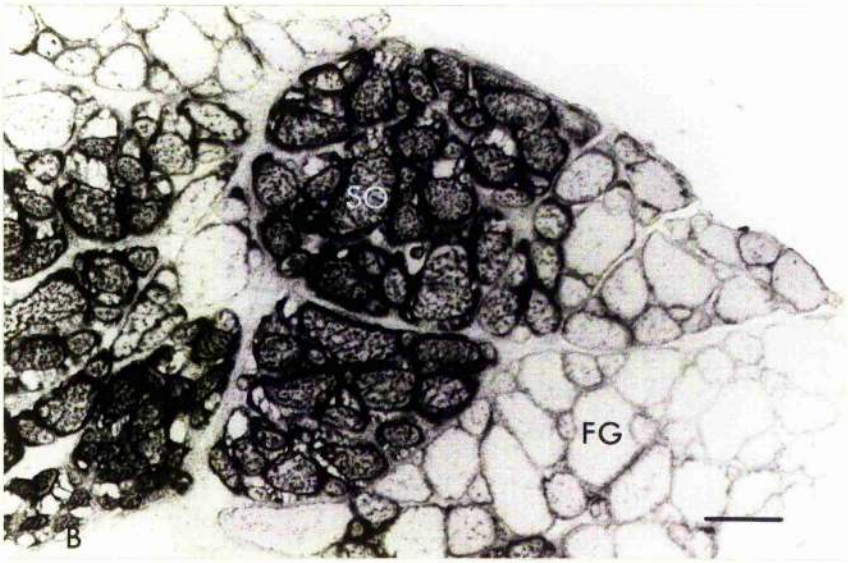
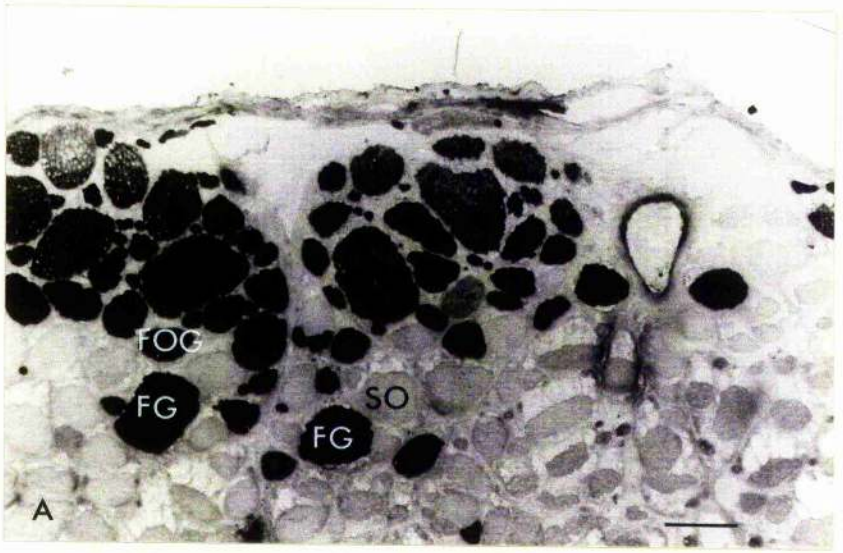

SO
FG
B

Fig. 5

High power serial transverse sections of m. hyohyoideus muscle from cold-acclimated carp stained for ATPase activity (A) and Succinic dehydrogenase activity (B). FG= fast glycolytic fibres; FOG= fast oxidative fibres; SO= slow oxidative fibres. Scale bars =60 µm


FOC
FG
SO
FG
A

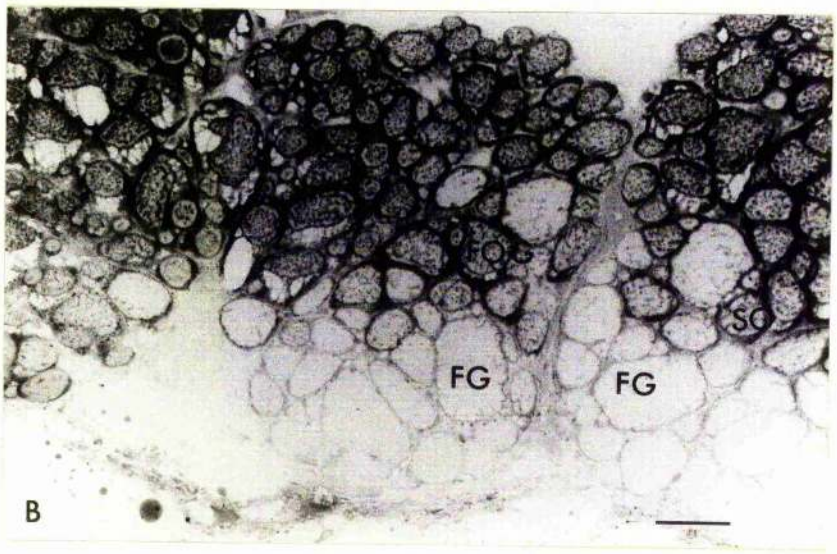
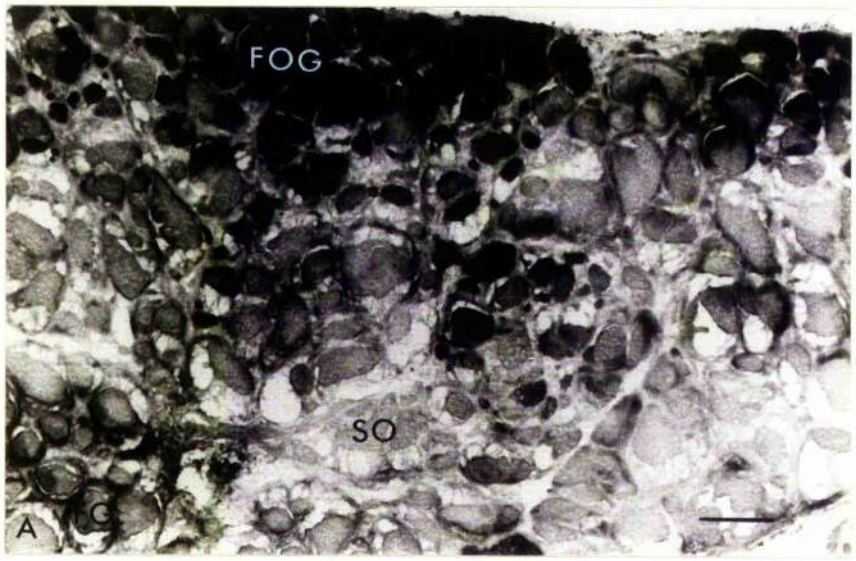
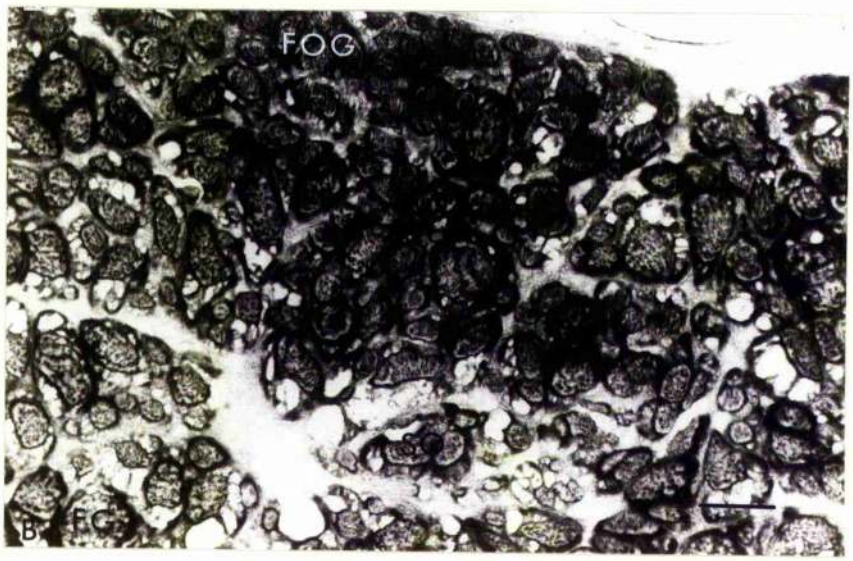

SO
FG
FG
B

Fig. 6

High power serial transverse sections of m. hyohyoideus muscle from cold-acclimated (hypothyroid) carp stained for ATPase activity (A) and Succinic dehydrogenase activity (B). FG= fast glycolytic fibres; FOG= fast oxidative fibres; SO= slow oxidative fibres. Scale bars =60 µm


FOG
SO
A
G


FOG
B
FG

in muscles from euthyroid compared with hypothyroid fish ($P<0.05$, Table 1). The percentage cross-sectional area of FOG fibres was unaffected by hypothyroidism.

The results (Table 1) suggest that the level of thyroid hormone may be involved in determining the percentage cross-sectional area of SO and FG fibres.

The effects of temperature acclimation on the ultrastructure of slow muscle from the carp

The cross-sectional area of fibres sampled from warm- and cold-acclimated fish was not significantly different ($P>0.05$, Table 2). Mean diameter for fibres from warm- and cold-acclimated fish was approximately 20μm.

The volume fraction of myofibrils was slightly higher in fibres from cold- than warm-acclimated fish ($P<0.01$, Table 2. Fig. 8 and 9).

The total volume fraction of mitochondria was 1.3-fold higher in fibres from cold- than warm-acclimated fish ($P<0.001$, Table 2). The volume fraction of intermyofibrillar mitochondria was 1.5-fold higher in fibres from cold- than warm-acclimated fish ($P<0.01$). Muscle fibres from cold-acclimated fish also had a higher volume fraction of mitochondria in the sub-sarcolemmal zone than fibres from warm-acclimated fish ($P<0.05$, Table 2, Fig. 8 and 9).

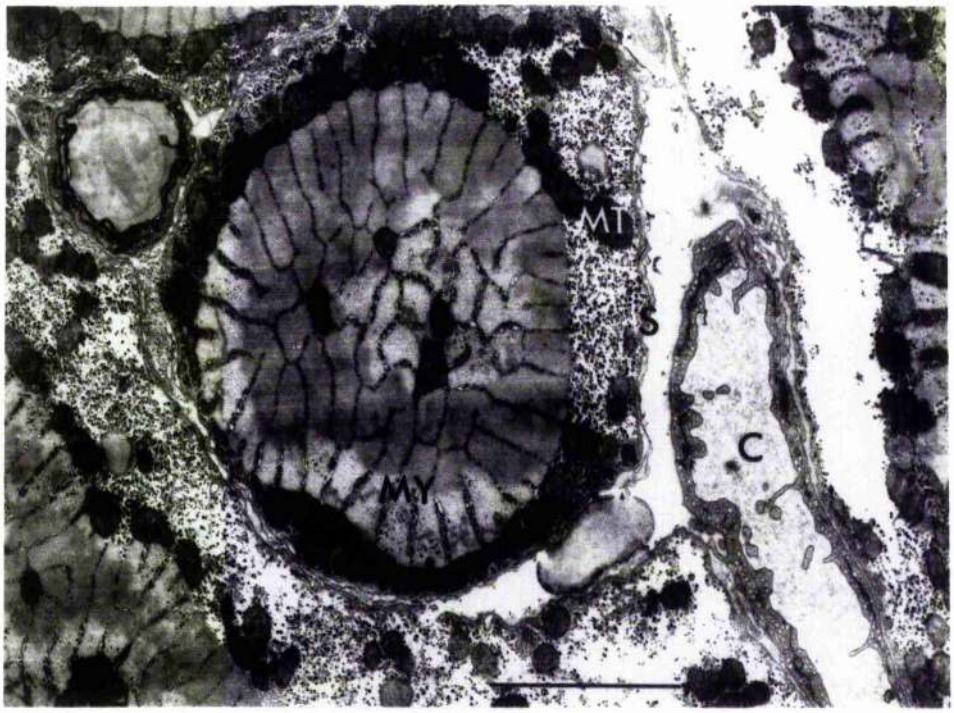
Table 2
The effects of temperature acclimation on the volume fraction of myofibrils and mitochondria in slow muscle fibres from carp.

Parameter	Cold-acclimated fish	warm acclimated fish
A(f), (μm^2)	284.5 $\pm$30	261.0 $\pm$29 ns
V_v(myf,f) (%)	63 $\pm$1	61 $\pm$1 **
$V_v(mit_{inter}, f)$ (%)	7.15 $\pm$0.45	4.43 $\pm$0.66 **
$V_v(mit_{sub}, f)$ (%)	17.56 $\pm$0.78	14.39 $\pm$1.1 *
$V_v(mit_{tot}, f)$ (%)	24.59 $\pm$0.79	18.42 $\pm$1.1 ***

A(f), mean cross-sectional area of fibres. V_v, entries are fraction of cell volume (volume density) occupied by V_v(myf,f), myofibrils; $V_v(mit_{inter}, f)$, mitochondria in intermyofibrilar zone; $V_v(mit_{sub}, f)$, mitochondria in subsarcolemmal zone; $V_v(mit_{tot}, f)$, total mitochondria. * = statistically significantly different at $P<0.05$; ** = $P<0.01$; *** = $P<0.001$; ns = not statistically significant at $P<0.05$ level. All values are mean $\pm$ s.e. of 24 electron micrographs from 5 fish.

Fig. 8

Electron micrographs of transversely sectioned slow muscle fibres from warm-acclimated carp. Abbreviations for this and figures 9 and 10: MY= myofibrils; MT= mitochondria; SR= sarcoplasmic reticulum; C= capillary; N= nucleus; S= sarcolemma. Scale bars= 5 µm.


MT
S
C
MY

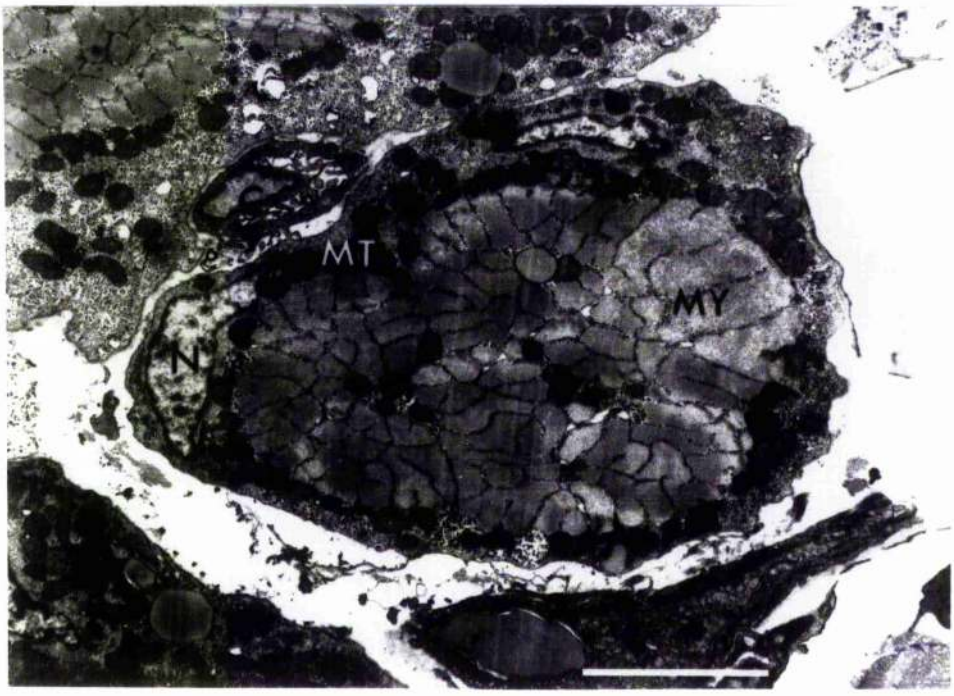
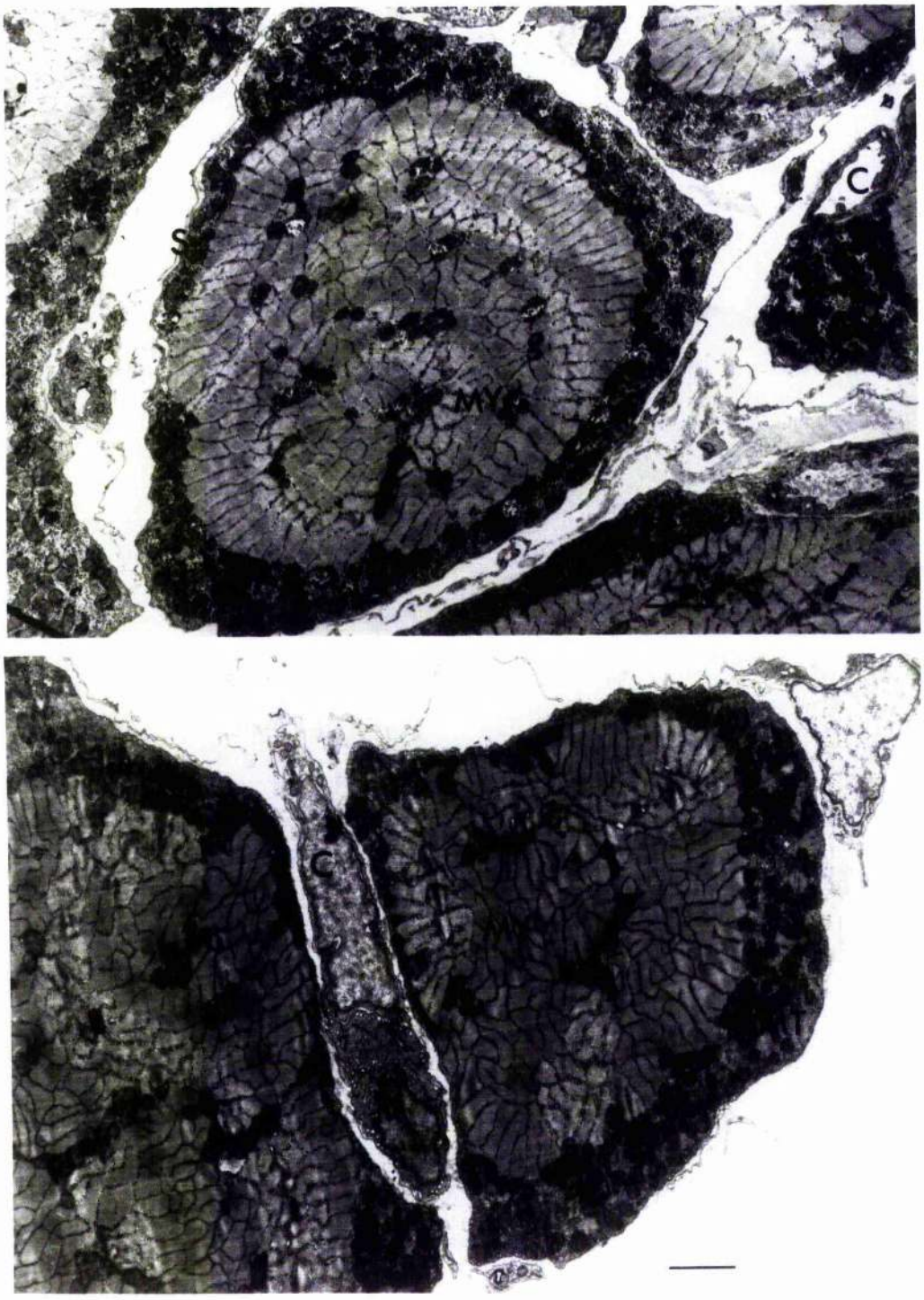

MT
MY
N

Fig. 9

Electron micrographs of transversely sectioned slow muscle fibres from cold-acclimated carp. Note the higher volume fraction of mitochondria compared with fibres from warm-acclimated fish. Scale bars= 5 μm.


S
MY
C
C

The effects of temperature acclimation on the dimensions of myofibrils

The mean radius of myofibrils was 8% lower for cold- than warm-acclimated fish ($P<0.001$, Fig. 7 A and B).

The mean cross-sectional area ($\mu m^2 \times 10^{-3}$) of myofibrils in fibres from warm- and cold-acclimated fish was 406 $\pm$ 8 (546) and 373 $\pm$ 7 (539) respectively (mean $\pm$ s.e. number of myofibrils for all measurements in brackets). The mean cross-sectional area of myofibrils in fibres from cold-acclimated fish was therefore 9% lower than for myofibrils in fibres from warm-acclimated fish ($P<0.001$). This result indicates that the higher volume fraction of myofibrils found in cold-acclimated fish (Table 2) must consist of an increased number of myofibrils compared to fibres from warm-acclimated fish.

The similarity between the mean perimeter values for myofibrils from warm- and cold-acclimated fish (2.6 $\mu m \pm$ 0.02 and 2.5 $\mu m \pm$ 0.02 respectively, mean $\pm$ s.e.) suggests that myofibrils from cold-acclimated fish have a higher surface area to volume ratio than myofibrils from warm-acclimated fish.

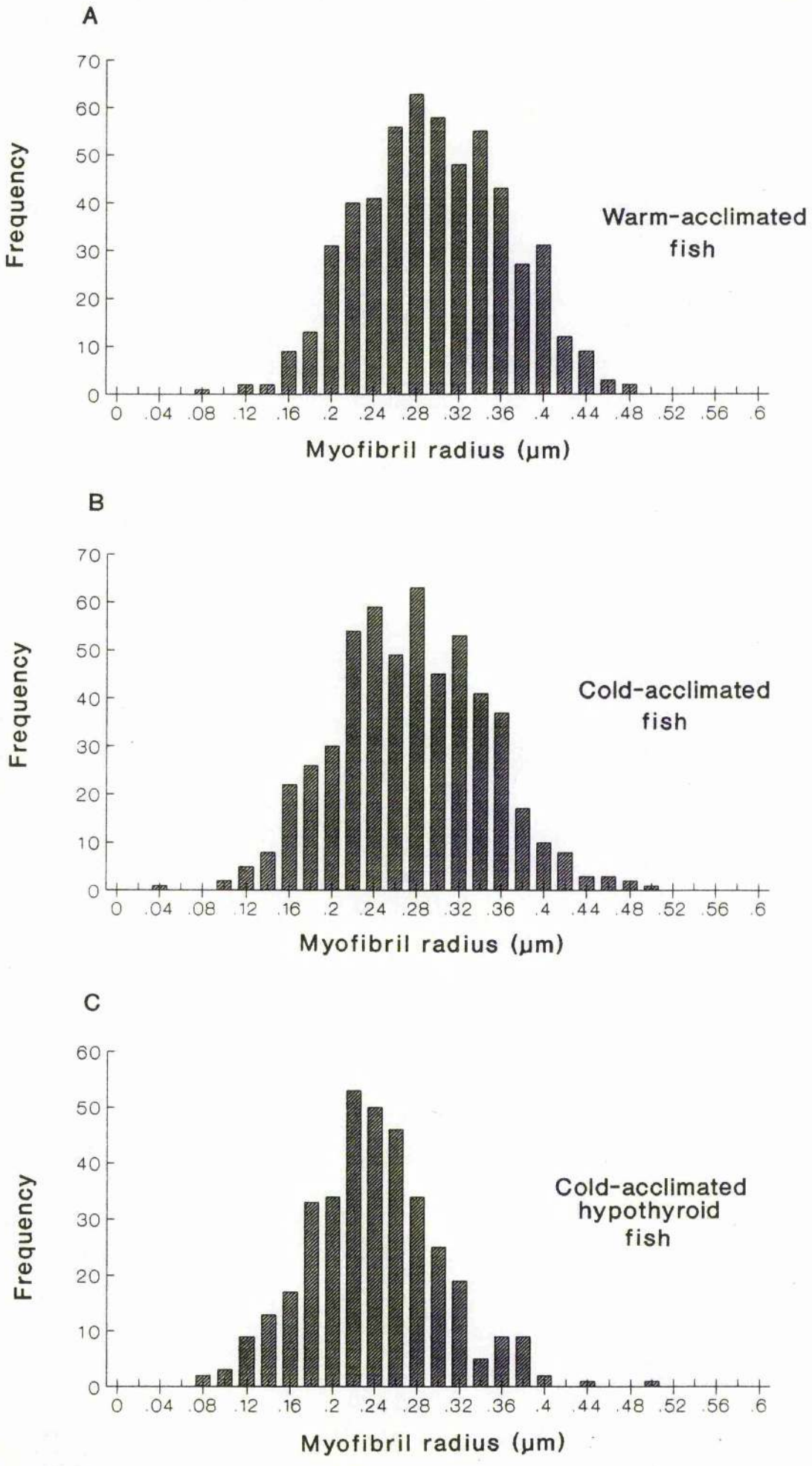

The frequency distribution of myofibril radius
A
Frequency
70
60
50
40
30
20
10
0
0 .04 .08 .12 .16 .2 .24 .28 .32 .36 .4 .44 .48 .52 .56 .6
Myofibril radius (μm)
Warm-acclimated fish
B
Frequency
70
60
50
40
30
20
10
0
0 .04 .08 .12 .16 .2 .24 .28 .32 .36 .4 .44 .48 .52 .56 .6
Myofibril radius (μm)
Cold-acclimated fish
C
Frequency
60
50
40
30
20
10
0
0 .04 .08 .12 .16 .2 .24 .28 .32 .36 .4 .44 .48 .52 .56 .6
Myofibril radius (μm)
Cold-acclimated hypothyroid fish

Fig 7

The effects of hypothyroidism on the ultrastructure of slow muscle fibres from cold-acclimated carp

Fibres analysed from euthyroid and hypothyroid fish had similar cross-sectional areas (μm^2) of 284 $\pm$ 30 and 371 $\pm$ 57 respectively (mean $\pm$ s.e.).

The volume fraction of myofibrils was 9% higher in fibres from euthyroid than hypothyroid fish ($P<0.001$, Table 3. Fig. 9 and 10).

The total volume fraction of mitochondria in fibres from euthyroid and hypothyroid fish was not significantly different ($P>0.05$, Table 3). However the volume fraction of intermyofibrillar mitochondria in muscle fibres from euthyroid fish was 1.4-fold higher than for fibres from hypothyroid fish ($P<0.01$, Table 3). This result may be affected by the lower volume fraction of myofibrils in hypothyroid compared with euthyroid fish. The sub-sarcolemmal zone contained around 75% of the total volume fraction of mitochondria in fibres from both euthyroid and hypothroid fish.

The effects of hypothyroidism on the dimensions of myofibrils

Without exception all dimensions of myofibrils from hypothyroid fish were lower than those observed for

Table 3
The effects of hypothyroidism on the volume fraction of myofibrils and mitochondria in cold-acclimated carp.

Parameter	Cold-acclimated fish	Cold-acclimated (hypothyroid) fish
A(f), (μm^2)	284.5 $\pm$30	371.0 $\pm$57 ns
V_v(myf,f) %	63 $\pm$1	54 $\pm$2 ***
V_v(mit_{inter}, f) %	7.15 $\pm$0.45	5.24 $\pm$0.54 **
V_v(mit_{sub}, f) %	17.56 $\pm$0.78	17.46 $\pm$0.66 ns
V_v(mit_{tot}, f)	24.59 $\pm$0.79	22.73 $\pm$0.98 ns

A(f), mean cross-sectional area of fibres. V_v, entries are fraction of cell volume (volume density) occupied by V_v(myf,f), myofibrils; V_v(mit_{inter}, f), mitochondria in intermyofibrilar zone; V_v(mit_{sub}, f), mitochondria in subsarcolemmal zone; V_v(mit_{tot}, f), total mitochondria. ** = statistically significantly different at $P<0.01$; *** $P<0.001$; ns = not significant at $P<0.05$. Values are mean $\pm$ s.e. of 24 electron micrographs from 5 fish.

euthyroid fish. For example, the mean radius (nm) of myofibrils from hypothyroid fish was 249 ± 3 (365) (mean ± s.e. number of myofibrils in brackets), 17% lower than for myofibrils from euthyroid fish ($P<0.001$, Fig. 7C).

The frequency distribution of myofibril radius for hypothyroid fish showed a pronounced shift towards lower values compared with results from euthyroid fish (Fig. 7 B and C).

The mean cross-sectional area of myofibrils from hypothyroid fish was 279 ± 7 $\mu m^2 \times 10^{-3}$ (mean ± s.e). This was 34% lower than the mean cross-sectional area of myofibrils from euthyroid fish ($P<0.001$). The mean perimeter of myofibrils from hypothyroid fish was 2.15 µm ± 0.03 (mean ± s.e); 17% lower than for myofibrils from euthyroid fish ($P<0.001$).

The effects of temperature acclimation and hypothyroidism on the morphology of mitochondria

The volume and surface densities of mitochondria cristae, relative to mitochondria volume, were about 28% and 31 $\mu m^2/\mu m^3$ respectively. The volume and surface density of mitochondria cristae were not affected by either temperature acclimation or hypothyroidism (Table 4, Fig. 11).

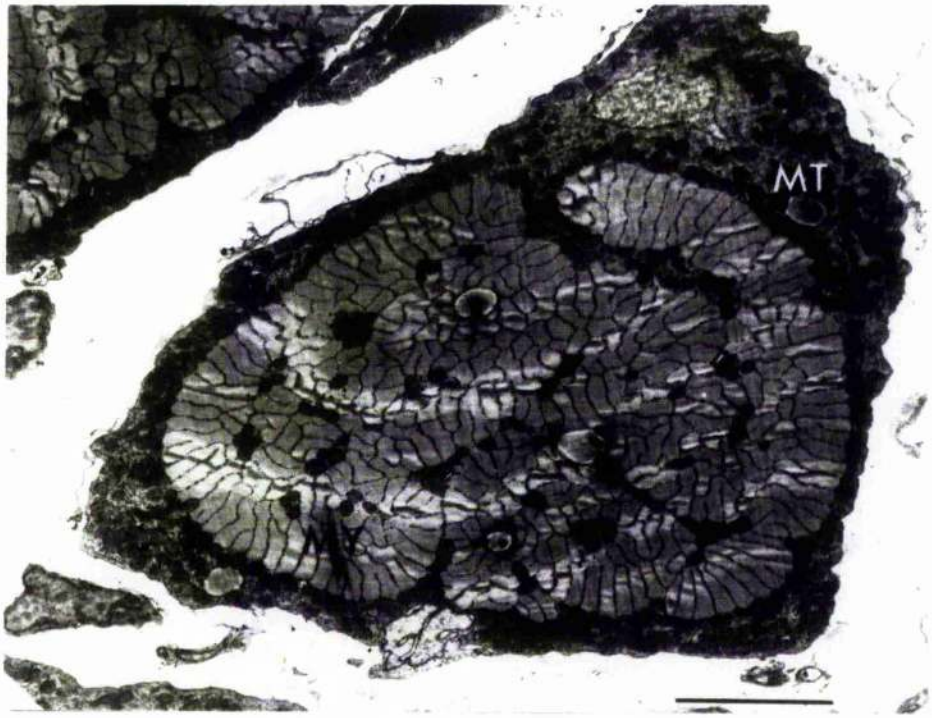
Table 4
The effects of temperature acclimation and hypothyroidism on the volume and surface density of cristae in mitochondria from slow muscle fibres of the carp.

Parameter	Acclimation group	mean ± s.e.
Volume	Cold	26.1 ±2.0 (30)
density	Warm	31.3 ±2.6 (25)
cristae	Cold (hypothyroid)	27.4 ±1.8 (24)
%		
Surface	Cold	33.1 ±2.1 (29)
density	Warm	32.2 ±2.1 (25)
cristae	Cold (hypothyroid)	30.8 ±1.6 (25)
(μm^{-1})		

Number of mitochondria analysed from 5 fish is shown in brackets. Values are mean ± s.e. Comparisons of the volume and surface density of cristae, expressed per unit volume of mitochondria were not statistically significant at $P<0.05$ level.

Fig. 10

Electron micrographs of transversely sectioned slow muscle fibres from cold-acclimated hypothyroid carp. Scale bars= 5 µm.


MT
MY

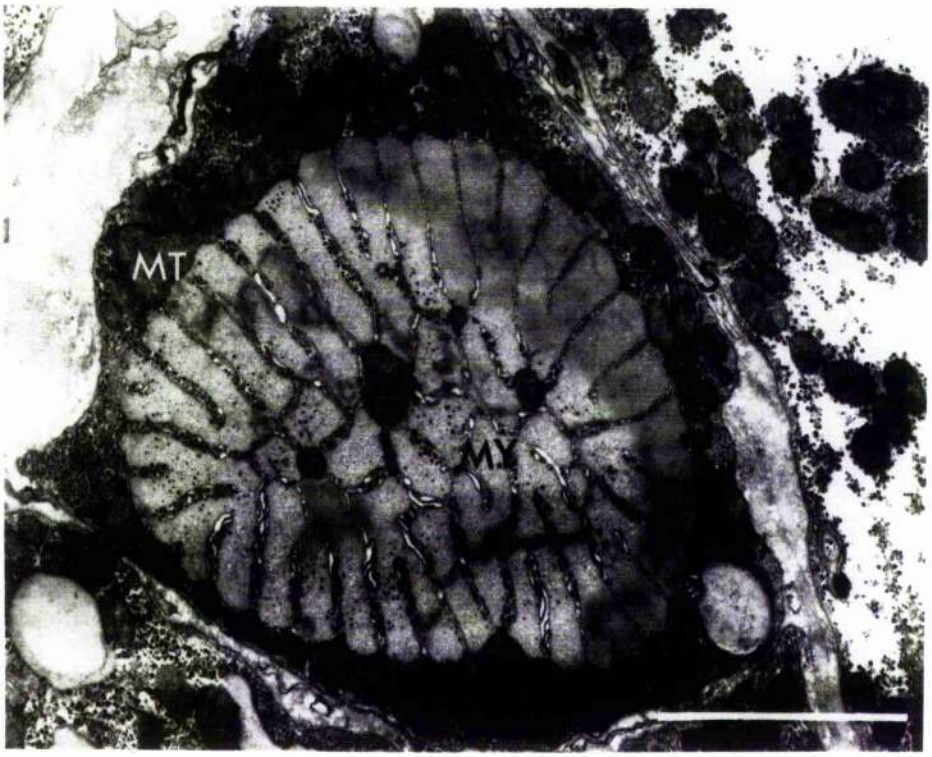
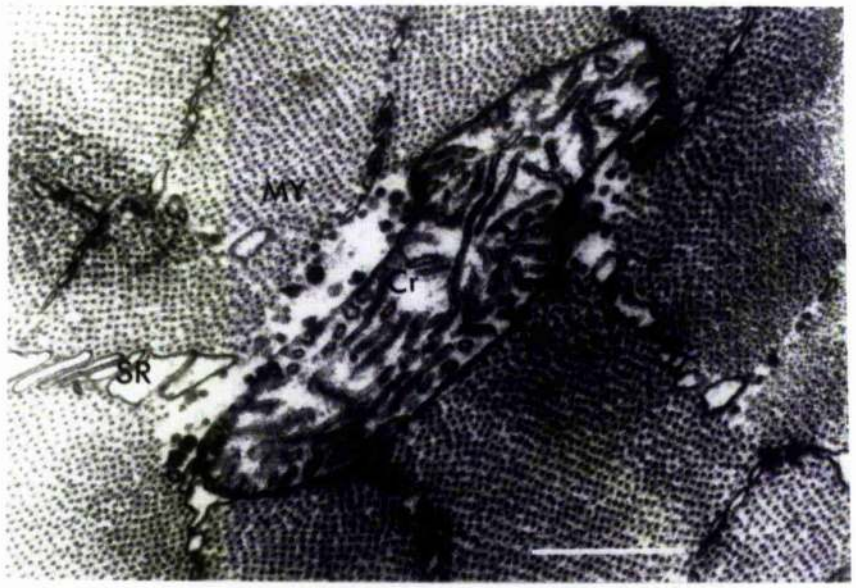
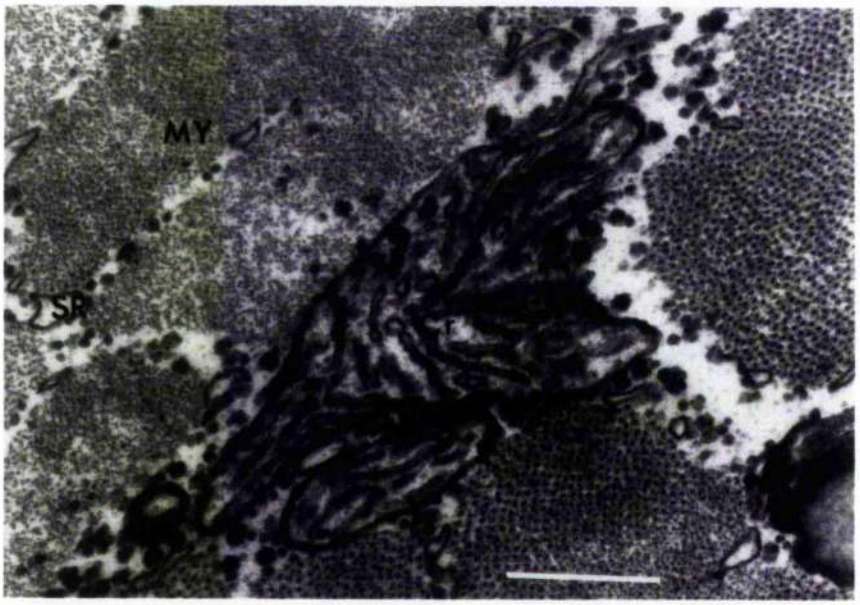
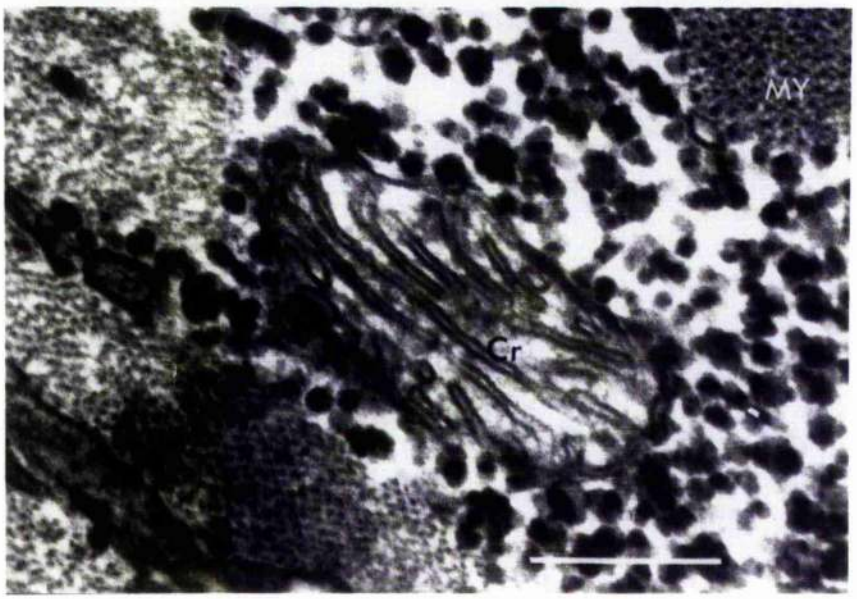

MT
MY

Fig. 11

Electron micrographs of mitochondria from fibres of (A) warm- (B) cold- and (C) cold-acclimated hypothyroid fish. Cr= Cristae; SR= sarcoplasmic reticulum; MY= myofibrils. Scale bars= 0.25 μm.


MY
Cr
SR


MY
SR


MY
Cr

Discussion

Considerable research into the structure and function of the thyroid gland in teleosts has been unable to elucidate its role, if any, in coordinating physiological responses to environmental stimuli (reviewed by Eales, 1979; Higgs *et al.*, 1982). Studies have shown that the teleostean thyroid is basically similar to that of the mammal.

In mammals and fish, blood plasma contains at least three thyroid hormones, L-thyroxine (T4), 3,5,3' -triiodo-L-thyronine (T3) and 3,3',5' -triiodo-L-thyronine or reverse T3 (rT3). Stimulation of the thyroid releases T4 into the blood by the hydrolysis of colloidal thyroglobin stores present within the thyroid cell. Small amounts of T3, are either derived directly from thyroglobin hydrolysis, or from intrathyroidal monodeiodinaton of T4 (Chopra, 1978). However, most T3 is generated extracellularly by monodeiodination of T4 in tissues such as the kidney and the liver.

In the present study mean levels of T4 were similar for warm- and cold-acclimated carp at around 2.5-3.5 ng/ml suggesting that T4 does not have a role in temperature acclimation. In every case the level of T4 was higher than the corresponding level of T3. This is consistent with the view that the thyroid gland of fish produces mainly T4.

Levels of thyroid hormones in the present study are low but comparable with those reported in the literature. For

example, Hurlburt (1977) reported T4 levels for the goldfish of 5-10 ng/ml and Higgs and Eales (1973) found levels of T4 of less than 10 ng/ml in 10 species of freshwater teleost. Some of the highest levels of thyroid hormones were found in immature Oncorhynchus kisutch with levels of T4 and T3 reaching 24 ng/ml and 17 ng/ml respectively. These values are among the highest reported for any fish (Leatherland and Sonstegard, 1980). Reported levels of T4 in rainbow trout vary from 13 ng/ml (Leatherland, Cho and Slinger, 1977), to values as low as <0.1-5 ng/ml (Brown and Eales, 1977).

There is often considerable variation in the level of thyroid hormones between individual fish and there is growing evidence supporting diel variation. Several studies suggested that diel fluctuations in T3 and/or T4 occur in salmonids and goldfish, with peak levels of thyroid hormone occuring during the light phase of the photoperiod (White and Henderson, 1977; Spieler and Noeske, 1979). In contrast, Osborn et al. (1978) reported a diurnal rhythm in plasma T4 of rainbow trout held in out-door ponds. Peak levels of T4 occured from 1 to 5 hours after sunset according to season. In the present study, samples of blood were taken at the same time each day. This should minimise variation in the level of throid hormone due to diurnal fluctuation.

In addition to short term variation, levels of thyroid hormone may fluctuate seasonally, perhaps due to an involvement of the thyroid gland in spawning. For example,

levels of T4 and T3 in brook trout varied between 0.8-3.7 ng/ml and 2.4-8.6 ng/ml respectively over a 12 month period with maximal levels of hormones occuring in mid spring and minimal levels in early November at the time of spawning (White and Henderson, 1977).

In trout, up to 70% of plasma T4 may be rapidly deiodinated extrathyroidally to T3. This is consistent with the hypothesis that T4 plays a pro hormone role (Eales, 1979). In the present study, the mean level of T3 was 2-fold higher in warm- than cold-acclimated carp suggesting that the level of T3 may be involved in temperature acclimation.

Equally, an increase in the level of T3 at higher temperature may be due to the effects of temperature on the peripheral monodeiodination of T4 to T3 (Eales, 1979; Eales et al., 1980). Recent in vitro studies on liver and kidney homogenates from Salmo gairdneri showed that the catalysis of T4 to T3 is dependent on both temperature and pH (Leatherland, 1981). Other factors may also modify the extrathyroidal conversion of T4 to T3 including the size of ration and composition of food (Higgs and Eales, 1978, 1979), and the synergistic effects of other hormones (e.g. testosterone) (Hunt and Eales, 1979). All of these factors will tend to increase the level of T3 in warm- than cold-acclimated fish.

An increase in acclimation temperature increases the metabolic rate in poikilotherms. This results from the

direct effects of temperature on the rate of numerous biochemical reactions including those of the thyroid gland itself. Thus increased rates of radioiodide uptake in warm-acclimated fish (Eales, 1964; Drury and Eales, 1968; Smith and Eales, 1971) may, as Eales (1964) suggested, simply reflect a response of the thyroid to temperature without an effect mediated *via* the hypothalamus and pituitary.

Several studies have shown an inverse relationship between the height of epithelial cells (thought to be positively correlated with secretory activity) and ambient temperature (Swift, 1960; Drury and Eales, 1968; Eales, 1964; Leatherland *et al.*, 1977, 1980). Eales (1964) suggested that the height of epithelial cells may simply indicate the degree of TSH stimulation. Thus at lower temperatures the thyroid gland is stimulated by the hypothalamus-pituitary axis to produce T4 and T3 to satisfy the requirements of the animal whereas at higher temperature the thyroid gland is "free-running" (ie independent of TSH stimulation) and temperature regulated.

This mechanism may also explain discrepancies between the histological appearance of thyroid cells and thyroid activity assessed by other methods (Gorbman, 1969).

There have been very few studies of appropriate goitrogen doses for fish (Chua and Eales, 1971; Kinnear, 1960). Malyusz and Thiemann (1976) found that thiourea administered *in vitro* to gill and kidney of *Scylinhorinus*

inhibited almost all enzymes studied. In the present study methimazole effectively reduced the level of T3 below the limit of detection in 50% of the hypothyroid fish without any visible signs of distress. The level of T4 was not affected but since methimazole inhibits the iodoperoxidase that catalyses the deiodonation reactions converting T4 into T3 it is probable that fish treated with methimazole were hypothyroid. No side effects have been reported following the use of methimazole to induce hypothyroidism in fish.

The effects of temperature acclimation on the percentage cross-sectional area of fibre types in the m. hyohyoideus muscle

The higher percentage cross-sectional area of slow fibres in muscles from cold- compared with warm-acclimated fish coupled with a significant lowering of the level of T3 suggests that the level of T3 may be a factor in determining the fibre composition of this muscle. However a further reduction in the level of T3 in hypothyroid compared with euthyroid fish resulted in a lowering of the percentage cross-sectional area of slow fibres and a significant increase in the proportion of fast fibres to values similar to muscles from warm-acclimated fish. This result suggests that the level of T3 is not directly determining the composition of the muscle.

However recent studies suggest that a minimum level of T3 may be required to induce the poliferation/ growth of

muscle tissue. Recently, Matty et al., (1982) showed that the effect of thyroid hormones on protein and nucleic acid metabolism in liver and muscle tissue from tilapia and carp was biphasic and enhanced by an increase in temperature. Single injections of T3 and T4 were anabolic at lower doses (0.5µg/g body weight) while at high (2µg/g) doses they became catabolic on the level of both protein and RNA in liver and muscle.

Other reports have shown that the sensitivity of tissues to T3 and T4 may vary depending on the physiological status of the animal. For example, Regard, Taurog and Nakashima, (1978) showed that during prometamorphosis of the toad Rana catesbeiana, levels of T4 and T3 were at their lowest but sensitivity to thyroid hormone was at its highest. Yoshizato and Frieden (1975) suggested that increased sensitivity to T3 might be associated with an increased concentration of T3 nuclear receptors.

The changes in the percentage cross-sectional area of fibre types observed in the m. hyohyoideus muscle with temperature acclimation may be explained by a mechanism that assumes that the synthesis of proteins associated with slow fibres is more reponsive to T3 than the synthesis of proteins characteristic of fast fibres.

In warm-acclimated fish the level of T3 may increase due to the effects of high temperature on the biochemical reactions involved in hormone production. TSH production will be supressed by the action of T3 on the hypothalamus.

Following a drop in temperature the level of thyroid hormone will drop, stimulating the production of TSH which will then stimulate the thyroid gland to produce levels of T3 characteristic of the cold-acclimated state. If the production of proteins characteristic of slow fibres is relatively more responsive to lower (anabolic) levels of T3 there will be an increase in the proportion of slow compared to fast fibres (Table 1). This mechanism may also explain the percentage composition of muscles from hypothyroid fish as a chronic low level of T3 will tend to reduce the growth of all muscle (see below) but the effect will be relatively greater for slow fibres resulting in a lowering of the proportion of slow to fast muscle.

Several studies have reported an increase in the proportion of oxidative fibres relative to fast-glycolytic fibres in muscles from hypothyroid rats (McKeran et al., 1975; Wiles et al., 1979; Nwoye and Mommaerts, 1981; Nwoye et al., 1982; Everts, 1983). Similar results were reported for myotomal muscle of cold- compared with warm-acclimated goldfish (Johnston and Lucking, 1978; Sidell, 1980) and striped bass (Jones and Sidell, 1982). Further work is required to determine if thyroid hormones have a role in determining the fibre composition of muscles following temperature acclimation.

It is conceivable that T3 and T4 have no effect on muscle per se, but instead influence the activity of the motor neurons supplying the muscle. Many experiments with

mammalian twitch fibres have implicated the pattern of motor neurone activity as a major determinant of muscle phenotype. For example, chronic electrical stimulation with a slow motor firing pattern (10Hz) transformed the contractile membrane and metabolic properties of fast-glycolytic muscles toward that of a slow muscle (Lomo, 1976; Pette, 1980).

Electromyographical studies of myotomal muscles from common carp showed an increase in the electrical activity of fast fibres at low temperature and at relatively low swimming speeds compared to similar fibres in carp at higher temperature (Rome et al., 1984). An increase in the electrical activity of fast muscle in the cold could partially explain the relative increase in the proportion of slow to fast fibres in the m. hyohyoideus muscles from cold- compared with warm-acclimated fish.

In contrast, deinnervation experiments on muscles from the rat suggest that thyroid hormones act directly on muscle, perhaps by altering the rate of transcription of mitochondrial and other proteins, rather than through the nervous system (Winder et al., 1980). It is likely that several mechanisms are involved in determining the composition of muscle following temperature acclimation but more work is required to show whether there is a causal relationship between the level of thyroid hormones and the cellular reponses observed.

The effects of temperature acclimation on the ultrastructure of slow myotomal muscle fibres

The increase in the volume fraction of myofibrils in fibres from cold- compared with warm-acclimated fish (Table 2) is at variance with the results presented by Johnston and Maitland (1980) for 28 °C- and 2 °C-acclimated crucian carp. However a higher volume fraction of myofibrils following cold-acclimation may partially explain the 2-fold increase in power output of skinned fibres from cold- compared with warm-acclimated common carp (Johnston et al., 1985).

The increase in the volume fraction of mitochondria following cold-acclimation (Table 2) was less than previously reported (crucian carp, Johnston and Maitland, 1980; Johnston, 1982; goldfish, Tyler and Sidell, 1984). Some authors have suggested that mitochondria in the subsarcolemmal and intermyofibrillar zones represent biochemically and functionally distinct populations (Pette, 1966; Muller, 1976). Mitochondria in the subsarcolemmal zone may provide energy for active transport of glucose and amino acids etc. across the sarcolemma while those in the intermyofibrillar matrix supply ATP for muscle contraction (Pette, 1966; Kubista et al., 1971; Muller, 1976). The 2-fold increase in the volume fraction of intermyofibrillar mitochondria in fibres from cold- compared to warm-acclimated carp (Table 2) may increase the rate at

which ATP is made available for muscle contraction and thus may partially explain the near doubling of the rate of contraction, at low temperature, in fibres from cold- compared to warm-acclimated common carp (Johnston et al., 1985).

An increase in the total volume fraction of mitochondria may reduce the diffusion path length of oxygen and other metabolites in fibres from cold- compared with warm-acclimated fish (Egginton and Sidell, 1989). This may offset the effects of increased viscosity of the cytosol and a reduction in the rate of diffusion of small molecules that will occur following a drop in temperature (Kushmerick and Podolsky, 1969; Tyler and Sidell, 1984; Egginton and Sidell, 1989).

The volume and surface density of cristae per mitochondrion was unaffected by temperature acclimation. Similar results were found for myotomal muscle from warm- and cold-acclimated goldfish (Tyler and Sidell, 1984). Enhanced aerobic capacity following cold-acclimation is partially due to an increase in the oxygen consumption of isolated mitochondria (Thillart and Modderkolk, 1978). A similar surface density of cristae in mitochondria from warm- and cold-acclimated carp suggests that either enzymes are more densely packed within the cristae following cold-acclimation or homeoviscous adaptation of the mitochondrial membrane permits higher enzyme activity at low temperature (Wodtke, 1980, 1981).

The effects of temperature acclimation on the dimensions of myofibrils from slow fibres of the carp

A reduction in the mean radius and mean cross-sectional area of myofibrils following cold-acclimation may reduce the distance between the SR and the contractile proteins. This may enhance the rates of contraction and relaxation of muscle by speeding the rate of diffusion of Ca^{2+} between SR and myofibrils. Penney and Goldspink, (1980) interpreted similar results for fast fibres of Carassius carassius L. as showing an increase in the surface density and distribution of SR throughout the myofibrillar mass in fibres from cold- compared with warm-acclimated fish (see chapter 2).

The effects of hypothyroidism on the ultrastructure of slow myotomal muscle following cold-acclimation

The lower volume fraction of myofibrils in fibres from hypothyroid fish may reduce the maximum force generated per fibre compared to fibres from euthyroid fish (Table 3). The effects of altered thyroid status on the mechanics of fish muscle are unknown but in the soleus muscle of the rat force was either decreased (Fitts et al., 1980) or increased (Nicol and Bruce, 1981) by 25-40% after 6 weeks treatment

with T3. In contrast, an alteration in the state of the thyroid did not affect the generation of force in either man (Takamori et al., 1971) or the rat (Grossie et al., 1978; Nicol and Bruce, 1981; Everts, 1983). More research is needed to assess the effects of hypothyroidism on the performance of fish muscle.

The observation that muscles used during prolonged effort were affected more by thyroid disease than muscles involved in burst activity has directed attention towards mitochondria as possible targets for thyroid hormones (Gustafsson et al., 1965). The results (Table 3) show a lower volume density of intermyofibrillar mitochondria in hypothyroid compared with euthyroid fish. This may indicate that T3 is involved in the proliferation of intermyofibrillar mitochondria following cold-acclimation but since the volume densities of both subsarcolemmal and total mitochondria are similar in hypo-and euthyroid fish, it is more likely that the volume fraction of intermyofibrillar mitochondria has been indirectly lowered by the decrease in the fractional volume of myofibrils.

The volume and surface density of cristae within mitochondria from hypothyroid and euthyroid fish remained similar (Table 4). In mammals, an increase in the population of mitochondria resulting from altered levels of T3 or T4 is invariably accompanied by an increase in the surface density of cristae. This indicates both a relative and absolute enlargement of the entire surface of the

cristae per unit volume of fibre (Everts, 1983; Frey and Skjorten, 1967; Gustafsson et al., 1965; Meijer, 1972). These studies may provide further evidence that higher fractional volumes of mitochondria in fibres from cold- compared with warm-acclimated fish are not due to changes in the state of the thyroid.

The effects of hypothyroidism on the dimensions of myofibrils from cold-acclimated fish

Proteins of animal tissue exist in a dynamic steady state, undergoing continuous synthesis and degradation (Goldberg et al., 1974). The lower mean radius, cross-sectional area and perimeter of myofibrils in fibres from hypothyroid compared with euthyroid fish are consistent with a lowering in the rate of synthesis compared to degradation of myofibrillar protein.

Thornburn and Matty, (1963) and Narayansingh and Eales, (1975) followed the incorporation of labelled amino acids into tissue of goldfish and rainbow trout and concluded that both T4 and T3 stimulate protein synthesis.

Although thyroid hormone receptor sites have been claimed to be present on mitochondrial and plasma membranes (Sterling et al., 1977; Pliam and Goldfine, 1977), most thyroid action is thought to depend on the stimulation of DNA-dependent RNA synthesis and subsequent protein

synthesis. This stems from a direct interaction of T3 with a saturable high-affinity acidic-protein receptor on the nuclear chromatin (Oppenheimer and Dillerman, 1978; Bernal and DeGroot, 1980). In this respect T4 is regarded largely as an inactive precursor, or prohormone for T3. The stimulatory mechanism of T3 and T4 on protein synthesis is not clear although Neilsen and Haschmeyer (1977a, b) report that T3 elevates elongation factor 1 in liver post ribosomal supernatants of Opsanus.

The higher levels of T3 in warm- than cold-acclimated fish may stimulate the synthesis of protein and result in an increase in the mean radius of myofibrils in warm- compared with cold-acclimated fish (Fig. 7A, B). The results (Fig. 7) clearly show that hypothyroidism shifts the whole distribution of myofibril radius to lower values. This shift is consistent with a lowering of protein synthesis at reduced levels of T3. Furthermore, Matty et al., (1982) reported that neither T3 nor T4 induced protein synthesis in goldfish at low (15 °C) temperature. Lone et al., (1983) showed that this was not due to low temperature blocking the translocation of T4 from cytoplasm to nucleus by injecting juvenile carp with ^{125}I-T4. Perhaps the coupling of T4 (T3) with chromatin is dependent upon a temperature sensitive nuclear enzyme system (Lone et al., 1983).

In conclusion, thyroid hormones may be involved in determining the percentage composition of fibre types in fish muscles following cold-acclimation. On the other hand

it is doubtful that thyroid hormones have a role in the proliferation of mitochondria in fish following acclimation to the cold. The lower mean radius of myofibrils from cold- compared with warm-acclimated fish may be due to a reduction in the rate of synthesis relative to degradation of myofibrillar protein as a result of low levels of thyroid hormone. More research is required to determine how the responsiveness of different tissues to thyroid hormones varies with temperature and temperature acclimation.

CHAPTER 5

General discussion

Large seasonal fluctuations in temperature experienced by eurythermal temperate zone fishes such as the carp present major metabolic challenges that require physiological compensation if relatively constant biological activity is to be maintained.

A change in temperature directly affects the rate of diffusion and the viscosity of cytoplasm, itself an important determinant of the rate of intracellular diffusion of small compounds (Wojcieszyn et al., 1981; Mastro, Babich, Taylor and Keith, 1985; Gershon, Porter and Trus, 1985).

The importance of these factors to fish that experience significant changes in body temperature becomes apparent when the thermal sensitivity of viscosity of aqueous solutions is considered. For example, the viscosity of pure water (Weast, 1971) and cytosol prepared from fast glycolytic muscle of white perch (Morone americanus) (Sidell and Hazel, 1987) increased by 71% and 80% respectively following a decrease in temperature from 25°C to 5°C. As a result, the diffusive flux of critical metabolites and regulatory ions will be greatly slowed at body temperatures experienced during winter (2 °C-6 °C) compared to temperatures typically experienced during the summer (14-20

°C).

It is theoretically possible that cold-acclimation could result in a concomitant reduction in the viscosity of muscle cytoplasm by alterations in the hydration state of the tissue (Sidell and Hazel, 1987). An increase in the water content of muscle tissue following cold-acclimation is consistent with data reported by Johnston and Maitland (1980) who found that the fractional volume of myofibrils was higher in fibres from 28 °C- than 2 °C-acclimated Crucian carp.

For solutes such as Ca^{2+} that show significant thermal sensitivity of diffusion (Sidell and Hazel, 1987) an increase in the viscosity of the cytosol can account for a significant portion but not the entire temperature effect on their diffusion coefficients (Sidell and Hazel, 1987). Other factors that may contribute to the observed depression of diffusion at lower temperatures include a reduction in the kinetic energy of the system and an increase in the bond strength of ionic and dipole interactions which accompany decreasing temperature. Interactions of this sort may be either increased or decreased depending on intracellular pH. According to the alphastat hypothesis the intracellular pH of muscle will increase with decreasing temperature with a Δ pH /°C temperature coefficient of approximately -0.018 pH unit/° C (Reeves, 1977). However, changes in intracellular pH with temperature are generally lower than this value ranging from -0.003 (Walsh and Moon, 1982) to around -0.018

(Heisler et al., 1976). Alterations in pH may also cause the reversible deprotonation of other ionisable groups within the polyelectrolytic cytosol, making the interaction between charged groups more likely and thereby slowing the diffusion of ions such as Ca^{2+} through the cytoplasm further.

Ca^{2+} released from the sarcoplasmic reticulum (SR) of muscle tissue must be able to diffuse to binding sites on troponin-C to activate contraction (Potter and Gergely, 1975). Similarly, the rate of relaxation of muscle is also dependent upon the rate of diffusion of Ca^{2+} from troponin-C to pump sites located within the SR. A restructuring of the SR that compensates for the effects of low temperature on the diffusion of Ca^{2+} is clearly an attractive hypothesis but this thesis showed that the volume and surface density of SR does not increase in either fast or slow muscle fibres of the carp following cold-acclimation.

McArdle and Johnston (1981) explained higher rates of calcium uptake into the SR of fast fibres by showing that fast fibres had a higher density of calcium pump protein per unit membrane area of the SR compared with slow fibres. A similar mechanism was proposed in the present study to explain the more rapid rate of relaxation, at low temperature, of muscles from cold- than warm-acclimated fish (chapter 2; Heap et al., 1987). More work is needed to elucidate the exact mechanism.

Changes in the complement of regulatory proteins

(Johnston, 1979; Crockford and Johnston, 1989) and in the activity of myofibrillar ATPase following temperature acclimation have been reported for a number of species of fish (Johnston et al., 1975; Johnston and Goldspink, 1980; Heap et al., 1985) and may serve to reduce the effects of low temperature on the processes of contraction and relaxation of muscle.

The present study, showed that the pCa-tension relationship was similar for fibres from warm- and cold-acclimated carp (chapter 3) suggesting that changes in the regulatory proteins following cold-acclimation do not affect the pCa-tension relationship. Reducing temperature from 15 °C to 0 °C at alkaline pH, shifted the pCa-tension relationship to higher concentrations of Ca^{2+}. This shift may enhance the rate of relaxation at low temperature provided the rate of Ca^{2+}-uptake into the SR is maintained in the cold.

Martyn and Gordon, (1988) used glycerinated psoas fibres from the rabbit to investigate the role of myofilament charge and lattice spacing in determining the sensitivity of the contractile apparatus to calcium. A reduction in the spacing of the myofilament lattice and an increase in ionic strength lowered the sensitivity of the contractile apparatus to Ca^{2+}. A lowering in the ionic strength of the cytosol in carp muscle following cold-acclimation may increase the sensitivity of myofibrils to calcium and thus compensate for a reduction in the rate of diffusion of Ca^{2+}.

Further studies into changes in the ionic strength of the cytosol in muscle fibres following temperature acclimation are required to test this hypothesis.

In recent years, several laboratories have reported major changes in the ultrastructure of muscle tissue from fish following temperature acclimation (Johnston and Maitland, 1980; Penney and Goldspink, 1980; Tyler and Sidell, 1984; Egginton and Sidell, 1986; Egginton and Sidell, 1989). These results have prompted the suggestion that cold-induced proliferation of organelles in fish muscle may serve to compensate for the effects of temperature on the rate of diffusion of small compounds between cellular compartments (Sidell, 1983).

Quantitative changes in the populations of muscle organelles during cold-acclimation in fish include increases in 1) the volume density of mitochondria (carp, Johnston and Maitland, 1980; chapter 4; goldfish, Tyler and Sidell, 1984; striped bass, Egginton and Sidell, 1986), 2) the surface density of the SR (Penney and Goldspink, 1980) and 3) a reduction in the mean diameter of myofibrils (Penney and Goldspink, 1980; chapter 4). Such restructuring of muscle will decrease the mean diffusion path length for small molecules and increase the area of contact between membranes from cytoplasmic and organelle compartments. During processing for electron microscopy a greater degree of shrinkage was observed in fibres from warm- than cold-acclimated fish. This is a potential source of error

in the quantification of cellular organelles and should be taken into account before drawing conclusions. Furthermore osmium ferricyanide stain should be prefered for staining membranes since it reveals more of the SR than uranyl acetate / lead citrate stain. Both of these findings have implications for estimating characteristics of muscle involving the quantification of membranes e.g. the T-system, T-SR contact (Akster et al., 1985) and the surface density of cristae within mitochondria.

Many of the compensatory changes in the ultrastructure and protein content of muscle following temperature acclimation can be explained either by considering the differential effect of temperature on the rate of synthesis and degradation of proteins found in muscle (Sidell, 1977) or by the indirect effects of temperature on muscle proteins mediated through systems associated with homeostasis such as the endocrine system.

Studies correlating cyclic structural changes in teleost thyroids with annual environmental cycles of temperature and photoperiod suggest that there are sensory-evoked nervous efferents to the hypothalamus of teleosts that can alter the secretion of thyroid stimulating hormone (TSH) by the pars distalis (Gorbman, 1969). Studies into the variation of thyroid function with changes in temperature are contradictory. For example, the activity of the thyroid parallels changes in temperature in the goldfish (Hoar and Eales, 1963) while in common carp and tench the activity of

the thyroid is unaffected by a change in temperature (Olivereau, 1955). In the present study levels of T4 in carp remained unchanged following temperature acclimation while the level of T3 was significantly lower in cold- than warm-acclimated fish (chapter 4). In vivo, thyroid hormones enhance the incorporation of amino acids into tissues that are otherwise T4-responsive. The synthesis of protein was stimulated at the transcriptional level in studies of hypothyroid animals given exogenous hormone. An increase in the activity of RNA-polymerase was followed by an increase in the synthesis of RNA, particularly ribosomal RNA (Tate and Widnell, 1966). Subsequently, it was demonstrated that thyroid hormones affected the synthesis of protein at the level of translation by enhancing the transfer of amino acid bound to tRNA to ribosomal protein (Sokoloff et al., 1968).

The present study did not find any conclusive evidence to support the involvement of thyroid hormones in temperature acclimation. However, the effects of thyroid hormones on protein synthesis may be important in the restructuring of muscle fibres following cold-acclimation. The lower volume fraction of myofibrils in fibres from hypothyroid than euthyroid fish could be explained by a lowering in the rate of synthesis relative to degradation of myofibrillar proteins. A similar mechanism could also explain the reduction in the mean radius of myofibrils from cold-acclimated and hypothyroid fish compared with their respective controls. Further work needs to be done to

establish if the rates of synthesis and degradation of myofibrillar proteins are altered in hypothyroid compared to euthyroid fish.

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