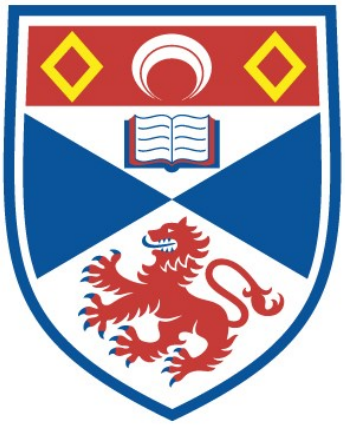
THE INFLUENCE OF TEMPERATURE ON THE MECHANICS AND ENERGETICS OF CONTRACTION IN FISH MUSCLE

Timothy Paul Johnson

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



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THE INFLUENCE OF TEMPERATURE ON THE MECHANICS AND ENERGETICS OF CONTRACTION IN FISH MUSCLE

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

by

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DECLARATION

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

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

Signed: Date: 14/11/90.

CERTIFICATE

I hereby certify that Timothy P. Johnson 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 is qualified to submit the accompanying thesis for the Degree of Doctor of Philosophy.

Signed: Date: 14/11/90

Dedicated to the late Joanna Copley

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SUMMARY

Chapter 1.

A general introduction into the adaptation of teleost fish to changing thermal environments. The underlying mechanisms at all levels of organization are reviewed in relation to adaptive change over evolutionary and seasonal time-scales.

Chapter 2.

1. The contractile properties of swimming muscles have been investigated in marine teleosts from Antarctic (Trematomus lepidorhinus, Pseudochaenichthys georgianus), temperate (Pollachius virens, Limanda limanda, Agonis cataphractus, Callionymus lyra) and tropical (Abudefduf abdominalis, Thalassoma duperreyi) latitudes.

2. Small bundles of fast twitch fibres were isolated from anterior myotomes and/or the pectoral fin adductor profundis muscle (m.add.p). Live fibre preparations were viable for several days at in vivo temperatures, but became progressively inexcitable at higher or lower temperatures. The stimulation frequency required to produce fused isometric tetani increased from 50Hz in Antarctic species at 0°C to around 400 Hz in tropical species at 25°C. Maximum isometric tension (P_o) was produced at the normal body temperature (NBT) of each species (Antarctic, 0-2°C; North Sea and Atlantic, 8-

10°C; Indo-West Pacific, 23-25°C). P_o values at physiological temperatures (200-300 kN m^{-2}) were similar for Antarctic, temperate and tropical species.

3. A temperature induced "tension hysteresis" was observed in muscle fibres from the temperate and Antarctic species. Exposure to temperatures below 0-2°C resulted in the temporary depression of tension over the whole experimental range, an effect reversed by incubation at higher temperatures.

4. At NBT's the half-times for activation and relaxation of twitch and tetanic tension increased in the order Antarctic > temperate > tropical species. Relaxation was generally much slower at temperatures < 10°C in fibres from tropical than temperate fish. Q_{10} values for these parameters at NBT's were 1.3-2.1 for tropical species, 1.7-2.6 for temperate species and 1.6-3.5 for Antarctic species.

5. The force-velocity (P-V) relationship was studied in selected species using iso-velocity releases and the data below $0.8P_o$ iteratively fitted to Hill's equation. The P-V relation at NBT was found to be significantly less curved in Antarctic than temperate species.

6. The unloaded contraction velocity (Vmax) of fibres was positively correlated with NBT increasing from about 1 muscle lengths s^{-1} in an Antarctic fish (Trematomus lepidorhinus) at 1°C to around 16 muscle lengths s^{-1} in a tropical species (Thalassoma duperreyi) at 24°C.

7. It was concluded that although muscle contraction in Antarctic fish shows adaptations for low temperature function, the degree of compensation achieved in shortening speed and twitch kinetics is relatively modest.

Chapter 3.

1. Fast and slow muscle fibres were isolated from the myotomes of atlantic cod (Gadus morhua L.) and short-horned sculpin (Myoxocephalus scorpius L.).

2. Epinephrine was found to have no effect on twitch or sub-tetanic contractions in fast muscle fibres.

3. Isoprenaline (10^{-6}M) had no effect on the contractility of slow muscle fibres.

4. In contrast, epinephrine elicited a dose-dependent decrease in the half-time for twitch relaxation ($t_{\frac{1}{2}}r$), and in most cases a decrease in twitch amplitude. The maximum decrease in $t_{\frac{1}{2}}r$ was around 5-20% of control values (at 10^{-6}M epinephrine), with a half maximal response at about 30 nmol l^{-1}.

5. Responses to epinephrine were unaffected by propranolol and reversed by phentolamine, consistent with the stimulation of α-adrenoreceptors.

6. 10^{-6}M epinephrine produced a rise in cAMP levels from 1.8 to 3.1 pmol mg dry wt^{-1} in cod slow fibres. However, the cellular mechanism underlying the action of ephinephrine is unclear since forskolin, a potent activator of adenylate cyclase activity where it has been

investigated, was found to increase not decrease twitch duration and amplitude.

7. The responses of fast and slow fibres to epinephrine and its antagonists were similar in summer (13°C) and winter acclimatized (5-6°C) sculpin.

8. It is suggested that epinephrine may act to modulate the active state of slow muscle fibres at high cruising speeds and thereby increase swimming performance.

Chapter 4.

1. Fast muscle fibres were isolated from the abdominal myotomes of the short-horned sculpin, Myoxocephalus scorpius L. Sinusoidal length changes were imposed about resting muscle length and fibres stimulated at a selected phase during the strain cycle. The work output per cycle was calculated from the area of the resulting force-position loops.

2. The strain amplitude required for maximum work per cycle had a distinct optimum at ± 5% resting length which was independent of temperature.

3. Maximum positive work loops were obtained by retarding the stimulus relative to the start of the length-change cycle by 30° (full cycle = 360°). The maximum negative work output was obtained with a 210° stimulus phase shift. At intermediate stimulus phase shifts, work loops became complex with both positive (anti-clockwise) and negative (clockwise) components. The relationship between stimulus phase shift and work

output was similar for summer and winter acclimatized fish.

4. The number and timing of stimuli were adjusted, at constant strain amplitude (± 5% resting muscle length), in order to optimize net positive work output over a range of cycle frequencies. The cycle frequency that was required for maximum power output (work per cycle times cycle frequency) increased from around 5-7 Hz at 4°C to 9-13 Hz at 15°C.

5. The maximum tension generated per cycle at 15°C, was around 2-times higher at all cycle frequencies in summer relative to winter acclimatized fish. Fast muscle fibres from summer fish also produced consistently higher tensions at 4°C, but the differences were only significant at 15 Hz.

6. The phase delay between peak length and peak force was prolonged at 15°C and high cycle frequencies (9-20 Hz) and reduced at 4°C and low cycle frequencies (3 Hz) in summer relative to winter acclimatized fish.

7. The maximum power output of muscle fibres showed little seasonal variation at 4°C and was in the range 20-25 W kg^{-1}. In contrast, at 15°C, maximum muscle power output increased from 9W kg^{-1} in the winter to 30 W kg^{-1} in the summer acclimatized fish.

Chapter 5.

1. Bundles of 20-30 fast muscle fibres were isolated from the abdominal myotomes of the short-horned sculpin (Myoxocephalus scorpius L.).

2. The energy cost of contraction was measured during oscillatory work at 4°C and 15°C following treatment with iodoacetate and nitrogen gas to block glycolysis and aerobic metabolism.

3. Isolated fibres were subjected to sinusoidal length changes about in situ resting length and stimulated at a selected phase in the strain cycle. Preliminary experiments with untreated preparations established the strain amplitude and stimulation parameters required to maximize work output over a range of cycle frequencies at 4°C and 15°C.

4. Following oscillatory work treated preparations were rapidly frozen, freeze-dried and the concentrations of phosphocreatine, creatine, ATP, ADP, AMP and IMP measured by High Performance Liquid Chromatography.

5. The concentration of phosphocreatine declined in proportion to the total work done for up to 64 cycles without a significant change in ATP.

6. Maximum power output was produced at a cycle frequency of 5Hz at 4°C (14-18 W/kg) and 17Hz at 15°C (23-27 W/kg). The rate of utilization of phosphocreatine per cycle was independent of temperature. However, since work per cycle was higher at 4°C (2.7-3.7 mJ/g wet weight) than 15°C (1.2-1.6 mJ/g wet weight), the energetic cost of contraction tended to decrease with increasing temperature.

Chapter 6.

The results of the thesis are discussed, particularly in relation to their evolutionary and locomotory significance. From the principles and ideas formulated, future lines of research are suggested.

CHAPTER 1

GENERAL INTRODUCTION

Adaptation refers to specific properties or traits affecting fitness as well as to the processes by which these properties adjust to local conditions. In one sense, adaptation represents the selection of genes coding for traits affecting fitness, but equally the process of adjustment may simply involve the "phenotypic plasticity" of pre-existing traits (Wells, 1990).

The "adaptationist" is faced with numerous pitfalls in the interpretation of biological function and habitat. The organism is not simply a collection of separable compartments independently sensitive to environmental change, but is an integrated whole (Pogson 1987; Wells 1990). Thus, one must be cautious in the simplistic interpretation of individual physiological traits to isolated environmental factors. Not all physiological traits are necessarily adaptive and although some traits may seem non-adaptive this does not render them unintelligible (Wells, 1990). For example, the lack of correlation with environmental factors may simply reflect plasticity of functional capacity. The adoption of the idea that all animals are optimally adapted has been extensively critisized, the aim of adaptive research should be to understand specific examples in terms of

selective forces and the developmental and historical constraints operating (Parker and Maynard Smith, 1990). It is well known that constraints are imposed by phyletic inertia, structure and ontogeny which influence the direction and "tempo" of adaptive change (Pogson, 1987; Wells, 1990). For example, Von Baer (1828) in his fundamental laws of embryology, stated that "the early stages of development are conservative and restrictive of later development". Thus, adaptation should not simply be viewed in the context of an organism's struggle for survival in the face of environmental pressure, but also in its ability to overcome the constraints imposed by the conservatism of certain fundamental physiological mechanisms. On the one hand selective pressure conserves critical properties, whilst on the other drives properties towards change; thus adaptation refers to the best or optimal compromise "in the face of conflicting alternatives" (Hochachka, 1988a).

All physico-chemical processes of living organisms responsible for maintaining the functional activity of cells are influenced to a greater or lesser extent by the all-pervading factor, temperature (Alexandrov, 1977). In the course of evolution a variety of adaptive mechanisms have developed to maintain the relative independence of living processes from changes in temperature over geological and seasonal timescales.

Evolutionary temperature adaptation

Teleosts have successfully exploited a wide range of habitats at temperatures from -1.9°C in polar seas to 44°C in geothermal hot springs. High Antarctic species face body temperatures of as low as -1.9°C and may vary by as little as 0.2°C annually whilst in others such as the mummichog, Fundulus heteroclitus (Sidell et al. 1983), body temperature may exceed 30°C and fall 15°C in less than an hour.

Adaptations occur at all levels of organisation ranging from the molecular to the biocenotic one in response to environmental change (Alexandrov, 1977).

Metabolism, growth and development

Krogh (1916), suggested that cold-adapted animals would increase their metabolic rates to compensate for the effects of low temperature, a phenomenon referred to as "metabolic cold adaptation". Several studies have made measurements of oxygen consumption in fingerlings and adults of polar fish species (Scholander et al. 1953; Wohlschlag 1960, 1964; Ralph and Everson, 1968; Holeton, 1974; Everson, 1977; Smith and Haschemeyer, 1980; Morris and North, 1984). Earlier studies were criticised for failing to account for the effects of stress and activity on oxygen consumption (Holeton, 1974; Clark, 1983; Johnston, 1990). It is likely that a direct test of metabolic cold adaptation is not possible in its present form. For example, tropical and temperate fish either

die or become comatosed at polar temperatures, or alternatively the extrapolation of data from these species to polar temperatures results in a wide range of predicted oxygen consumptions (Johnston et al. 1991). The prediction of elevated rates in cold-adapted species depends upon comparing species of similar life-styles, life-histories and energetic strategies (Cossins and Bowler, 1987). Recent observations of ecologically equivalent fish species from Antarctic, temperate and tropical latitudes have provided some evidence for the partial elevation of metabolic rate (Torres and Somero, 1988; Johnston et al. 1991). Elevated respiratory rates, consistent with cold adaptation have also been found for isolated tissues, including muscle (Lin et al. 1974), brain and gill filaments (Somero et al. 1968). In addition, it would appear that aerobic and lipid catabolizing pathways of energy metabolism in Antarctic fishes are metabolically cold-adapted (Crockett and Sidell, 1990).

It has been suggested that elevated metabolic rates may result from the additional costs incurred at sub-zero temperatures (see below; Johnston et al. 1991). For example, the upward adjustment of functional, transmembrane ion pump densities in polar fishes is thought to have a role in the elevation of metabolic rate (Hochachka, 1988b). However, even with these additional costs the resting consumption of polar fish is still less than that of temperate and tropical fish at normal environmental temperatures (Johnston et al. 1991). By

assuming a simple factorial relationship between maximum and resting metabolic rate, Johnston et al. (1991) found that the energy available for sustained activity is also constrained by low temperature, a factor which may have played a key role in the abscence of fast cruising pelagic fish in Antarctic ecosystems.

Despite the possible small increases in the rates of metabolism and protein synthesis (eg. Smith and Haschmeyer, 1980), from earlier studies it would appear that growth rate in Antarctic fish is still slower than warmer water species (DeVries and Eastman, 1981; Everson, 1984). However, slow growth is not simply a consequence of the direct limitation by temperature. The early life history characteristics of Antarctic Notothenioidei are similar to those reported for fish from other regions, differing only in the size at hatching and metamorphosis and in the duration in the pelagic larval phase (North, 1990). Larval growth of polar fish varies widely from season to season although in some species it is fully comparable to larval fish from warmer waters (North, 1990). Thus it is suggested that overall slow growth rates of polar fish may be due to severe seasonal limitation of food (Clarke, 1983; North, 1990). Recent studies have also suggested that the reliance on muscle fibre hypertrophy for muscle growth in Antarctic fish limits maximum rates, a limitation that may have been set before the radiation of the highly endemic Notothenioidei (Battram and Johnston, 1990). Thus, it is suggested that constraints on growth rate may be phylogenetic, in

addition to food limitation and low temperature (Battram and Johnston, 1990).

Oxygen transport

The blood of Antarctic fishes has fewer erythrocytes and less haemoglobin than tropical and temperate fishes, resulting in a decrease in viscosity and subsequent reduction in the energetic costs of circulation at low temperature (Wells et al. 1980, 1990; Macdonald et al. 1988). Fish from high Arctic latitudes have also been shown to have reduced blood viscosities (Graham et al. 1985). In the Antarctic fish, family Channichthyidae (Ice-fish), haemoglobin and myoglobin are completely absent and the number of erythrocytes is very low (Hureau et al. 1977). Adaptations to compensate for the lack of respiratory pigments include high cardiac output in part acheived through increases in relative heart weights (Johnston et al. 1983b), high blood volume (Hemmingsen and Douglas, 1970) and large capillary cross-sectional area to minimize vascular resistance (Fitch et al. 1984).

Freeze-avoidance; antifreeze proteins

It is known that elevated metabolism may be associated with the synthesis of antifreeze proteins (see above; Macdonald et al. 1987; Johnston et al. 1991). Fishes living in ice-laden water avoid freezing by the depression of the freezing point of body fluids, in part by the increase in blood sodium chloride levels, but mostly through the production of antifreeze proteins.

The initial discovery of these proteins was made by DeVries in the mid-1960's (DeVries, 1970, 1971), and to date five major classes have been identified. The antifreeze proteins depress the freezing point of water via a non-colligative mechanism termed "adsorption-inhibition" (see DeVries, 1988). In contrast to non-Antarctic notothenioids such as Notothenia angustata which cannot be induced to form antifreezes, the kidneys of Antarctic notothenioids are completely aglomerular (Dobbs et al. 1974). It would appear that aglomerular kidneys represent an adaptation to prevent the excretion of antifreeze proteins (DeVries, 1982). In the absence of glomeruli, urine is formed by secretion (DeVries and Eastman, 1981), a process which is likely to contribute to the increased metabolic costs incurred over and above those found in warm water species (see above; Johnston et al. 1991).

The production of antifreeze proteins represents an evolutionary adaptation in marine teleosts from both hemispheres (Scott et al. 1986), although in contrast to Antarctic species (O'Grady et al. 1982a), production is more seasonal in northern species (O'Grady et al. 1982b; Scott et al. 1986; DeVries, 1988; Davies et al. 1988). The diversity of their structure points to disparate evolutionary origins (Davies et al. 1988).

Molecular adaptation

The basic principle underlying adaptive responses to temperature is in the relative semistability or

semilability of main cellular constituents, ie. proteins, nucleic acids and lipids (Alexandrov, 1977). Adaptive change at the molecular level represents the "optimal compromise" between stability and efficiency, the limitations to this compromise being set by the conservatism of certain molecular sub-units critical for basic function (see above; Hochachka, 1988a). Thus, the cold-adaption of an enzyme is acheived by the adoption of a less constrained and rigid structure, whilst maintaining the same basic molecular design required for its specific function. The result is an increase in susceptibility to heat denaturation, although the thermal energy required for enzyme activation is decreased and enzymatic rate increased in the cold (reviewed in Hochachka and Somero, 1984). For example, studies have shown a marked correlation between cell temperature and the thermostability and catalytic activities of different $Mg^{2+}Ca^{2+}$ myofibrillar ATPases isolated from fish skeletal muscle (Johnston et al. 1975; Johnston and Walesby, 1977; Hashimoto et al. 1982); evidence suggests that this mechanism of adaptation involves selective modifications in the structure of heavy and light chains of myosin (Johnston and Walesby, 1979).

The heterogeneity, structure, charge and amino acid composition of Antarctic fish tubulins have been shown to differ from temperate fish species (Detrich and Overton 1986, 1988). Modifications in the hydrophobic interactions of Antarctic fish tubulins have been shown to represent an evolutionary adaptation to enhance their

assembly at low temperature (Detrich and Overton, 1988; Detrich *et al*. 1990). The rate of polymerisation of filamentous actin from globular actin has also been found to be greater at low temperature in polar species (Somero, 1990).

Interestingly, the differences in amino acid composition of Antarctic fish and bovine tubulins although detectable, are only very subtle (Detrich and Overton, 1988). Given the high degree of unity in the structure of molecules in nature such as these, the mechanisms of diversification to some extent still elude us. What is generally accepted is that the explanation for diversity lies in the regulatory, not the structural gene part of the genome (Hochachka, 1988a). The production of heat shock proteins (HSP's) following sudden increases in temperature is a universal feature of eukaryotes and represents a model system with much potential for the study of gene regulation and evolutionary change. Maresca *et al*. (1988) reported heat shock gene activation in *Notothenia rossi* at $5^{o}C$, the lowest temperature at which the heat shock response has been recorded in any eukaryote. Investigations are in progress, for example on the high latitude species, *Chionodraco hamatus*, to correlate heat shock gene regulation with the ability to live at different water temperatures (Harrison *et al*. 1990). In contrast, threshold temperature for the initiation of HSP synthesis in teleost hepatocytes has been shown to be unaltered by acclimation (Koban *et al*. 1987).

Adaptations at the molecular level have also been demonstrated in membrane fluidity, a phenomenon referred to as "homeoviscous adaptation" (Sinensky, 1974). At a given temperature, unsaturated lipids tend to be less viscous than saturated lipids of the same molecular weight. The proportion of unsaturated lipid in cellular membranes of Antarctic nototheniids is greater than that of warmer water species (eg. Bottino et al. 1967) and is strongly correlated with environmental temperature in membrane preparations from northern hemisphere species (Cossins and Prosser, 1978). The resulting increase in membrane fluidity in cold-adapted fish has played an important role in the adaptation of neuromuscular transmission at low temperature in Antarctic species (Macdonald et al. 1987). For example, in the extraocular muscle of Pagothenia borchgrevinki, the decay of minature end plate potentials at ambient temperature (-1.9°C) is 2-6 times faster than extrapolated values for the temperate carangid Trachurus novaezelandiae (Macdonald and Montgomery, 1986). The function of peripheral nerves and the quantal release of neurotransmitter also conform to the widespread pattern of neural cold adaptation in Antarctic fish (Macdonald et al. 1987).

Compensation of motor systems

Eye movements

The coordination of eye movements in opposition to head movement during locomotory activity (vestibulo-ocular reflex) is required to stabilize retinal images

(Walls, 1962). In Antarctic fish eye movements are only partially compensated for at low temperature (Macdonald et al. 1987). At -1.9°C the fastest eye movements of Antarctic species are about half that of temperate species at 14°C (Montgomery and Macdonald, 1984), although eye movements of warm temperate fish extrapolate to zero at 3-4°C and cease at 5°C (Montgomery et al. 1983). Gain, defined as the degrees of eye movement divided by frequency of impulse in the abducens nerve, is actually increased at low temperature (see Macdonald et al. 1987, 1988). Much of the increased low frequency gain may be the consequence of slower twitch responses in extraocular muscles but in addition may result from a decreased viscous coupling in the eye socket and an increase in the elasticity of muscle and connective tissue (Macdonald et al. 1987). The increased neuromuscular gain may also be a consequence of large fibre diameter, high input resistances and probably low internal resistances contributing to membrane constants of 1.5-2 mm in the extraocular muscles of Antarctic fishes (Macdonald et al 1987).

Swimming

Antarctic notothenioids swim at low speed by the sculling movements of pectoral fins (labriform locomotion) powered mainly by the recruitment of slow oxidative fibres (Walesby and Johnston, 1980a). Sub-carangiform locomotion (tail-fin propulsion) powered by fast muscle fibres (Johnston, 1983), is reserved for

burst swimming activity (Montgomery and Macdonald, 1984; Archer and Johnston, 1989).

In slow muscle fibres the density of mitochondria varies with activity and possibly with latitude (Johnston et al. 1988). The increased volume density of mitochondria in Antarctic species may represent an adaptation to overcome the deleterious effects of low temperature on ATP synthesis and the rate of diffusion of metabolites from capillaries to mitochondria and mitochondria to myofibrils (Johnston and Battram, 1990). It is suggested that the maintenance of high mitochondrial volume densities may also contribute to additional metabolic costs in cold compared to warm water adapted species (see above; Johnston et al. 1991).

In some demersal species, including Notothenia neglecta and Chaenocephalus aceratus the energy supply for burst swimming is provided by the utilization of phosphocreatine in fast twitch fibres, the scope for anaerobic glycogenolysis being significantly reduced (Dunn and Johnston, 1986). However, high activities of glycolytic enzymes have been reported in Notothenia gibberifrons (Dunn et al. 1989) and it is therefore likely that the pattern of energy production is determined by lifestyle and locomotory habit, rather than temperature (Dunn, 1988; Johnston, 1989).

Despite the compensation of a number of physiological processes, maximum swimming performance of Antarctic fish shows only partial compensation at low temperature (Montgomery and Macdonald, 1984; Archer and Johnston,

1989). The constraints imposed on swimming ability at low temperature are more noticeable in juvenile and larval fish where maximum tail-beat frequencies and levels of performance are found (Archer and Johnston, 1989; Johnston et al. 1990b). Although tail-beat frequency is low, the compensation of maximum swimming speed in juvenile specimens of Notothenia neglecta may in part result from an increase in tail-beat amplitude (Archer and Johnston, 1989). However, it is suggested that poor burst swimming performance probably reflects incomplete adaptation of the myotomal muscle to low temperature (Archer and Johnston, 1989).

Acclimatization and acclimation

Acclimatization represents compensation of biological functions in response to seasonal change of several interacting factors (temperature, food, salinity etc), acclimation is simply reserved to describe adaptations in response to laboratory induced changes (Prosser, 1973). The distinction is critical, conclusions based on field and laboratory studies may differ significantly. For example, in the golden ide (Leuciscus idus) although adaptive responses in the histological, morphometrical and biochemical properties of liver during winter were in part attributable to temperature and nutrition, the overall response differed profoundly in fish maintained in outdoor ponds and fish subjected to selected

environmental variables in the laboratory (Segner and Braunbeck, 1990). What is generally accepted is that the effects of temperature acclimation are most marked in species living in environments where seasonal variations in temperature are large.

Metabolism; cells, tissues and organisms

Patterns of acclimation have been classified by Precht (1958): 1) over-compensation; 2) perfect compensation, the acclimated rates being the same regardless of acclimation temperature; 3) partial compensation; 4) no compensation, rates simply following a Q_{10} relationship, and; 5) inverse or paradoxical compensation where the acclimated rate in the cold is lower than that resulting from an equivalent, but acute drop in temperature.

The acclimation of whole animal oxygen consumption as described by types 2 and 3 have been observed in several fish species (reviewed in Cossins and Bowler, 1987). In this way, selective advantage is conferred by ensuring the relative constancy of various rate processes despite seasonal fluctuations in temperature.

Inverse or paradoxical adaptations (type 5) have also been reported in several teleost species (reviewed in Hazel and Prosser, 1974; Cossins and Bowler, 1987). This strategy is viewed as a mechanism to reduce energy expenditure during periods of extreme cold and reduced food availability. For example, eel (Walsh et al. 1983), bullhead and striped bass (Crawshaw, 1984) remain torpid and burrow into the mud for up to 6 months in the winter.

Adaptation temperature affects both basal and maximal energy flux, although to date a significant compensation of both standard and active metabolic rate has only been demonstrated in the goldfish Carassius auratus (eg. Fry and Hochachka, 1970). Activity produces a greater effect on total energy output than changes in acclimation temperature (Johnston and Dunn, 1987). Thus to determine the significance of, and underlying mechanisms involved in compensatory adjustments of oxygen consumption it is important to separate the changes in maintenance metabolism and in the capacity of the cellular apparatus to perform work from changes in the level of activity (Cossins and Bowler, 1987). The separation is not always easy, changes in spontaneous activity, muscular tone and irritability complicate interpretation (Cossins and Bowler, 1987).

Despite the problems, compensatory adjustments in oxygen consumption are also observed at the tissue and cellular levels of organization. For example, measured at acclimation temperature, the rate of oxygen consumption for isolated skeletal muscle of striped bass (Morone saxatilis) is greater in cold- compared to warm-acclimated fish (Jones and Sidell, 1982). Enhanced aerobic capacity of skeletal muscle with cold acclimation is also reflected by increases in mitochondrial densities (see below) and oxygen consumption of isolated mitochondria (van den Thillart and Modderkolk, 1978). Changes in mitochondrial oxygen consumption are associated with concomitant modifications of aerobic

enzyme activity (reviewed in Johnston and Dunn, 1987). For example, in flounder (Platichthys flesus L.) the activity of cytochrome oxidase, a marker enzyme for mitochondrial metabolism, is approximately 2.8 times higher in muscles of cold-acclimated (5°C) compared to warm-acclimated (23°C) fish (Johnston and Wokoma, 1986). Recent studies have demonstrated higher activities of mitochondrial enzymes in the axial muscles of nine-spine sticklebacks (Pungitius pungitius) acclimated to 3°C compared to 20°C and spring- compared to summer-acclimatized specimens (Gurderley and Foley, 1990). It has also been suggested that the changes in adenine nucleotides and phosphocreatine concentrations observed in brook trout muscle (Salvelinus fontinalis) may be an important factor in producing an enhanced metabolic rate in cold-acclimated fish (Walesby and Johnston, 1980b).

Thermal acclimation is often associated with changes in the pattern of cellular metabolism ie, alterations in the relative capacities of different metabolic pathways to switch between different energy sources. It has been suggested that changes in the utilization of different energy sources may result from the differential effects of temperature on certain regulatory enzymes at branchpoints in metabolism (Hochachka, 1968). However, changes in the patterns of metabolism are not necessarily universal to all tissues and/or species. For example in the liver of striped bass, glycogen is preferentially used in the cold and fats in the warm (Stone and Sidell, 1981) or vice versa in the skeletal muscle of the same

species (Jones and Sidell, 1982) or liver of rainbow trout, Salvelinus gairdneri (Hazel, 1979). The situation is clearly complex, with changes in tissue/cellular metabolism not necessarily reflecting the acclimation pattern of the whole animal.

Transport of ions and metabolites

Ultrastructural modifications to cell and tissue morphology may occur to compensate for the limitations imposed by reduced diffusion at low temperature. In goldfish skeletal muscle there is an increase in the surface area of the sarcoplasmic reticulum (SR) and a more intimate relationship between the SR and the myofibrils following cold acclimation (Penny and Goldspink, 1980). However, this is not a universal feature of teleost fish muscle (eg. carp fast muscle, Fleming et al. 1990). The increase in mitochondrial volume densities of skeletal muscle from cold-acclimated fish has been reported in a number of species (reviewed in Johnston and Dunn, 1987) and may be accompanied by increased densities of mitochondria between myofibrils at the expense of those at the periphery of a cell (Sidell, 1983). Thus in cold acclimation the capacity for ATP production increases, particularly in the region of the myofibrils and diffusion distances for metabolites and ions such as Ca^{2+} and ATP are decreased (Cossins and Bowler, 1987). For example, by measuring the harmonic mean of mitochondrial spacing in slow muscle fibres of goldfish acclimated to 5^{o}C and 25^{o}C, Tyler and Sidell

(1984) found that diffusion path length was reduced by 23% in cold- compared to warm-acclimated fish. Similar increases in the surface area of sarcoplasmic reticulum have also been observed in the cardiac cells of cold- compared to warm-acclimated perch (Perca fluviatilis), although mitochondrial volume density remained unchanged (Bowler and Tirri, 1990).

Partial and complete compensations of absorptive function in response to thermal acclimation have also been observed in the epithelial cells of goldfish (Smith, 1966, 1970, 1976). In addition, adaptations may involve considerable structural modifications. For example, in carp, mucosal surface area is increased by up to 100% following cold acclimation (Lee and Cossins, 1988). There is also substantial evidence for the perfect compensation of gut enzyme activity (Owen and Wiggs, 1971; Hofer, 1979) and secretion of gastric HCl (Smit, 1967) with thermal acclimation in fish (see Cossins and Bowler, 1987).

The diffusion of oxygen through muscle decreases by 1.4% per ^{o}C (Prosser, 1973) due to the direct effects of temperature on diffusion rate and solvent viscosity within a cell (Johnston, 1982). In crucian carp, to compensate for reduced oxygen delivery at low temperature, cold acclimation results in an increase in the surface and volume density of muscle capillaries, with the result that the capillary surface supplying $1\mu m^3$ of mitochondria is independent of acclimation temperature (Johnston, 1982). Adjustments in oxygen transport also

involve changes in ventricular contractility. For example, cold acclimation of goldfish (Tsukuda et al. 1985) and yellow perch (Perca flavescens)(Bailey and Driedzic, 1990) improves cardiac output relative to an acute transfer to low temperature.

Enzyme function

As discussed above the same basic principle applies for compensatory adjustments to temperature change at the molecular level, ie. the adoption of less constrained and rigid tertiary structures at low temperature to maintain activity, acheived at the expense of thermal stability. For example, myofibrillar ATPase activity at 1^{o}C was three times higher in goldfish acclimated to 1^{o}C than 26^{o}C, although became more susceptible to heat denaturation following cold adaptation (Johnston et al. 1975).

There are several mechanisms by which enzyme function is regulated in response to thermal acclimation: 1) change in concentration; 2) differential expression of isoforms; 3) factors influencing enzyme microenvironment; 4) conformational changes ('instantaneous isozymes'); 5) conversion of enzyme from active to inactive form (eg. phsphorylation); 6) altered post-translational processing (Johnston and Dunn, 1987).

The skeletal muscle concentrations of cytochrome c of green sunfish, Lepomis cyanellus (Sidell, 1977) and cytochrome c oxidase of goldfish (Wilson, 1973) increase with acclimation temperature (5^{o}C to 25^{o}C). There is

evidence to suggest that the changes in the concentration of cytochrome c of green sunfish result from alterations in the relative rates of synthesis and degradation (Sidell, 1977). Alternatively, the changes in the concentration of other enzymes has been linked to changes in the activity of enzymes responsible for protein synthesis. For example, rates of polypeptide elongation in vivo increase following cold acclimation (Haschmeyer, 1969).

The altered expression of enzyme isoforms may result from the 'on-off' switching of particular genes (eg trout brain acetylycholinesterase, Baldwin and Hochachka, 1970), or more commonly involves the change in sub-unit composition (eg. goldfish lactate dehydrogenase, Hochachka, 1965). Despite the evidence from a number of studies of trout and goldfish, some doubt exists about how widespread this response is (Cossins and Bowler, 1987).

An important modulative effect on enzyme function is exerted by changes in intracellular pH, associated with temperature change. For example, regulated changes in blood and cytosolic pH have been widely observed in animals following thermal acclimation (Reeves, 1977). Changes in the microenvironment of enzymes with acclimation include the modification of membrane fluidity (discussed below).

It has been suggested that temperature induced changes in protein conformation provides a mechanism for producing enzyme variants, although evidence for such

'instantaneous enzymes' is still very limited (see Hochachka and Somero, 1984; Johnston and Dunn, 1987). The importance of phosphorylation and post-translational modification of proteins as a mechanism of modulating enzyme function with temperature acclimation has yet to be fully assessed (Johnston and Dunn, 1987). The modulation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity by phosphorylation has been demonstrated in liver microsomes of catfish (Hunter and Rodwell, 1980) and carp (Teichert and Wodtke, 1987) and may represent a short term regulatory mechanism *in vivo* (see Teichert and Wodtke, 1987). However, recent evidence has shown that the activation state (ratio of expressed/total activity) of this enzyme in carp liver is not affected by acclimation, long term regulations of HMG-CoA reductase activity being primarily mediated by changes in the quantity of enzyme protein (Teichert and Wodtke, 1987).

Membrane fluidity; homeoviscous adaptation

The observations made by Teichert and Wodtke (1987) suggest that there is an acclimatory compensation in hepatic cholesterol synthesis, which in turn may control changes in membrane fluidity with thermal acclimation (eg. Anderson *et al*. 1981). Compensatory adjustments in membrane fluidity also result from the fatty acid substitutions in the phospholipids of membranes observed in a number of fish species and tissue types (Cossins, 1983). Decreased membrane fluidity is correlated with an

increase in the proportion of unsaturated fatty acids (see above; Cossins and Prosser, 1978). Precisely how this is regulated is not known, although increases in the activity of Δ^9 desaturase of liver microsomes in cold-acclimated carp (Schüenke and Wodtke, 1983), the enzyme responsible for the production of unsaturated fatty acids, implicates the protein synthetic system and perhaps gene expression (Cossins and Bowler, 1987). Adaptive responses in membrane fluidity are not universal and may differ even within membranes of single organelles. For example, the outer and inner brain mitochondrial membranes from temperature acclimated goldfish differ in their response to maintain membrane fluidity at temperature extremes (Chang and Roots, 1988), the fluidity of outer membranes being regulated by cholesterol (Chang and Roots, 1989). The compositional re-distribution of phospholipids with a change in acclimation temperature has also been implicated in stabilising the bilayer conformation of goldfish brain mitochondrial membranes, in concurrence with the maintenance of membrane fluidity (Chang and Roots, 1989). The mechanisms responsible for the thermal compensation of membrane function may also differ in response time. For example, in plasma membranes of trout kidney, rapid adjustments occur in phospholipid headgroup and molecular species composition, whilst changes in the proportion of long chain polyunsaturated fatty acids are significantly slower (Hazel and Landrey, 1988).

The adaptive significance of these changes includes modifications in the activities of membrane bound enzymes (reviewed in Cossins and Bowler, 1987). Increased activities of Na^+K^+-ATPase in goldfish intestinal mucosa (Smith and Ellory, 1971) and cytochrome in carp mitochondria (Wodtke, 1981) without any change in the number of active enzyme molecules support this hypothesis. In addition, it has been shown that the reconstitution of lipid-free extracts of succinic dehydrogenase from goldfish muscle mitochondria is increased in the less restricted environment of lipid extracts of cold- compared to warm-acclimated fish (Hazel, 1972).

Homeoviscous adaptation may also be important in the movement of electrolytes and non-electrolytes across cell/organelle membranes. For example, artificial membranes and mitochondria show increased permeabilty prepared from cold-acclimated compared to warm-acclimated animals (Cossins, 1983; Cossins and Bowler, 1987).

Endocrine systems

Almost nothing is known about changes in endocrine status with temperature acclimation in teleost fish (see Hazel and Prosser, 1974; Johnston and Dunn, 1987). The pituitary-thyroid axis of adult teleost fishes is known to be responsive to environmental variables including temperature (Leloup and DeLuze, 1985). For example, recent investigations have shown that thyroxine levels are higher in pre-metamorphic larvae of striped bass

(Morone saxatilis) reared for 10 days at 15°C compared to those at 20°C (Parker and Specker, 1990). The function of thyroid hormones in regulating mitochondrial turnover rates and tissue aerobic capacity has been demonstrated in amphibia (Jankowsky, 1960). However, in goldfish (Klicka, 1965) and hyophysectomized mud minnows Umbra limi (Hanson and Stanley, 1970) there is no correlation between thermal acclimatory adjustments in oxygen consumption and thyroid or adrenal activity. In contrast, hormonal involvement has been implicated in metabolic temperature compensation of carp. Although the exact nature of the humoral agents were unknown, Precht (1964) found that blood from cold-acclimated carp increased the oxygen consumption of muscle isolated from warm acclimated fish.

The nervous system

Modifications in membrane fluidity play a significant role in the compensation of neural function in fish, in response to a drop in temperature over seasonal as well as evolutionary timescales. In common with all other membranes, myelin is subject to significant alterations to allow change in fluidity and permeability in response to thermal acclimation (Selivonchick and Roots, 1976). Significant differences in the morphology of optic nerves have been observed in goldfish acclimated to 5°C and 25°C, morphological characteristics which are known to be important determinants of conduction velocity (Matheson and Roots, 1988 a,b). However, conduction velocities in

the peripheral nerves of brown bullhead (Ictalurus nebulosus) were insensitive to temperature acclimation, although the duration of slow negative potentials in the facial lobes of the brain following electrical stimulation of the barbels, exhibited perfect temperature compensation (Bass, 1971). These and other studies suggest that although there may be changes in the conduction properties of axons, it is at synaptic and neuromuscular junctions that the most important changes probably occur (Cossins and Bowler, 1987).

Locomotory activity

First it is important to distinguish between cruising or sustainable swimming which is powered by the recruitment of slow, oxidative muscle fibres and burst or non-sustainable swimming which is powered by the recruitment of fast, glycolytic muscle fibres (Bone, 1966; Johnston, 1981).

Fry and Hart (1948) using goldfish acclimated to different temperatures, demonstrated that sustainable swimming performance increases at low temperature and decreases at high temperature in cold compared to warm acclimated fish. More recently, similar observations have been made in another cyprinid, the carp (Rome et al. 1985), although the response is by no means universal to all teleost fish. For example, thermal sensitivity of sustained swimming speed to acute temperature change in striped bass (Morone saxatilus) has been shown to be independent of a fish's thermal history (Sisson and

Sidell, 1987). It therefore remains to be determined how widespread and universal the mechanisms of compensatory adjustments in swimming performance are in teleost fish.

Compensatory adjustments to acute changes in temperature in swimming performance have been shown to result from changes in muscle recruitment patterns. In carp (Rome et al. 1984) and striped bass (Sisson and Sidell, 1987), white fibre recruitment occurs at lower swimming speeds to compensate for the reduced power output of slow muscle fibres at low temperature, a phenomenon referred to as "compression of recruitment" (Rome et al. 1985). However, this is presumably acheived at the cost of lowered maximum burst swimming speed (Sidell and Johnston, 1985), although to date there is no direct evidence for changes in burst swimming performance with temperature acclimation.

Following a period of cold acclimation, white fibre recruitment at low swimming speed is reduced. For example, tested at 15°C, striped bass acclimated to 9°C are able to attain a speed of 2.46 ± 0.13 body lengths s^{-1} (BL s^{-1}) using exclusively red muscle, whilst fish acclimated to 25°C begin to recruit white muscle fibres at 1.84 ± 0.10 BL s^{-1} (Sisson and Sidell, 1987). The increase in the proportion of red fibres during cold acclimation in a number of fish species may also provide a mechanism for increasing the maximal cruising speed and thereby off-set the effects of cold (see Johnston and Dunn, 1987).

The mechanisms underlying temperature compensation of swimming performance involve the complex changes in nervous systems, muscles and other tissues discussed above (reviewed in Hazel and Prosser, 1974; Johnston and Dunn, 1987). However of specific interest in this study is the maintenance of locomotory capacity in response to temperature change by modifications in muscle contractile properties acheived through compensations of either energy metabolism or of the myofibrillar apparatus itself.

Temperature and muscle contractile properties

A number of common mechanisms operate to maintain the constancy of muscular activity in response to temperature change over evolutionary and seasonal timescales. These include changes in the thermal characteristics of myofibrillar ATPase, kinetics of calcium transport by the sarcoplasmic reticulum (SR), mitochondrial densities and in the activities of aerobic and fatty acid oxidative enzymes (see Johnston, 1989; Johnston et al. 1990a). For example, it has been suggested that changes in the kinetics and/or density of Ca^{2+} pumps of SR contribute to observed adaptations in relaxation rate with temperature acclimation of carp fast myotomal muscle (Fleming et al. 1990). Similar adaptations have been observed in SR isolated from fast muscle of Antarctic and tropical fish species (McArdle and Johnston, 1980). However, to date

evidence has suggested only modest compensation of contraction rates despite significant compensation of force generation in species adapted to different thermal environments (reviewed in Macdonald and Montgomery, 1987).

The mechanisms involved in adaptations of muscle contractility during acclimation are not universal. For example, qualitative alterations in the myofibrillar complex may not be widespread and it has been suggested that isozymic change during acclimation may be restricted to polyploid species (Sidell and Johnston, 1985). It would also appear that a number studies have failed to demonstrate similar adaptations in marine teleost species. For example, the ATPase activity of fast muscle myofibrils in mummichog (Sidell et al. 1983) and striped bass (Moerland and Sidell, 1986a,b) and the contractile properties of isolated muscle fibres of striped bass (Moerland and Sidell, 1986a,b) and flounder (Johnston and Wokoma, 1986) are unaltered by temperature acclimation.

It was therefore the aim of this study to determine the effects of evolutionary and seasonal temperature change on the performance of live skeletal muscle fibres of marine teleost species and to equate these findings to changes in locomotory capacity.

CHAPTER 2

TEMPERATURE ADAPTATION AND THE CONTRACTILE PROPERTIES OF LIVE MUSCLE FIBRES FROM TELEOST FISH

Introduction

The Antarctic marine fauna became gradually specialised to sub-zero conditions after the establishment of the Circum-Antarctic current around 20 million years ago (Kennett, 1977). In order to capture prey and avoid predators, fish need to swim at very high speeds for short periods. Feeding and escape responses are therefore critical for survival and it seems likely that natural selection would act to maximize burst swimming speed. However, inspite of a long period of evolution to cold conditions, there is evidence that maximum swimming speeds in polar fish are lower than in warm-water species, particularly for larvae and juveniles (Archer and Johnston, 1989; Johnston et al. 1990b). For example, yolk-sac larvae of the Antarctic fish Harpagifer antarcticus have mean escape speeds of only 5.2 bodylengths/s at 0°C, whilst similar sized northern anchovy larvae (Engraulis mordax) reach 14.5 bodylengths/s at 17°C (Johnston et al. 1990b). Slightly larger post-larvae (10.7 mm) of the flying fish (Hirundichthys affinis) from tropical seas have been

reported to swim at 35.9 bodylengths for short periods at 25°C (Davenport, 1990).

The power for rapid movements comes from the recruitment of fast twitch muscle fibres (Bone, 1978; Johnston, 1981). Demembranated or skinned fibre preparations have been used to investigate cold adaptation of contraction in fast muscles at the level of the cross bridge cycle (Johnston and Brill, 1984; Johnston and Harrison, 1985; Johnston and Altringham, 1985). Fibres from Antarctic fish generate high tensions at 0°C, but fail to relax completely following activations above 10°C, due to the formation of abnormal cross bridge linkages with very long cycle times (Johnston and Altringham, 1985). In contrast, the unloaded contraction velocity (Vmax) of fibres at low temperatures is not significantly higher in cold- than warm-water species (Johnston and Brill, 1984).

Although skinned fibres are useful for investigating some aspects of contraction their properties cannot be directly related to the function of live muscle fibres *in vivo*. During swimming, muscle fibres are phasically stimulated and undergo alternate cycles of shortening and lengthening (Hess and Videler, 1984). Power output during oscillatory work is a complex function of strain amplitude, cycle frequency and the number and timing of nervous stimuli (Johnston and Altringham, 1988; Altringham and Johnston, 1990a; van Leeuwen *et al*. 1990). Important parameters influencing power output under these

conditions include twitch duration, maximum stress and the force-velocity relationship.

In the present study, isolated live fibres from fast muscles have been used to investigate temperature adaptation of twitch contraction kinetics and isotonic shortening. Since inter-specific comparisons are complicated by differences in body length and swimming style, a range of marine fish have been examined from Antarctic, temperate and tropical latitudes.

Methods

Fish

Specimens of saithe (Pollachius virens L.), dab (Limanda limanda L.) and pogge (Agonis cataphractus L.) were caught in the Firth of Forth, Scotland. Dragonets (Callionymus lyra L.) were obtained from the University Biological Station at Millport. All specimens were maintained in seawater aquaria at ambient temperature (5-13^{o}C).

Hawaiian sergeants (Abudefduf abdominalis Quoy and Gaimard.) and saddle wrasse (Thalassoma duperreyi Quoy and Gaimard.) were caught by divers and hand-lines in inshore waters around Hawaii. They were maintained in seawater aquaria at ambient temperature (25-28^{o}C).

Trematomus lepidorhinus (Pappenheim) and a specimen of Pseudochaenichthys georgianus (Norman), were caught between 71^{o} and 75^{o}S during the expedition, "Antarktis VII/4" (EPOS leg 3) of RV Polarstern in the Eastern

Weddell Sea, Antarctica (January-March 1989). A combination of Agassiz, bottom and benthopelagic trawl were used (see Arntz et al. 1990). Notothenia neglecta (Nybelin), were caught at Signy Island, South Orkney Islands, British Antarctic Territories (60° 43" S, 45° 36" W) and maintained in aquaria at 0.5-1.5°C after shipment to St Andrews.

The number, standard lengths and weights for all specimens used are summarised in Table 1.

Chemicals

Chemicals were obtained from Sigma (Poole, Dorset);-

Pyruvic acid (α-ketopropionic acid; 2 -oxopropanoic acid), sodium salt,

α-Bungarotoxin,

d-Tubocurarine,

from BDH (Poole, Dorset);-

$NaH_2PO_4.2H_2O$ (Analar),

NaCl (Analar),

KCL (Analar),

$NaHCO_3$ (GPR),

$CaCl_2$ (Analar volumetric solution 1 mol l^{-1}),

from Aldrich (Gillingham, Dorset);-

$MgCl_2$ (Anhydrous 98+%).

Solutions

Muscles were dissected and experiments performed in a basic Ringer solution containing (in mmol l^{-1}): 132.2; NaCl, 10; Na pyruvate, 2.6; KCl, 1; $MgCl_2$, 18.5; $NaHCO_3$,

Table 1. The number (n), standard length (cm), weight (g), date of capture and environmental temperature range (ET) of the fish species studied. Values represent the mean ± SE.

Species	ET (°C)	Capture (month)	Number (n)	Weight (g)	Length (cm)
Indo-West Pacific					
Abudefduf abdominalis	24-30	Dec-Jan	11	35.8 ±2.5	9.6 ±0.2
Thalassoma duperreyi	24-30	Dec-Jan	9	42.8 ±2.5	12.9 ±0.3
North Sea and Atlantic					
Pollachius virens	5-15	May-Jun	6	301.3 ±21.6	30.1 ±0.6
Limanda limanda	5-15	Jun-Jul	4	224.3 ±35.4	22.3 ±1.5
Agonis cataphractus	5-15	Apr-Jun	9	11.0 ±1.0	11.0 ±0.4
Callionymus lyra	5-15	Aug-Oct	12	67.8 ±4.8	17.2 ±1.6
Antarctic					
Trematomus lepidorhinus	-1.9 (± 0.1)	Jan-Mar	12	nd	18.9 ±1.5
Pseudochaenichthys georgianus	-1.9 (± 0.1)	Jan-Mar	1	nd	95
Notothenia neglecta	-1.0-1 5	Feb-Mar	5	434.7 ±166.7	27.1 ±4.2

(nd, not determined as accurate measurement could not be made on board ship).

3.2; $NaHPO_4$, 2.7; $CaCl_2$, pH 7.2-7.4 set using 1M HCL/NaOH (Hudson 1969).

Although muscle fibre preparations were found to be relatively insensitive to pH changes over a wide range (pH 7-8), at high pH calcium is deposited. pH tends to increase as Ringer is circulated, especially above 15°C and therefore, is controlled by bubbling with 95% CO_2: 5% O_2 and by adding small quantities of 0.1M HCl during experimentation.

Preparation of fibre bundles

All fish were sacrificed by a blow to the head followed by pithing and decapitation. The standard length and weight were then recorded. Small bundles of fast fibres were isolated from anterior abdominal myotomes of P. virens, L. limanda, C. lyra, T. duperreyi, N. neglecta and P. georgianus. The abdomen of the fish was excised, pinned out to resting length on a silicone elastomer base (Sylgard 184, Dow Corning, Midland MI, USA), and immersed in Ringer. A thin section was taken (3-4 myotomes long, 0.5-0.7 cm wide), pinned to resting length on a Sylgard lined petri-dish and transferred to an aluminium dissection stage (cooled on ice for Antarctic and temperate species). Under a light microscope the skin was removed (underside) and the peritoneum of one central myotome removed, carefully avoiding contact with surface muscle fibres. The selected myotome was pared down to leave a bundle of undisturbed, surface fibres (10-20 fibres). Adjacent

myotomes were pared down to leave the peritoneum, to which foil clips were secured by folding.

In some species good mechanical preparations could not be isolated from the myotomes and therefore fast muscle fibres bundles from the adductor profundis muscle (m.add.p) were studied. This muscle has an important role in adducting the pectoral fin in species that utilize labriform locomotion (C. lyra, A. cataphractus, T. duperreyi, A. abdominalis, N. neglecta and T. lepidorhinus). The skin from the underside of the pectoral fin was severed and the fin drawn back to reveal the major adductor superficialis (m.add.s) and the m.add.p. The complete fin was excised by severing the clethridium beyond the point of insertion of the m.add.p (Shann, 1920; Harrison et al. 1987). A strip of the m.add.p was removed leaving the attachment to the clethridium and fin rays intact, immersed in Ringer and pinned to Sylgard. Care was taken not to disturb the surface of the preparation to avoid damage to the underlying fast muscle fibres. The strip was pared down to a small bundle of fast fibres and foil clips attached to the tendons.

Dissections of fish were performed at 4-5^{o}C in temperate, 1-2^{o}C in Antarctic and 15-25^{o}C in Hawaiian species, with the Ringer being changed frequently.

Apparatus

The apparatus consisted of a perspex chamber (5x1x1 cm), designed to hold a constant level of circulating

Ringer. A peristaltic pump (Watson-Marlow) circulated aerated Ringer (approx. 250 ml) from a reservoir, through a coil immersed in a water bath (Grant LTD 6) then passed through the chamber to control temperature (± 0.1°C). Additional temperature control was facilitated by a cooling-jacket supplied with coolant (70% alcohol) from the Grant LTD 6. Temperature was measured with a digital thermometer (Digitron), the thermister being immersed in the chamber.

Force was measured using a silicon blade force transducer (AE 801) (sensitivity 0.5 mN V^{-1}, noise < 2 mV, drift < 1mV/hr) to which a stainless steel hook was glued (Araldite) and held in a screened aluminium tube waterproofed with silicon grease. This was mounted at one end of the chamber on a perspex and micromanipulator assembly to adjust muscle fibre length. The output from the transducer was modified for display by a bridge circuit and amplifier.

Isotonic releases were performed using a servo-controlled length transducer. This system consisted of a servo-motor (MFE R4) and an LED-photodiode assembly (relay) with control/amplifier circuitry. A hook was attached to the end of an aluminium beam mounted to the central spindle of the servo-motor and designed as light as possible to reduce inertia at the start of the release. Attached to the opposite end of the beam was a square flag inserted into the relay assembly, mounted on the back of the motor. The relay consisted of two Infra-red (IR) emitters and detectors (RS-components), mounted

in a light sealed plastic enclosure (see Altringham, 1981). The movement of the flag across this IR path allowed the measurement of hook displacement against time (sensitivity, 4.7 V/mm). The whole system was mounted adjacent to the chamber with the hook at the opposite end of the chamber to the force transducer.

Measurements of tension and length changes were made using either a Gould 1602 or Nicolet 3091 digital oscilloscope. Hardcopies were obtained directly from the Gould 1602 (internal printer facilities) or dumped to disc from the Nicolet 3091 to a BBC microcomputor and printed (Epson RX-80).

Muscle fibre preparations were attached to the hooks of the servo and force transducers using the foil clips. Preparations were stimulated by a Grass S48 stimulator, through platinum electrodes (Goodfellow) immersed in the chamber. Muscle fibre length was adjusted to give an optimal twitch contraction which corresponded to a sarcomere length of 2.3-2.4μm measured by He-Ne laser diffraction (Barr and Stroud, Hughes). The diffraction pattern was displayed on a screen, calibrated using Bragg's equation;

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

where s= sarcomere length, λ = wavelength of laser (0.6328 μm) and θ = angle sub-tended by the zero and first order diffraction patterns.

(Plate 1 and Fig. 1, illustrate an outline of the apparatus described above).

Plate 1. Photograph showing the chamber used in the muscle fibre experiments;

a) Relay

b) Servo-motor

c) Perspex chamber with "cooling jacket"

d) Servo-motor arm and hook

e) Tension transducer (AE801) and hook

f) Platinum electrodes

g) Micromanipulator arm to adjust fibre length

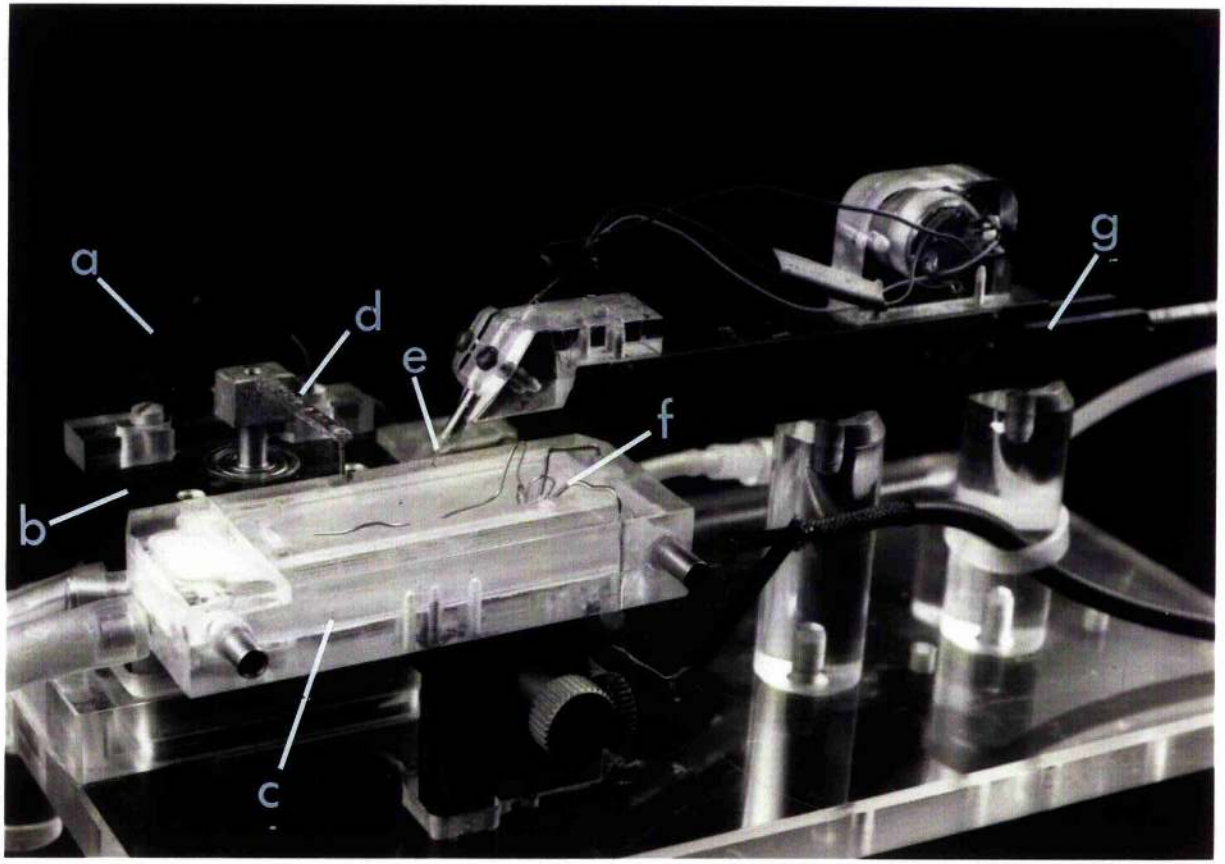
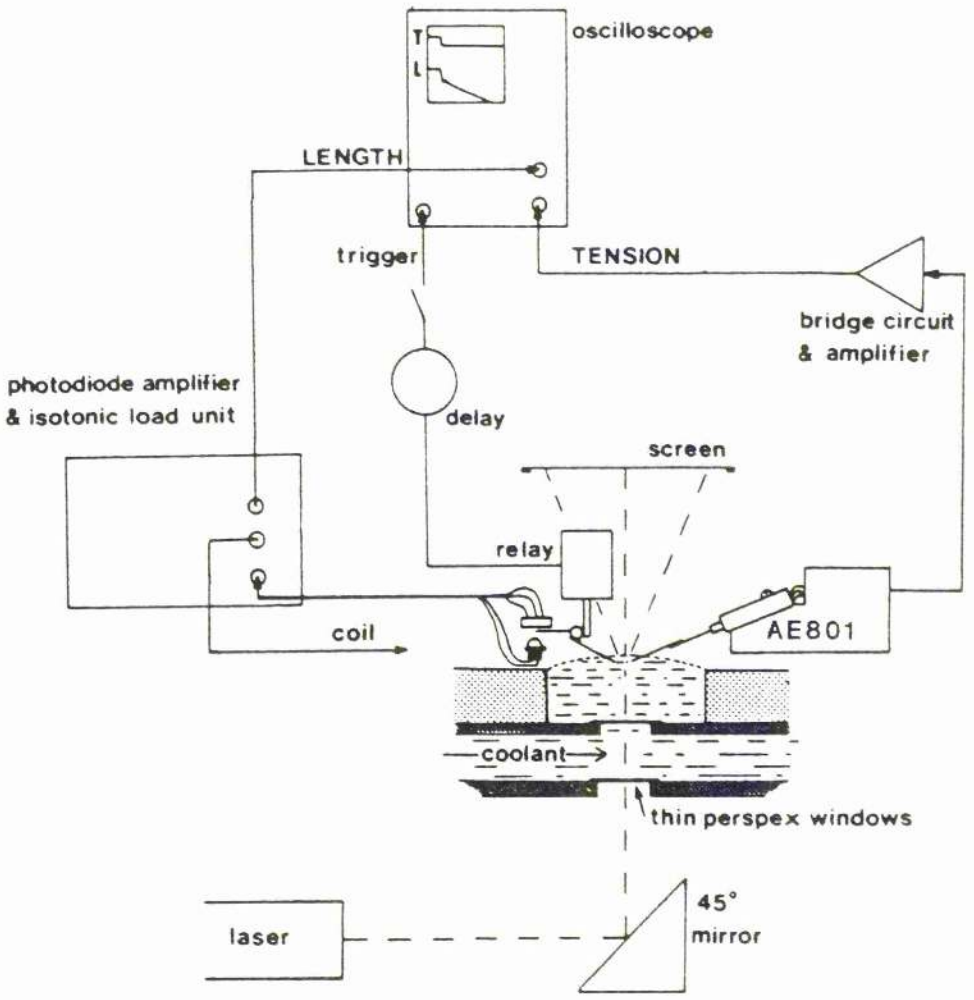

a
b
c
d
e
f
g

Fig. 1. Block diagram of the apparatus used in the measurement of isometric force and force-velocity characteristics of isolated muscle fibre bundles (from Altringham, 1981).


T
L
oscilloscope
LENGTH
trigger
TENSION
bridge circuit
& amplifier
photodiode amplifier
& isotonic load unit
delay
screen
relay
AE801
coil
coolant
thin perspex windows
laser
45°
mirror

Components

Fibre chamber;-

A.M.E Silicon blade force transducer AE801 (Horten, Norway),

M.F.E Servo-motor, R4 (USA),

RS electronic components (Corby, Northants, England),

Temperature control;-

Watson-Marlow peristaltic pump (101U/R)(Falmouth, England),

Grant LTD 6 water bath, (Cambridge, England),

Digitron, digital thermometer 3750-K.

Stimulation;-

Grass S48 stimulator (Quincy, Mass., USA),

Goodfellow, 99.9% platinum wire ,0.5 mm diameter (Cambridge, England),

Display and hardcopy facilities;-

Gould 1602, digital storage oscilloscope (Ilford, Essex),

Nicolet 3091 digital oscilloscope (Madison, Wisconsin, USA),

BBC Microcomputer, (London, England),

Epson RX-80 F/T+ printer (Middlesex, England),

Laser;-

Barr and Stroud, He-Ne laser power supply, type EL 103 LF (Glasgow, Scotland),

Hughes Aircraft Company laser type 3222H-D

(Carlsbad, California, USA).

Experimental protocol

i) Isometric contractions

Single or multiple supramaximal stimuli (50v, 1-2 ms duration) were used to elicit isometric twitch and tetanic contractions. An interstimulus interval of 10 minutes was allowed to ensure complete recovery and reproducible contractions for many hours. For a number of species the effects of temperature on the isometric properties of the preparations was determined over the widest range of temperatures that measurable contractions could be elicited. Changes in temperature were effected during the 10 minute interstimulus period temperature. In a number of species a "temperature induced tension hysteresis" was observed, ie. the relationship between tension and temperature was different when raising and lowering temperature (time dependent contraction parameters were unaffected). Low temperature was found to induce the hysteresis so measurements of tension were obtained whilst lowering temperature through the experimental range.

ii) Isotonic contractions

Contraction velocity was determined at various loads using iso-velocity releases. During the plateau phase of tetanus, an initial 2 ms release was given to reduce the tension (P_o). The velocity of a second release was adjusted to hold tension (P) constant after the step

(Fig. 2). This velocity (V) was plotted against relative tension over the first 10-20 ms interval after release (P/P_o), to construct a force-velocity curve. The tension was found to be relatively constant (< 0.1 Po) over this period. Force-velocity data for individual fibres was fitted to the Hill equation (1938):

$$V = b(P_o + a)/(P + a) - b$$

where a and b are constants, which was linearised as:

$$V = CZ - b$$

where $C = b(P_o + a)$ and $Z = 1/(P + b)$. Data points above 0.8 P_o were omitted since these are known to consistently deviate from the curve (Edman et al. 1976), an observation already supported by fast fibres from the teleost Myoxocephalus scorpius (Altringham and Johnston, 1988a). The remaining data were iteratively fitted to the equation without constraining the curve to go through P_o (Edman et al. 1976; Altringham and Johnston, 1988a). In tropical species the iso-velocity release required to hold tension constant at low load was too fast for accurate measurement. Maximum contraction velocity was therefore determined by the slack-test method (Edman, 1979). Muscle fibres were rapidly released (1 ms) to drop force to zero and the time required to take up the slack and begin to generate force (ΔT), was measured (Fig. 3a). A series of releases of increasing magnitude were given and the fibre was slowly extended to its original length between releases. The slack or maximum contraction velocity was determined from the slope of a least squares fit of ΔL versus ΔT (Fig. 3b).

Fig. 2. A series of isovelocity releases performed on a bundle of fast fibres isolated from the pectoral fin adductor profundis muscle of the pogge (Agonis cataphractus), to demonstrate the technique of load clamping.

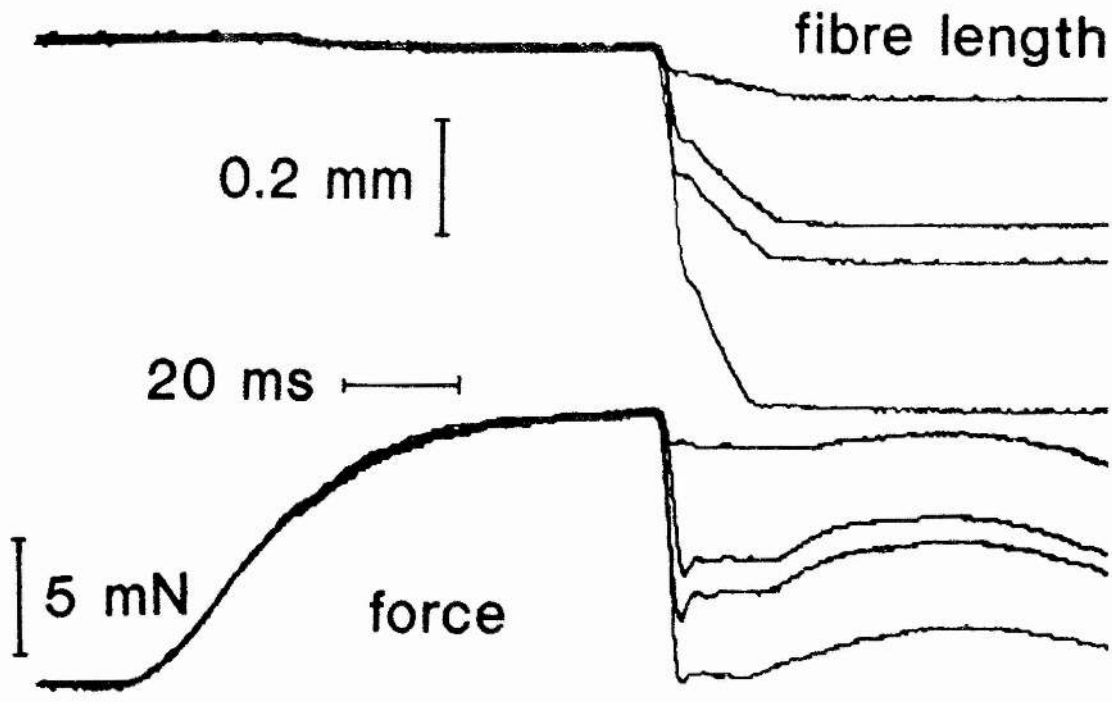
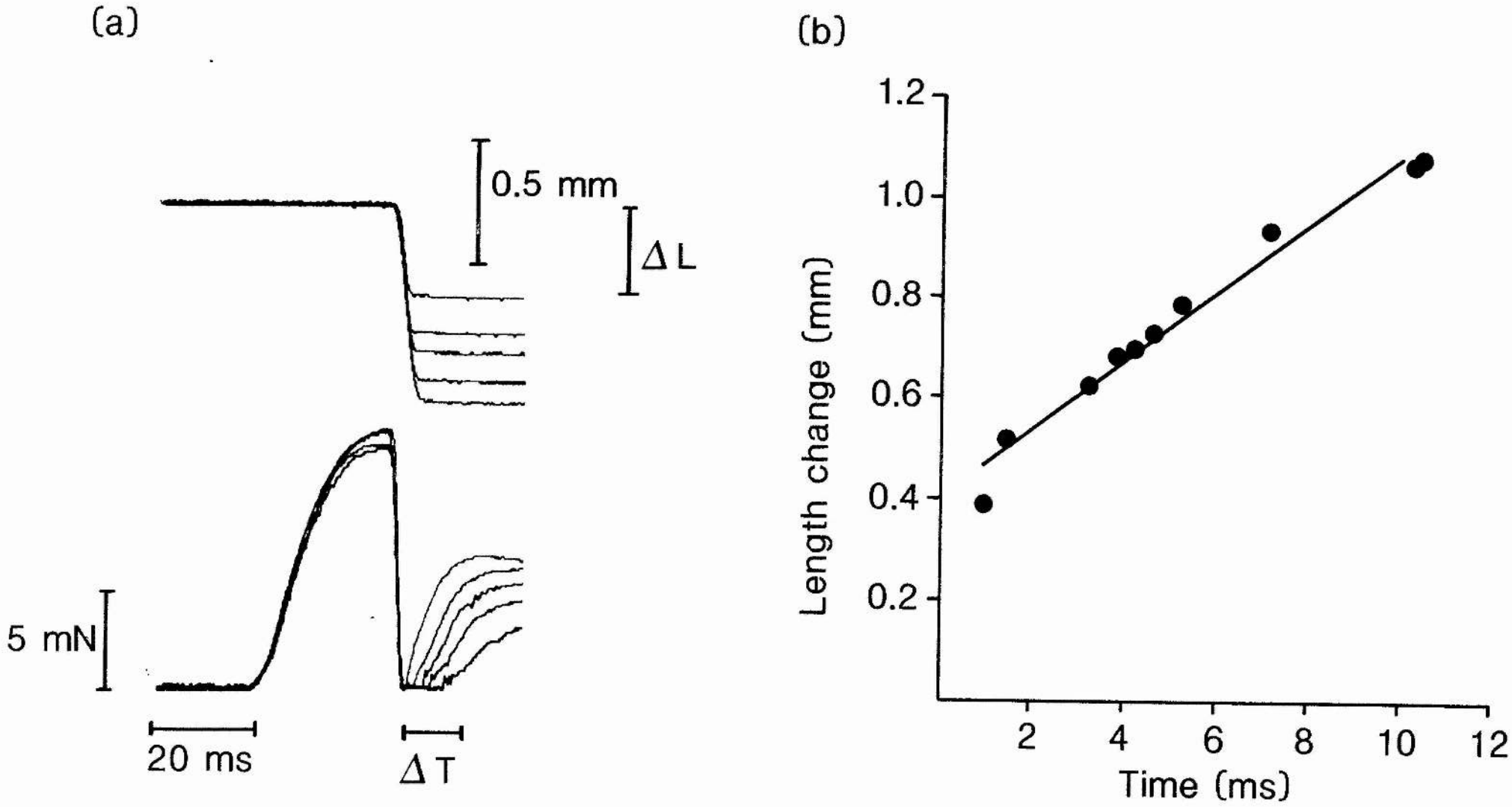

fibre length
0.2 mm
20 ms
5 mN
force

Fig. 3. a) A typical trace from a slack test performed on a myotomal preparation from the saddle wrasse (Thalassoma duperreyi). Stepwise length changes (ΔL) were given during the plateau phase of the tetanus to drop the force generated to zero. b) ΔL was plotted against the time required to take up the slack and begin to generate force (ΔT); unloaded contraction velocity was determined from the slope.


(a)
0.5 mm
ΔL
5 mN
20 ms
ΔT
(b)
Length change (mm)
1.2
1.0
0.8
0.6
0.4
0.2
2
4
6
8
10
12
Time (ms)

Statistical analysis

The standard error of the estimate for the force-velocity curves was determined for each preparation from the equation:

$$S.E.E. = \sqrt{RSS/(N-2)}$$

where RSS is the residual sum of squares and N is the number of values. The isometric and isotonic time dependent contractile properties for individual species were compared using a non-parametric Mann-Whitney test.

Results

Isometric contractions

Twitch duration decreases with normal body temperature (NBT) in the order Antarctic > temperate > tropical species (Fig. 4). The stimulation frequency required to obtain fused isometric tetani also increases from around 35-45 Hz in Antarctic species at 0°C, to 75-80 Hz in temperate species at 7-8°C and 400 Hz in sub-tropical species at 24°C (Figs. 4 and 5). Isolated preparations were found to deteriorate at temperatures outside their normal environmental temperature range (Fig. 6). The maximum twitch and tetanic tension was produced at the normal habitat temperature of each species (Fig. 6 and Table 2). Maximum isometric tension (kN m^{-2}) was 204.5 ± 16.4 for the Hawaiian sergeant (Abudefduf abdominalis) and 222.55 ± 13.18 for the saddle wrasse (Thalassoma duperryi) at 24°C (mean ± SE, n=4). This compares with

Fig. 4. Isometric tension records displayed with tetanic fusion frequency, from representative fast muscle preparations at normal body temperatures: North Sea/Atlantic; a) saithe (*Pollachius virens*, myotomal), b) dab (*Limanda limanda*, myotomal), c) pogge (*Agonis cataphractus*, m.add.p), d) dragonet (*Callionymus lyra*, myotomal), Indo-West Pacific; e) Hawaiian sergeant (*Abudefduf abdominalis*, m.add.p), f) saddle wrasse (*Thalassoma duperreyi*, myotomal), Antarctic; g) *Trematomus lepidorhinus* (m.add.p), h) *Notothenia neglecta* (m.add.p). Values represent the mean ± SE (n; refer to table 2).

North Sea and Atlantic

Indo-West Pacific

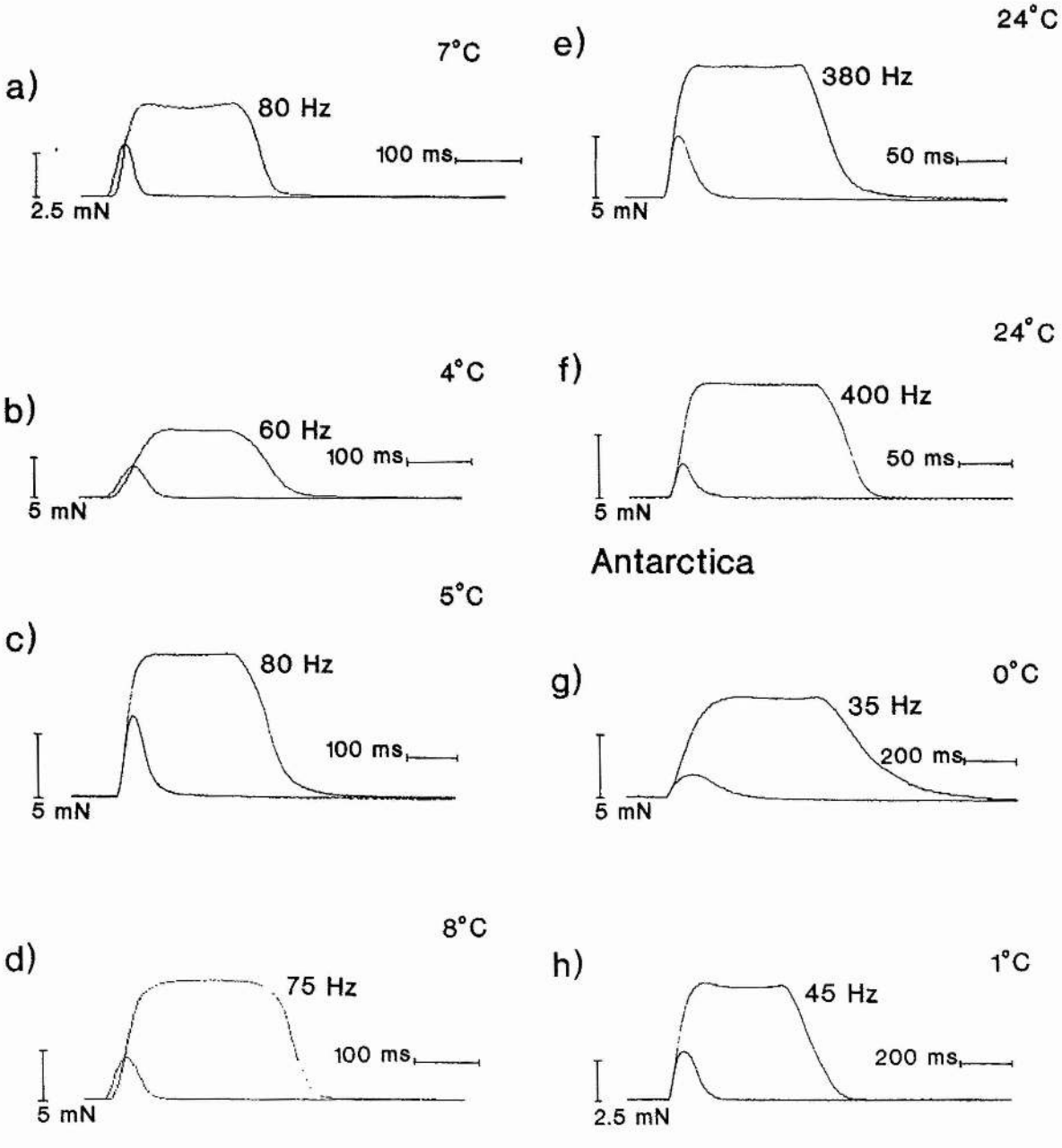


Fig. 5. The relationship between the stimulation frequency for tetanic fusion (Hz) and experimental temperature for fast muscle fibres isolated from the adductor profundis (m.add.p) and abdominal myotomes . a) M.add.p; symbols refer to the following species: Antarctic; ● Trematomus lepidorhinus, ■ Notothenia neglecta, Temperate; ○ pogge (Agonis cataphractus), Tropical; ▲ Hawaiian sergeant (Abudefduf abdominalis). b) Abdominal myotomes; symbols refer to the following species: Temperate; ○ saithe (Pollachius virens), △ dab (Limanda limanda), □ dragonet (Callionymus lyra), Tropical; ▲ saddle wrasse (Thalassoma duperreyi). Data represent mean ± SE (n; refer to Table 2). (·····;- the normal environmental temperature range).

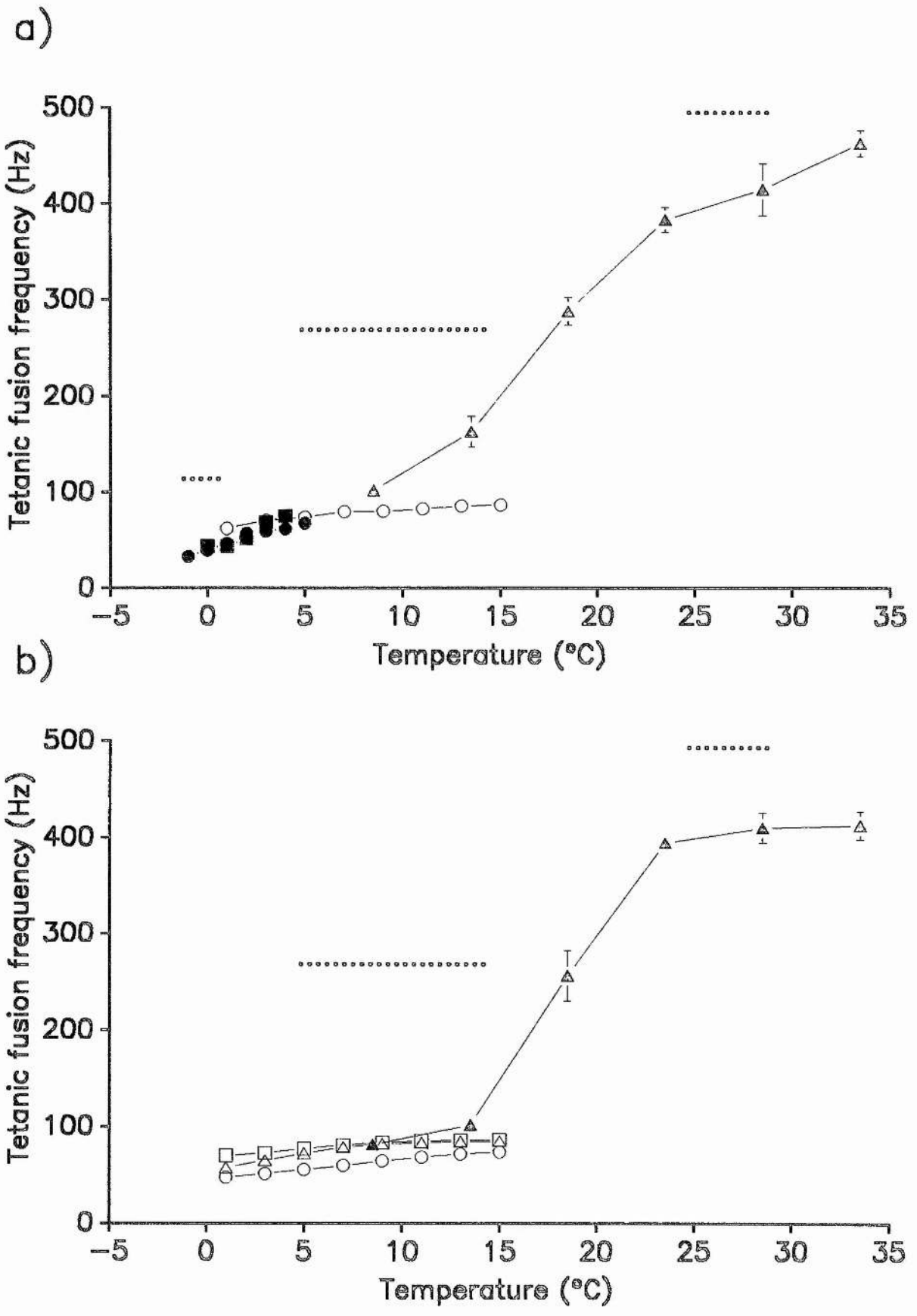
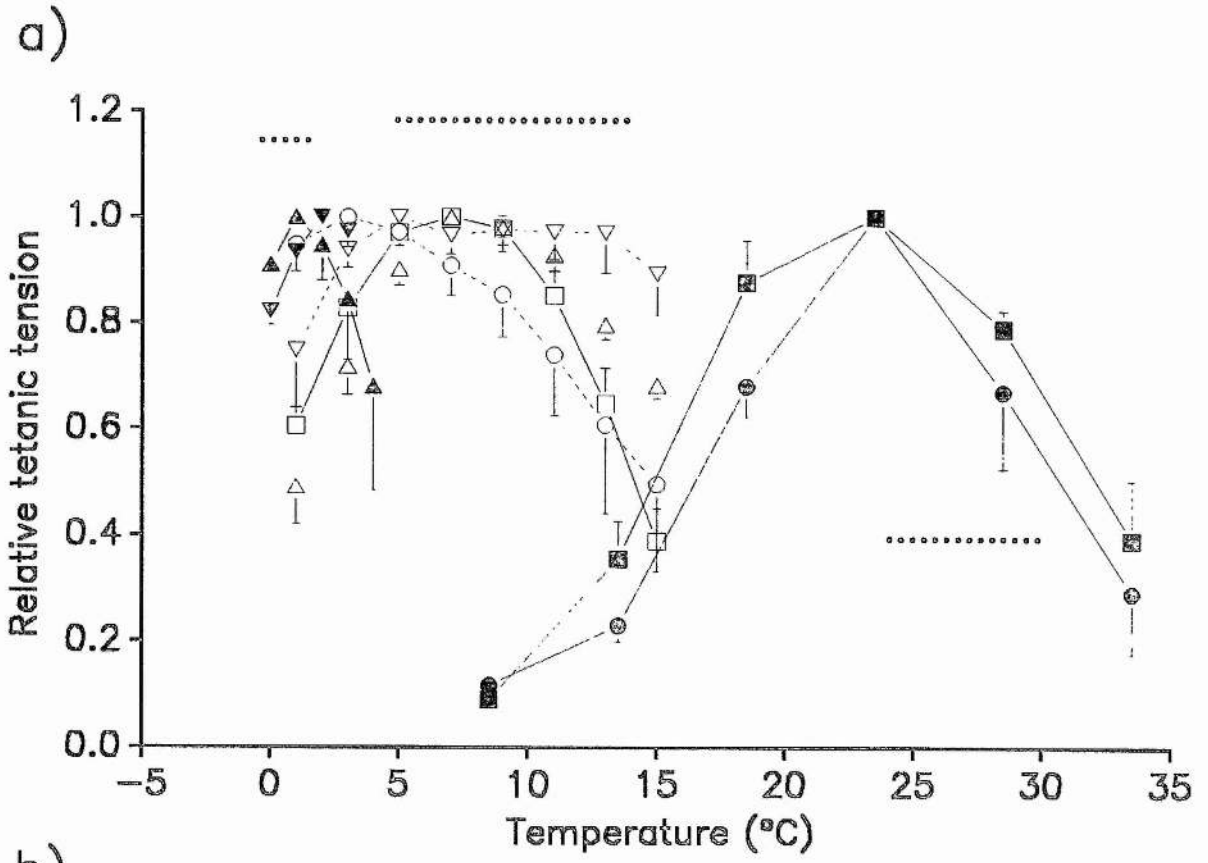

a)
Tetanic fusion frequency (Hz)
500
400
300
200
100
0
−5
0
5
10
15
20
25
30
35
Temperature (°C)
b)
Tetanic fusion frequency (Hz)
500
400
300
200
100
0
−5
0
5
10
15
20
25
30
35
Temperature (°C)

Fig. 6. The relationship between, (a) relative tetanic tension, (b) relative twitch tension, (normalised to the maximum at NBT) and experimental temperature for live, fast myotomal and fin (m.add.p) muscle preparations from Antarctic, temperate and tropical marine teleosts. The symbols refer to the following species: Antarctic; ▼——▼ Trematomus lepidorhinus,▲——▲(m.add.p)● ●(myotomal) Notothenia neglecta,◆——◆Pseudochaenichthys georgianus, Temperate;△·····△saithe (Pollachius virens),○·····○dab (Limanda limanda),□——□dragonet (Callionymus lyra),▽·····▽ pogge (Agonis cataphractus), Tropical;■——■Hawaiian sergeant (Abudefduf abdominalis),●——●saddle wrasse (Thalassoma duperreyi). Values represent the mean ± SE (n; refer to Table 2). (·····;- the normal environmental temperature range).


a)
Relative tetanic tension
1.2
1.0
0.8
0.6
0.4
0.2
0.0
−5
0
5
10
15
20
25
30
35
Temperature (°C)

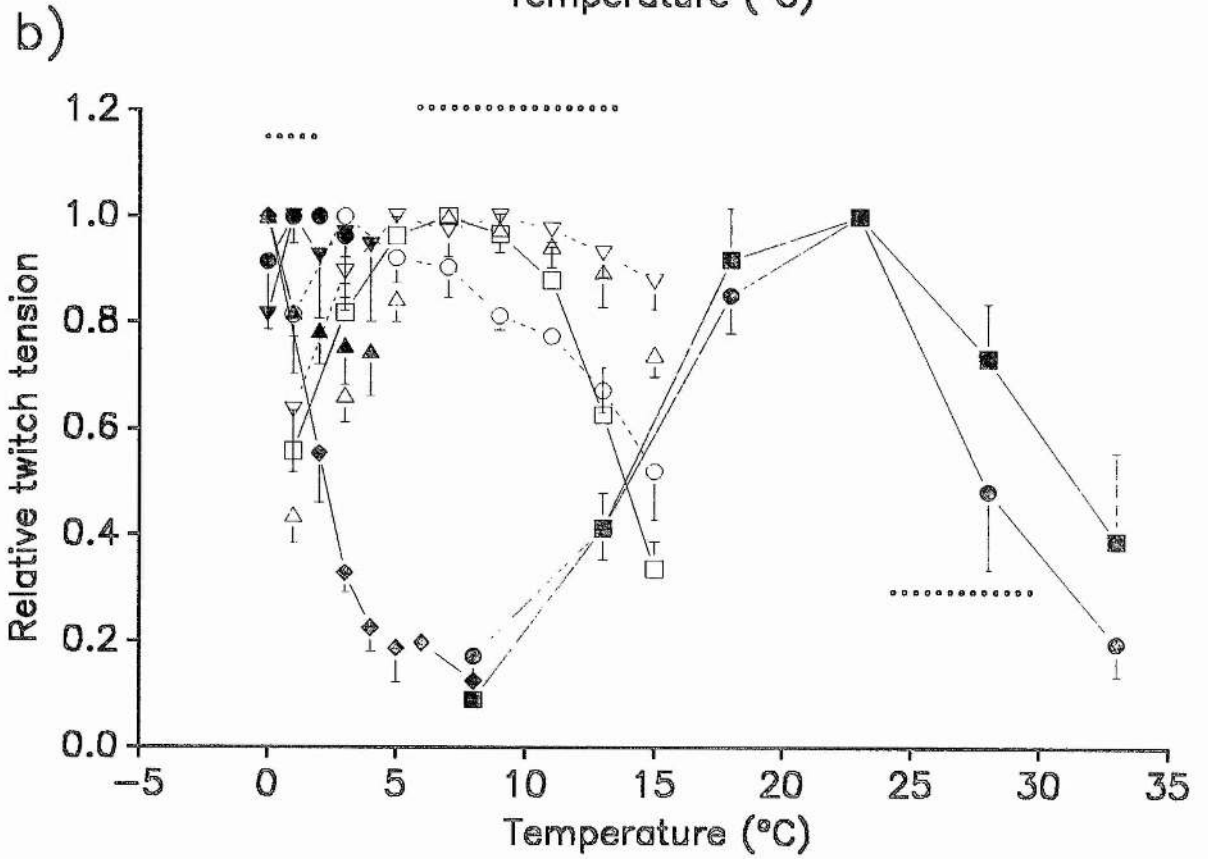

b)
Relative twitch tension
1.2
1.0
0.8
0.6
0.4
0.2
0.0
−5
0
5
10
15
20
25
30
35
Temperature (°C)

Table 2. Isometric properties of fast myotomal and pectoral fin (m.add.p) muscle fibres at normal body temperature (NBT). Values represent mean ± SE; (n, number of preparations; T_{max}, temperature for maximum isometric tension (^{o}C); $T_{½}a/r$, half activation/relaxation time (ms); FFq, tetanic fusion frequency (Hz)).

	(n)	T_{max}	Twitch $T_{\frac{1}{2}}a$	Twitch $T_{\frac{1}{2}}r$	Tetanus $T_{\frac{1}{2}}a$	Tetanus $T_{\frac{1}{2}}r$	FFq
Indo-West Pacific (NBT = 24°C)							
A.abdominalis (m.add.p)	16	21-26	6.8 ±0.1	15.1 ±1.2	12.3 ±0.5	24.7 ±0.9	381.9 ±12.7
T.duperreyi (myotomal)	13	21-26	6.0 ±0.2	10.4 ±0.8	13.7 ±0.6	30.1 ±1.8	394.0 ±3.9
North Sea and Atlantic (NBT = 6-8°C)							
P.virens (myotomal)	6	6-8	12.9 ±1.0	23.0 ±0.6	18.5 ±0.9	49.2 ±2.2	80.8 ±1.1
L.limanda (myotomal)	4	2-4	16.1 ±1.9	24.0 ±3.3	39.3 ±3.5	61.4 ±4.3	59.6 ±2.1
A.cataphractus (m.add.p)	7	4-6	16.9 ±1.0	39.2 ±4.0	23.6 ±2.0	81.2 ±6.8	79.3 ±1.9
C.lyra (myotomal)	7	6-8	14.6 ±1.2	31.6 ±4.6	24.4 ±2.2	66.9 ±4.8	78.6 ±0.7
Antarctica (NBT = 0.0) (NBT = -1.9°C)*							
T.lepidorhinus* (m.add.p)	7	2	44.6 ±5.2	118.9 ±10.9	110.2 ±5.6	190.4 ±5.8	33.3 ±2.4
P.georgianus* (myotomal)	1	0	39.5	34.0	NA	NA	NA
N.neglecta (m.add.p)	3	1	36.3 ±4.9	91.3 ±15.4	67.8 ±6.0	168.6 ±15.0	43.8 ±1.4
N.neglecta (myotomal)	8	0	20.7 ± 1.4	36.8 ± 2.2	NA	NA	NA

NA, not applicable; *,the lowest attainable temperature was used, 0°C for Pseudochaenichthys georgianus and -1°C for Trematomus lepidorhinus.

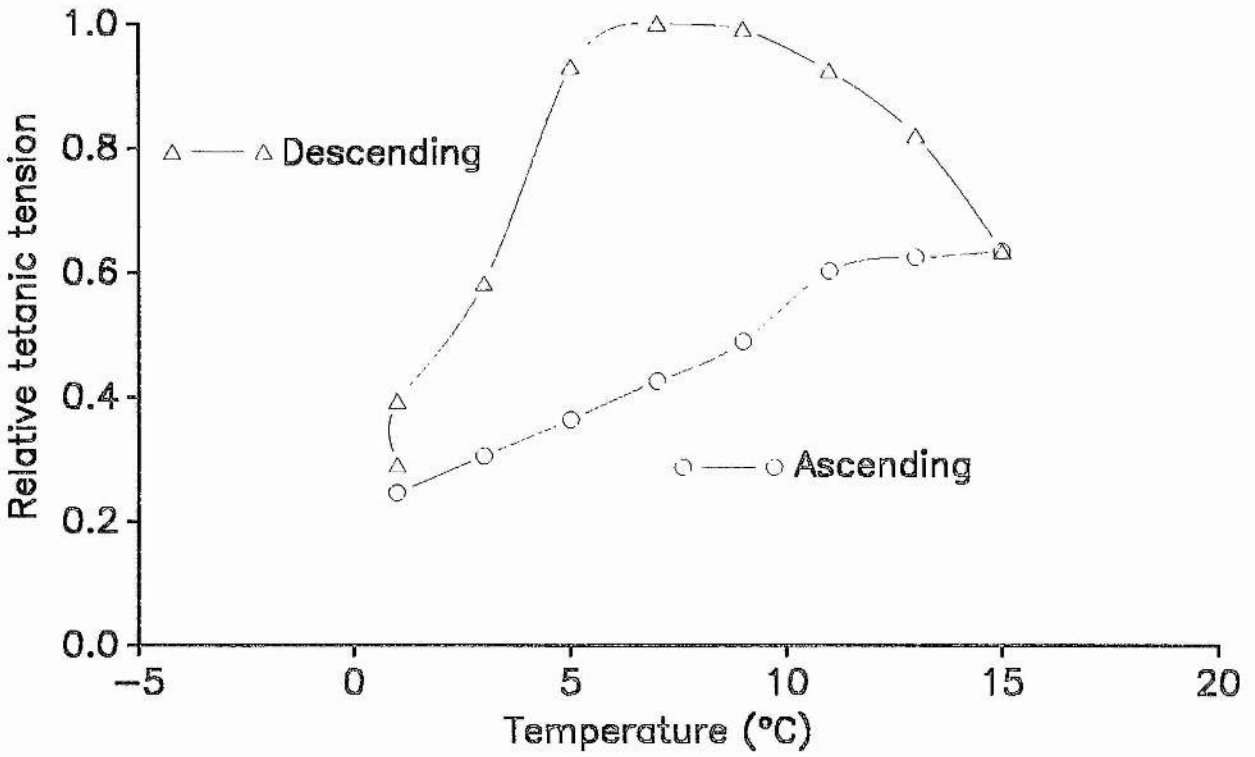
315.0 ± 17.9 kN m^{-2} for Myoxocephalus scorpius at 8°C (Langfeld et al. 1989) and 189 ± 21.4 kN/m^2 for the icefish (Chaenocephalus aceratus) at 0°C (Johnston, 1987). Thus, at NBT maximum isometric tension (P_o) for fast muscle fibres is similar in fish from different latitudes, ie. Po shows perfect temperature compensation.

The tension produced by muscle fibres from P. virens, C. lyra and T. lepidorhinus was greater for cycles of cooling than warming, ie. there was a "temperature induced tension hysteresis" (Fig. 7). Following exposure to low temperatures (< 0°C for T. lepidorhinus and < 2°C for P. virens and C. lyra) recovery to maximum tension occurred after approximately 20-30 minutes at the optimum temperature for tension development. Time dependent contractile parameters were similar for cycles of heating and cooling. A similar phenomenom with cooling has been described for whole muscles from four lizard species where the exposure of whole limb muscles to progressively colder temperatures and then re-testing at the initial temperature resulted in a 16% loss of tension (Putnam and Bennett, 1982). The mechanisms underlying this effect are unknown.

The rates of activation and relaxation of tension were measured as the time taken to rise/fall to half maximum tetanic or twitch tension ($T_{\frac{1}{2}}a$ and $T_{\frac{1}{2}}r$ respectively). Both parameters decreased with increasing experimental temperature (Figs. 8-11). For myotomal and m.add.p preparations, the half times for the fall of twitch tension were significantly prolonged at 7-8°C in tropical

Fig. 7. The effects of increasing and decreasing temperature on tension generation by fast muscle fibres from, a) saithe (Pollachius virens, myotomal) and, b) Trematomus lepidorhinus (m.add.p). Preparations were stimulated at 10 minute intervals over ascending (○——○) and then descending (△——△) temperature changes. Data represent tetanic tension normalised to the maximum.

a)


1.0
0.8
0.6
0.4
0.2
0.0
Relative tetanic tension
Descending
Ascending
−5
0
5
10
15
20
Temperature (°C)

b)

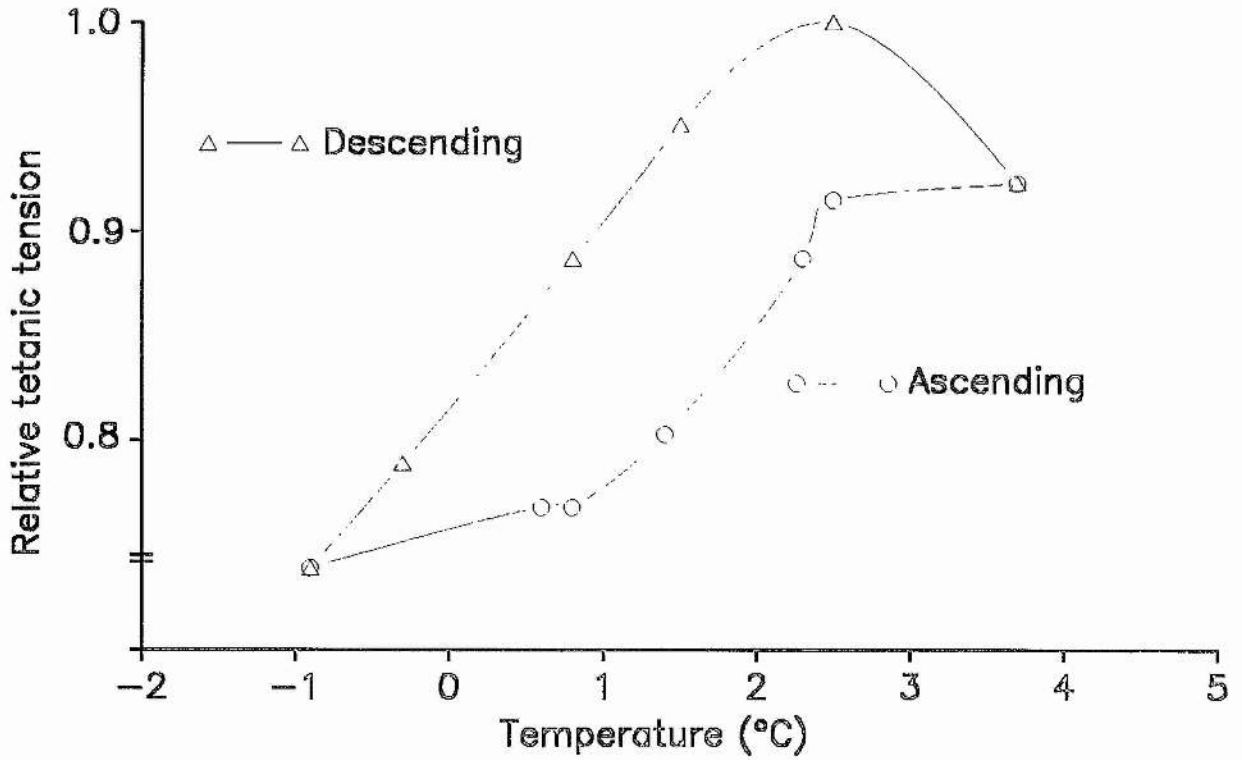

1.0
0.9
0.8
Relative tetanic tension
Descending
Ascending
−2
−1
0
1
2
3
4
5
Temperature (°C)

Fig. 8. The relationship between experimental temperature and (a) twitch half activation time (ms) and (b) twitch half relaxation time (ms) for live, fast myotomal muscle preparations from Antarctic, temperate and tropical marine teleosts labelled in Figure 5b and as follows: Antarctic; ● icefish (Pseudochaenichthys georgianus), ■ Notothenia neglecta. Data represent mean ± SE (n; refer to Table 2).

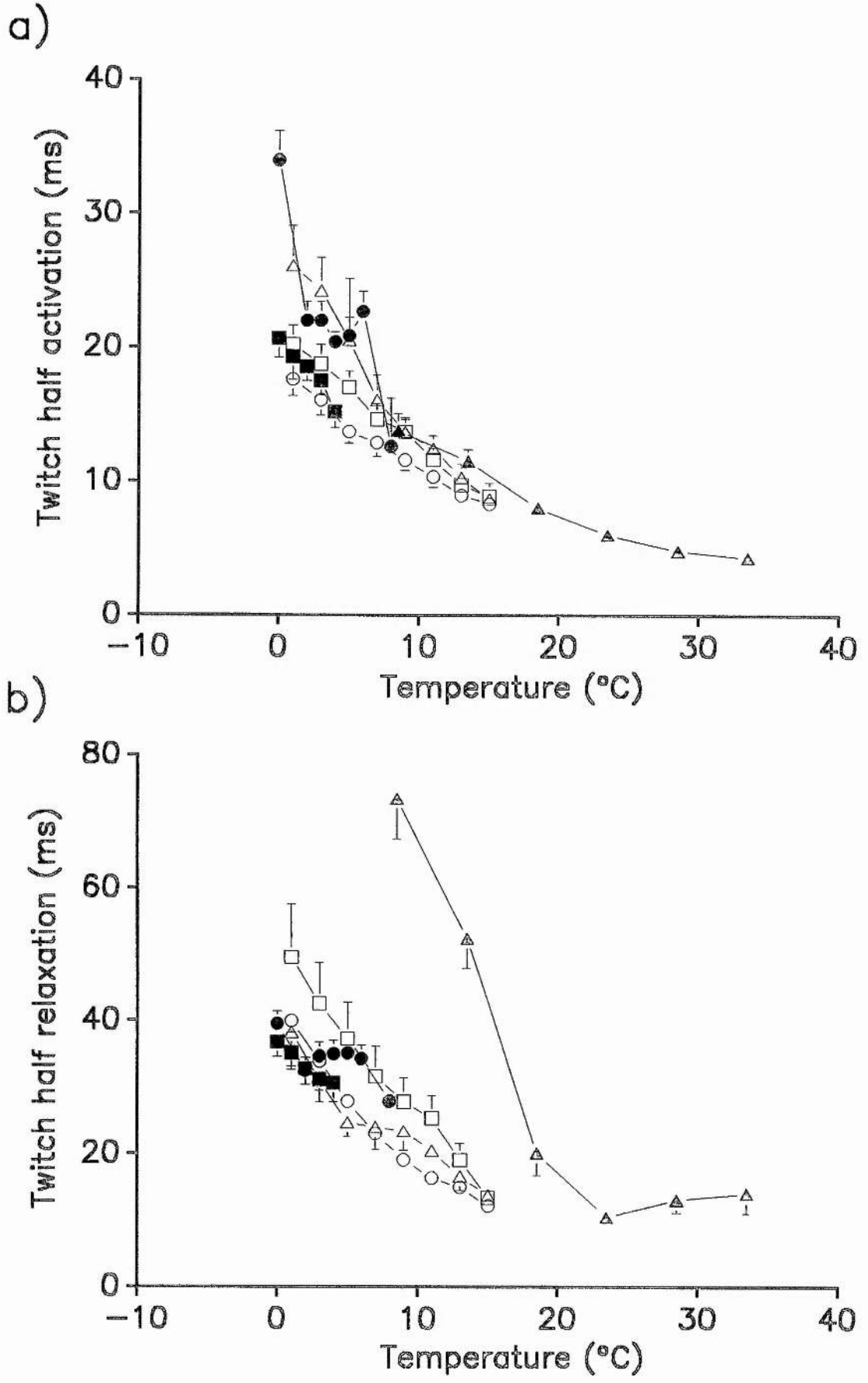

a)
Twitch half activation (ms)
40
30
20
10
0
−10
0
10
20
30
40
Temperature (°C)
b)
Twitch half relaxation (ms)
80
60
40
20
0
−10
0
10
20
30
40
Temperature (°C)

Fig. 9. The relationship between experimental temperature and (a) tetanus half activation time (ms) and (b) tetanus half relaxation time (ms) for myotomal preparations labelled as in figure 8a and 8b. Data represent mean ± SE. (n; refer to Table 2).

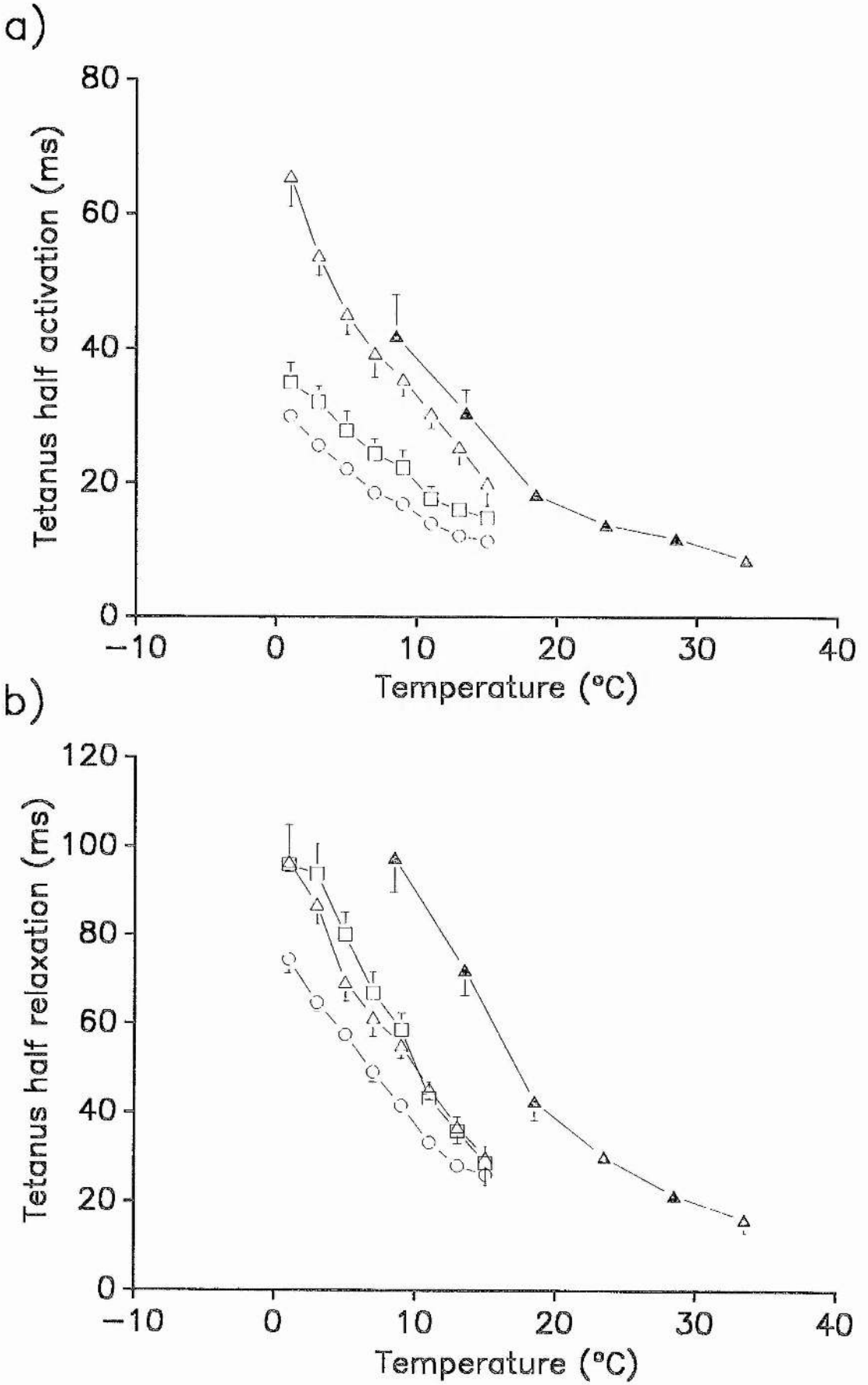

a)
Tetanus half activation (ms)
80
60
40
20
0
−10
0
10
20
30
40
Temperature (°C)
b)
Tetanus half relaxation (ms)
120
100
80
60
40
20
0
−10
0
10
20
30
40
Temperature (°C)

Fig. 10. The relationship between experimental temperature and (a) twitch half activation time (ms), (b) twitch half relaxation time (ms), for bundles of fast fibres isolated from the pectoral fin adductor profundis muscle of the Antarctic, temperate and tropical marine teleosts referred to in Figure 5a. Data represent mean ± SE (n; refer to Table. 2).

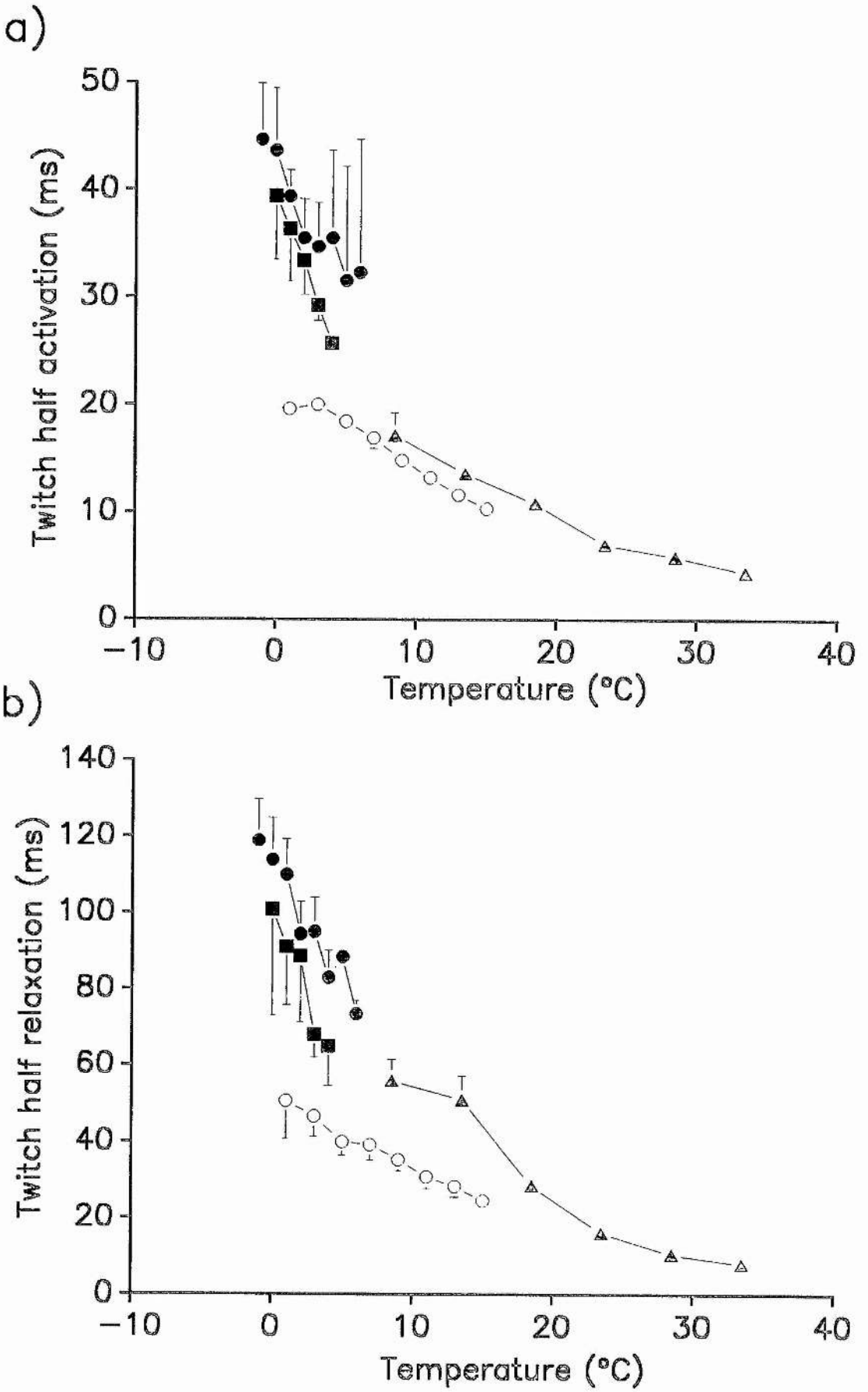
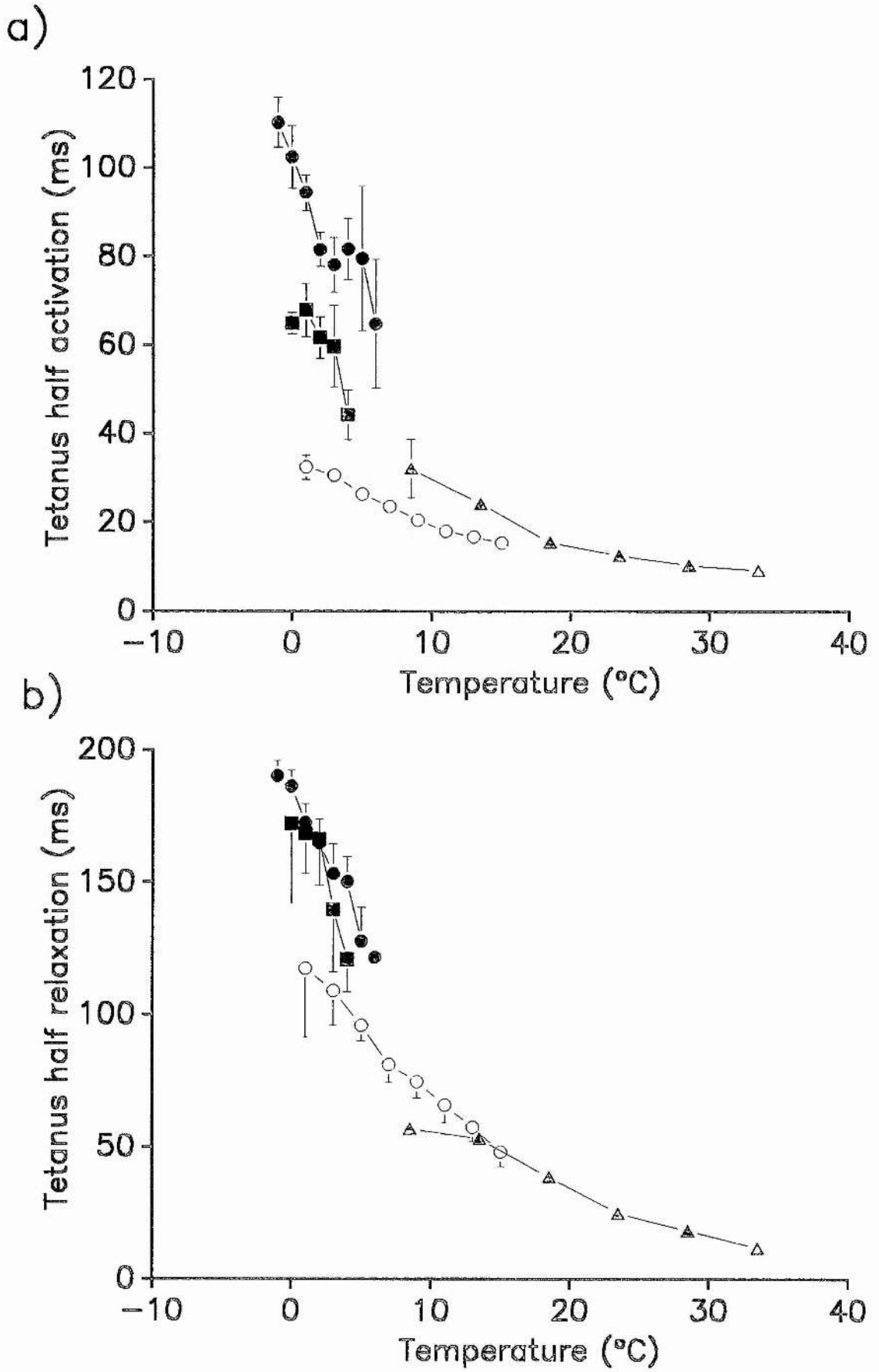

a)
Twitch half activation (ms)
50
40
30
20
10
0
−10
0
10
20
30
40
Temperature (°C)
b)
Twitch half relaxation (ms)
140
120
100
80
60
40
20
0
−10
0
10
20
30
40
Temperature (°C)

Fig. 11. The relationship between experimental temperature and, (a) tetanus half activation time (ms) and, (b) tetanus half relaxation time (ms), for pectoral fin preparations labelled as in figure 5a. Data represent mean ± SE (n; refer to Table 2.)


a)
Tetanus half activation (ms)
120
100
80
60
40
20
0
−10
0
10
20
30
40
Temperature (°C)
b)
Tetanus half relaxation (ms)
200
150
100
50
0
−10
0
10
20
30
40
Temperature (°C)

compared to cold temperate species ($P<0.01$) (Figs. 8b and 10b). Similar differences in half times for relaxation of tetanic contractions were only consistently found for myotomal preparations ($P<0.01$) (Figs. 9b and 11b). However, it is clear that there is at least a partial degree of temperature compensation of relaxation rate in some species/muscle types. In contrast, half activation times at 7-8^{o}C were relatively similar for all the species and preparations studied, indicating very limited or no temperature compensation in the rate of force development (Figs. 8-11a).

Measured at NBT, $T_{\frac{1}{2}}a$ and $T_{\frac{1}{2}}r$ were significantly shorter in the tropical species (24^{o}C) than in temperate species (7^{o}C, $P=0.001-0.01$) and Antarctic species (0^{o}C, $P<0.001$) (Table 2). With the exception of $T_{\frac{1}{2}}r$ for the twitch contractions of myotomal fibres from C.lyra, half activation and relaxation times for temperate and Antarctic species were significantly different at NBT ($P=0.001-0.05$) (Table 2). Thus, in general $T_{\frac{1}{2}}a$ and $T_{\frac{1}{2}}r$ (at NBT) are inversely related to habitat temperature and twitch duration only exhibits a modest degree of temperature compensation in cold-water species. However, it is important to note that the range of values of contraction time of muscle fibres at NBT, is far greater for the m.add.p than myotomal muscles. Therefore other factors, such as scaling and swimming style, in addition to the constraints incurred at low temperature may explain the slower contraction times of m.add.p fibres isolated from Antarctic specimens.

Measured at NBT's, the Q_{10} values for time dependent contractile parameters are comparable to those in the literature, ranging from 1.3-2.1 in Indo-West Pacific species (23.5-33.5°C) to 1.7-2.6 in North Sea/Atlantic species (3-13°C). The Q_{10} values for $T_{\frac{1}{2}}a$ and $T_{\frac{1}{2}}r$ of Antarctic species were more variable (1.6-3.5), probably due to the narrow experimental temperature range over which the measurements were made and the highly stenothermal nature of these animals.

Isotonic contractions

The P-V relationship described by the Hill equation was studied for a variety of specimens at a normally encountered body temperature. Representative curves from four m.add.p. preparations are shown in Figure 12 and a summary of the results are contained in Table 3 (for statistical analysis N.neglecta is omitted due to the small sample size, n=3). At high loads (>0.8P_o) the Hill equation was found to deviate from the P-V relationship (Edman et al. 1976; Altringham and Johnston, 1988a). The standard errors of the estimates for North Sea/Atlantic species were significantly different from the Antarctic species, T. lepidorhinus (P=0.02-0.05). Vmax increased with NBT in the order Antarctic < North Sea/Atlantic < Indo-West Pacific species (Fig. 13). The values from individual species, compared between each latitudinal category were significantly different at P=0.02-0.05 (Table 3). Hill's constant "a", which is a measure of the curvature of the P-V relationship, decreased with

Fig. 12. Force-velocity curves iteratively fitted to Hill's equation for representative pectoral fin *adductor profundis* muscle preparations from, *Trematomus lepidorhinus* (○, 1°C), *Notothenia neglecta* (●, 1°C), pogge (*Agonis cataphractus*.▲, 4°C) and dragonet (*Callionymus lyra*.△, 8°C). a/P_o= 0.55, 0.69, 0.28 and 0.17 respectively. Data $<0.8P_o$ was omitted from the curve fitting procedure (see text for further details).

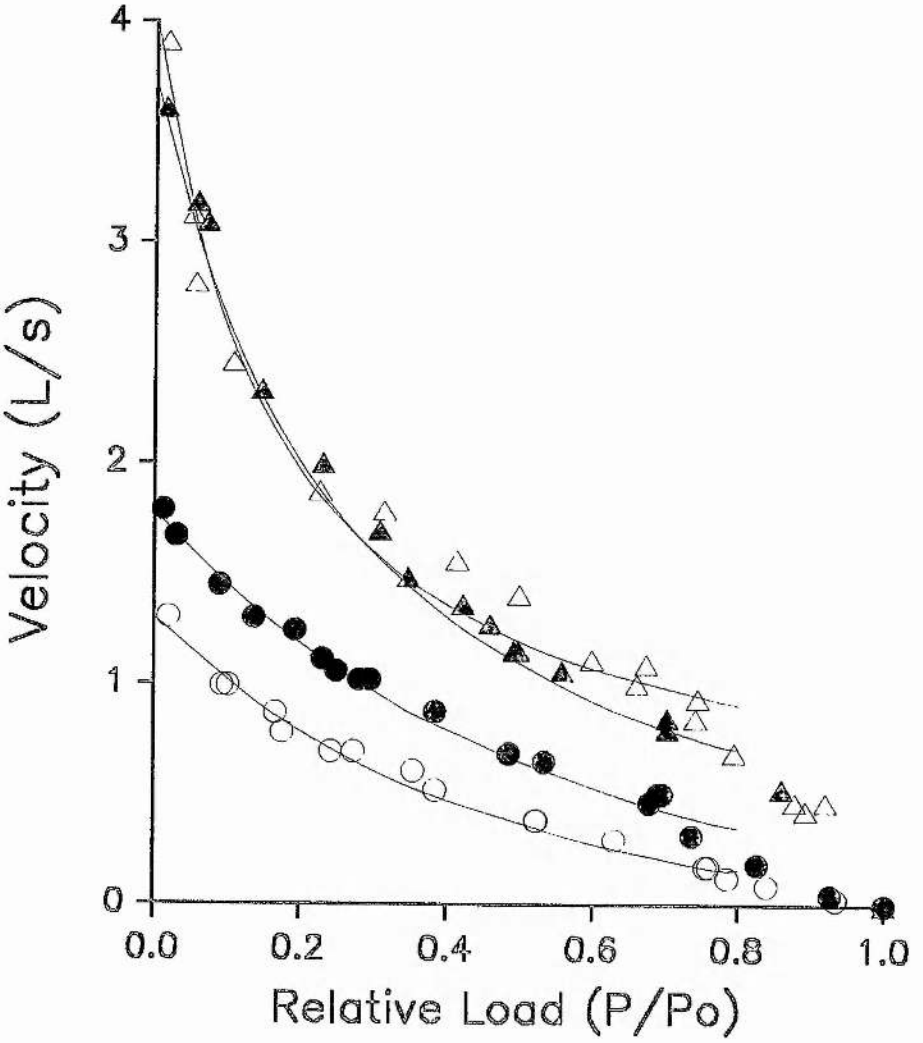

Velocity (L/s)
4
3
2
1
0
0.0
0.2
0.4
0.6
0.8
1.0
Relative Load (P/Po)

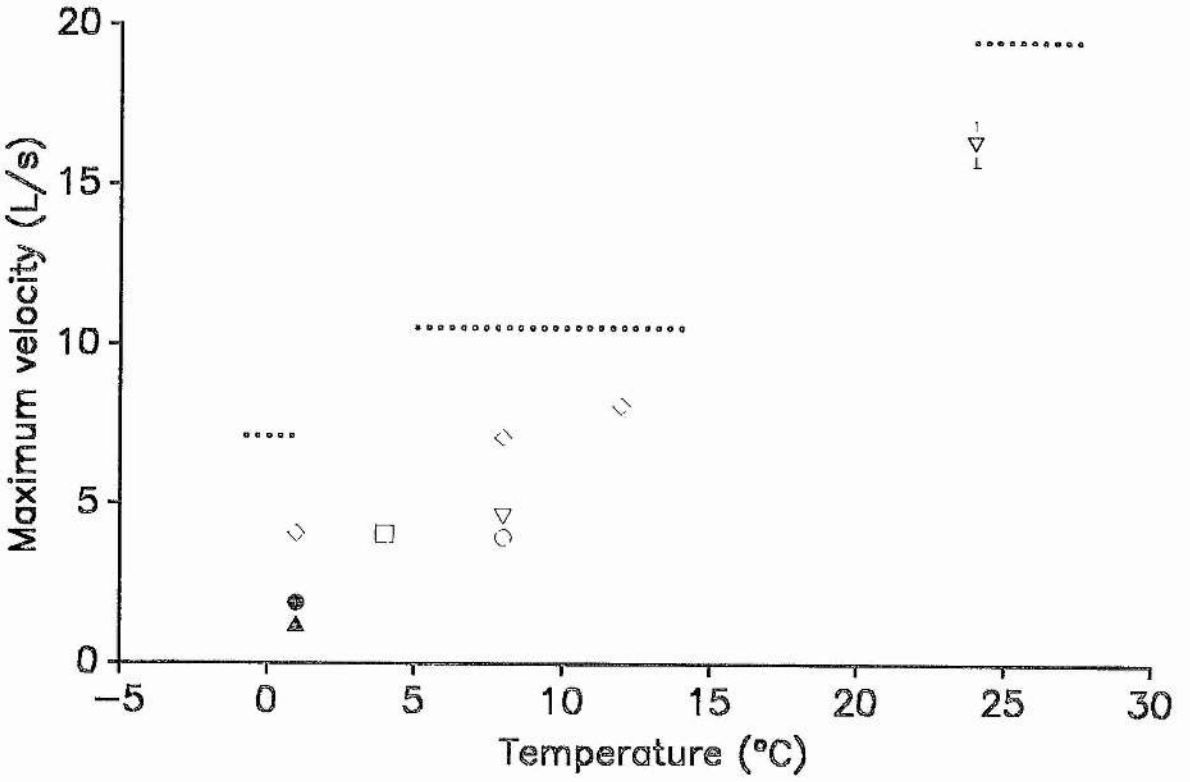
Table 3. Summary of the force velocity data of fast myotomal and pectoral (m.add.p) muscle fibres at appropriate physiological temperatures ($T^{o}C$). Data represent mean ± SE.

	n	T°C	Vmax	Hill's Equation a/P_o	$b(Ls^{-1})$	r^2	SEE
Indo-West Pacific							
T.duperreyi* (m.add.p)	4	24	16.36 ±0.72	-	-	-	-
North Sea and Atlantic							
A.cataphractus (m.add.p)	5	4	4.06 ±0.24	0.21 ±0.03	0.69 ±0.08	0.98	0.054 ±0.006
C.lyra (m.add.p)	4	8	3.96 ±0.27	0.20 ±0.04	0.58 ±0.07	0.97	0.072 ±0.007
C.lyra (myotomal)	7	8	4.62 ±0.10	0.30 ±0.07	1.13 ±0.24	0.97	0.051 ±0.004
Antarctica							
N.neglecta (m.add.p)	3	1	1.92 ±0.08	0.64 ±0.06	1.11 ±0.09	0.99	0.014 ±0.001
T.lepidorhinus (m.add.p)	4	1	1.23 ±0.04	0.62 ±0.08	0.73 ±0.09	0.98	0.005 ±0.002

Vmax, extrapolated maximum contraction velocity; a/P_o and $b(Ls^{-1})$ are constants from the Hill equation; SEE, standard errors of the estimates.

*Values were obtained for Vmax using the "slack test".

Fig. 13. Maximum contraction velocity, determined from the force-velocity relationship, plotted against experimental temperature for a range of species. Symbols refer to the following species: Antarctic; ▲ Trematomus lepidorhinus (m.add.p), ● Notothenia neglecta (m.add.p), Temperate; □ pogge (Agonis cataphractus, m.add.p), ▽ ,○ dragonet (Callionymus lyra, myotomal, m.add.p), ◇ sculpin (Myoxocephalus scorpius, myotomal)(from, Langfeld et al. 1989), Tropical; ▼ saddle wrasse*(Thalassoma duperreyi, myotomal). Data represent mean ± SE (n; refer to Table. 3). (The normal environmental temperature range of different species is indicated by a dotted line; *, determined from slack tests).


Maximum velocity (L/s)
20
15
10
5
0
−5
0
5
10
15
20
25
30
Temperature (°C)

increasing NBT (Table 3). Compared to the Antarctic species, T. lepidorhinus, the values of "a" from cold temperate species were significantly smaller at NBT ($P=0.025-0.05$).

Measured at 8°C, Vmax and SEE were significantly different for fast fibres isolated from the myotomes and m.add.p of C. lyra, Vmax being 1.2 times faster in the former ($P<0.05$) (Table 3).

Discussion

The thermal range over which live fibres produced maximum tension was highly correlated with habitat temperature (Fig. 6). At temperatures beyond the upper and lower lethal thermal limits of each species, isolated fibres become progressively inexcitable. The maximal force-temperature relationship of live fibres (Fig. 6), is significantly different than that described for skinned fibres. For example, the maximum Ca^{2+}-activated tension produced by skinned fast fibres from Myoxocephalus scorpius increased from 145 to 191 kN m^{-2} between 2°C and 15°C, and declined to 155 kN m^{-2} at 20°C (Johnston and Sidell, 1984). In contrast, maximum tetanic tension for live preparations from the same muscle increases from 242 to 315 kN m^{-2} between 2°C and 8°C, declined to 172 kN m^{-2} at 16°C, and was negligible at 20°C (Langfeld et al. 1989). At high temperatures, skinned fibres fail to relax completely following maximal activations and eventually undergo spontaneous

contractions in relaxing solution. Post-activation force is associated with a proportional increase in resting stiffness and a marked decrease in contraction velocity due to the formation of abnormal cross bridge linkages (Johnston and Altringham, 1985). Ca^{2+}-insensitive force development occurs at 8-10°C in Antarctic species, 16-20°C in cold-temperate species and > 30°C in tropical fish (Johnston and Brill, 1984; Johnston, 1985; Johnston and Altringham, 1985). All the fast muscles used in the present study are multiply innervated. In preliminary experiments, isolated live fibres from M. scorpius and C. lyra failed to contract when treated with the neuromuscular blocker, α-bungarotoxin, which suggests that they are being activated via the end plates (see Appendix 1). In contrast, α-bungarotoxin had no effect on the contractility of focally innnervated fast muscle fibres from two eel species, A. anguilla and C. conger (Appendix 1). It may be that the density of extra-junctional voltage sensitive channels is too low for the direct activation of the multiply innervated fibres. Thus, the reasons for the loss of excitability of live fibres at unphysiological temperatures are likely to be complex, involving a progressive failure of force generation, activation and/or excitation-coupling.

Fast muscle fibres from Antarctic, temperate and tropical fish produce similar absolute tensions at their normal body temperatures (Johnston, 1987; Langfeld et al. 1989; this study). Similar results have been obtained with skinned muscle fibres from a wider range of species

(Johnston, 1990). Thus, force generation shows perfect temperature compensation over evolutionary time scales. Evolutionary changes in the temperature-force relationship of muscle fibres are associated with adaptations in myosin structure. The myosin from cold-water fish belongs to an unstable type, relative to tropical fish and mammals, which rapidly aggregates on isolation, with a concomitant loss of ATPase activity (Connell, 1958, 1961; Richards et al. 1967). Sulphydryl groups are also more accessible to titration with DTNB in Antarctic than tropical fish myosins (Johnston et al. 1975). The ATPase activity of myofibrils from Antarctic fish is around 200-500 times more susceptible to thermal denaturation than the ATPase from tropical species under comparable conditions (Johnston and Walesby, 1977). These results suggest that adaptations in force production are associated with substantial differences in myosin tertiary structure between cold- and warm-adapted species.

Even relatively small differences in average body temperature are sufficient to produce resistance type adaptations in vertebrate skeletal muscle. For example, amongst Australian skinks the upper temperature for normal contractile function was higher for species in the genus Ctenotus, which have preferred body temperatures (PBT) of around 35^{o}C, than in the more cryophillic genera Sphenomorphus and Eremiascincus with PBTs of 30^{o}C and 25^{o}C, respectively (John-Alder and Bennett, 1987).

Time-dependent muscle contractile properties are highly temperature dependent, with Q_{10} values ranging from 1.3 to 3.5. Twitch relaxation rates are greatly prolonged in tropical species at low temperature (Figs. 8b and 10b). Temperature compensation of relaxation may be explained at least in part by adaptations in the kinetics of calcium transport by the sarcoplasmic reticulum. McArdle and Johnston (1980) found that at $0^{o}C$, SR isolated from the fast muscle of Antarctic fish accumulated Ca^{2+} at six-times the rate of SR isolated from tropical species. Activation enthalpies ($\triangle H$) of the SR ATPase ranged from 53-190 kJ $mole^{-1}$ for 15 species and were positively correlated with habitat temperature (McArdle and Johnston, 1980). However, at physiological temperatures the half-times for twitch activation and relaxation are significantly longer in cold- than warm-water species (Figs. 8-11; Table 2). Studies on the effects of temperature on live muscle fibres from amphibians and reptiles have reported broadly similar findings (Bennett, 1985). For example, rates of force development had Q_{10} values of 1.89-2.01, whilst force generating capacity remained relatively temperature independent in live muscle preparations of the salamander, Ambystoma tigrinum (Else and Bennett, 1987). Similar, very modest temperature compensation of twitch tension development and relaxation were noted for cryophillic species of skink (John-Alder and Bennett, 1987).

Studies with skinned, (Johnston and Brill, 1984) and now live fish muscle fibre preparations have shown no temperature compensation in unloaded contraction velocity (Vmax) between species from different latitudes (Fig. 13). However, the curvature of the P-V relationship increases with NBT and/or experimental temperature in fish (Table 3) (Johnston and Altringham, 1985; Langfeld et al 1989; this study) and in amphibians (Marsh and Bennett, 1985). Langfeld et al. (1989) normalized the P-V curves for live fibres from M. scorpius for P_o and Vmax at each temperature. They found that the change in curvature was sufficient to increase the relative power output of the muscle by around 15% on decreasing the temperature from 8°C to 1°C. This is because a less curved force-velocity relationship results in a higher velocity and hence greater power output, for a given load. However, any changes in the curvature of the P-V relation with temperature could only account for a modest temperature compensation of contraction velocity in vivo.

During swimming previous contractions will influence the dynamic properties of myosin cross bridges. Altringham and Johnston (1990b) determined the power output of fast muscle fibres from cod (Gadus morhua) undergoing oscillatory work. Using conditions for maximum net positive work they found that relative to isometric conditions, force and the rates of rise and fall of force, are increased and the duration of the contractile event is decreased. Although the time course of isometric contractions cannot be quantitatively

related to the *in vivo* situation, such data is useful for inter-specific comparisons. The present study has shown that twitch duration is significantly longer, and force development and contraction speed, are slower at *in vivo* temperatures in cold- than warm-adapted species (Table 2). The shorter duration of twitches in tropical species will enable their muscles to produce maximum power at higher tail-beat frequencies and hence work rates. The modest degree of temperature compensation observed for twitch duration is consistent with the relatively low maximum tail-beat frequencies reported for Antarctic fish (Montgomery and Macdonald, 1984; Archer and Johnston, 1989; Johnston *et al*. 1990b). Thus, although the contractile proteins in polar fish show substantial resistance type adaptations which enable contraction to proceed at very low temperatures, muscle power output remains significantly lower than in warm-water species. Temperature compensation of muscle power output would appear to largely involve adaptations in maximum force generation, with relatively minor contributions from time-dependent contractile properties.

CHAPTER 3

ACTIONS OF EPINEPHRINE ON THE CONTRACTILITY OF FAST AND SLOW SKELETAL MUSCLE IN TELEOSTS

Introduction

Several studies have shown that exercise produces an increase in the level of circulating catecholamines in teleosts (Butler et al. 1986). Butler et al. (1989) found that denervation of the head kidney in the cod (Gadus morhua L.) largely abolished any increase in epinephrine and norepinephrine during swimming. Denervation also resulted in a significant decrease in critical swimming velocity (U_{crit}), which was partially reversed by the infusion of catecholamines (Butler et al. 1989). Catecholamines have positive inotropic and chronotropic effects on the heart (Holmgren, 1977; Cameron, 1979), and have been shown to increase gill blood flow and oxygen uptake in isolated gill and head preparations (Wood, 1974; Perry et al. 1985). These actions of ephinephrine and norepinephrine would be expected to improve swimming performance by increasing oxygen delivery to the working muscles.

In mammals and amphibians catecholamines also have direct effects on the contractility of skeletal muscle fibres (see Bowman, 1980 for a review). For example, in mammalian slow muscles, catecholamines produce small

decreases in the duration and amplitude of twitches (Bowman and Zaimis, 1958; Bowman and Nott, 1969). This can result in a substantial decrease in the tension and the degree of fusion of subtetanic contractions (Bowman et al. 1985). Mammalian fast contracting muscles are much less sensitive to sympathomimetic amines and generally respond with an increase in twitch duration and amplitude (Holmberg and Waldeck, 1977). Epinephrine also increases twitch amplitude and the maximum rates of tension development and tension decay in frog semitendinosus muscle, but in this case without affecting twitch duration (Gonzalez-Serratos et al. 1981). There is evidence that these responses involve the stimulation of β_2-adrenoreceptors and a rise in 3'5'-cyclic monophosphate (cAMP) levels (Posner et al. 1965; Bowman and Nott, 1969). Isoprenaline effects are potentiated by phosphodiesterase inhibitors, which further supports the role of cAMP as a second messenger (Bowman and Nott, 1974). However, the precise mechanism remains uncertain since forskolin, a potent activator of adenylate cyclase activity where it has been investigated, was found to produce a 20-fold increase in cAMP concentrations in guinea-pig soleus muscle without changing the tension or degree of fusion of sub-tetanic contractions (Bowman et al. 1985).

In teleosts, levels of epinephrine range from 1 nmol l^{-1} at rest to 200-400 nmol l^{-1} following exhaustive exercise (Mazeaud and Mazeaud, 1985). The present study

is the first to investigate the effects of epinephrine and selective antagonists on fish skeletal muscle.

Methods

Fish

Atlantic cod (Gadus morhua L.) and short-horned sculpin (Myoxocephalus scorpius L.) were caught in the Firth of Forth, East Scotland, in late September/early October and maintained at ambient temperature (13-15^{o}C). The mean lengths and weights of the fish used were as follows: 33.2 ± 2.3cm, 480.4 ± 97.1g, n=5 for the cod and 27.6 ± 4.0cm, 449.2 ± 90.6g, n=9 for the sculpin (mean ± SD). Some experiments were also performed on winter-acclimatized sculpin caught in late November/December and maintained at the ambient sea-water temperature which was 5-6^{o}C.

Preparation of fibre bundles

Fish were sacrificed by a blow to the head followed by pithing and decapitation. Small bundles of fast muscle fibres were isolated from anterior abdominal myotomes at 4^{o}C, and aluminium foil clips placed around the trimmed peritoneum, previously described (Chapter 2). Slow fibre preparations were also prepared at 4^{o}C, by removing the skin from the lateral surface of the fish and excising a thin strip of muscle (see Johnston, 1981 for anatomical arrangement of fibres). Fibre bundles were isolated by further dissection and foil clips attached around the myosepta.

Sarcomere length was measured by laser diffraction and set to 2.3-2.4 μm (see Chapter 2). Single or multiple supramaximal stimuli (50v and 1ms duration pulses) were delivered via two platinum electrodes every 8 minutes; this inter-stimulation frequency allowed complete recovery so that reproducible contractions could be obtained for several hours. Force was measured with a silicon beam strain gauge (AE 801, AME Horten, Norway) (sensitivity 0.5 mN V^{-1}) and recorded using a Gould 1602 Digital oscilloscope (Chapter 2).

Drugs

The following drugs were used: (-) epinephrine (Sigma), dl-propranolol hydrochloride (Sigma), (-)isoprenaline bitartrate (Sigma), phentolamine mesylate BP (Ciba), forskolin (Sigma). Small quantities of epinephrine, propranolol and isoprenaline stock solutions were freshly prepared in Ringer, the pH of the isoprenaline stock was adjusted to 3.5 to enhance stability. Phentolamine was supplied as an aqueous solution (10 mg/ml). Stock solutions of forskolin were dissolved in 95% ethanol.

Experimental procedure

The effects of epinephrine and isoprenaline on muscle contractility were investigated using twitch and sub-tetanic contractions. The antagonists phentolamine and propranolol were used to characterize the receptor type. The effects of forskolin were also investigated where

adrenergic responses were demonstrated. Experiments on the cod were performed at 5°C. Studies with the sculpin were carried out at 8°C in winter- and summer-acclimatized fish (this was intermediate between winter and summer ambient temperatures).

Cyclic AMP determination

Slow fibre bundles from cod were incubated in either normal Ringer or in normal Ringer containing 1µmol l^{-1} epinephrine at 5°C for 20 minutes. Muscle fibres were rapidly frozen in iso-pentane cooled in liquid nitrogen and freeze dried. Using a Kontes Cell Disruptor the fibres were sonicated on ice (maximal frequency for 2-6 sec bursts) in 200 µl, 3.5 % PCA, neutralized with 45 µl of 1.5 M K_2CO_3 (checked with litmus paper) and microfuged prior to analysis. cAMP was analysed using the Amersham cAMP [^{3}H] assay kit (TRK.432).

Statistics

The concentrations of intracellular cAMP for agonist treated and control preparations were compared using a t-test for sample populations of unequal variance.

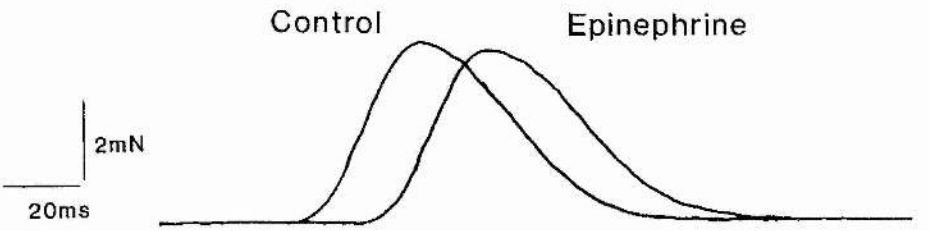
Results

Effects of drugs on contractility

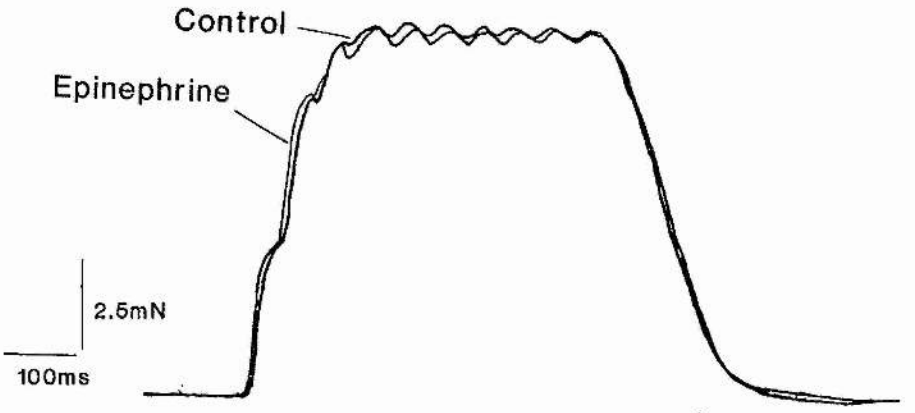
Epinephrine and isoprenaline (10^{-11} to 10^{-5} M) failed to induce any measureable change in twitch or sub-tetanic contractions in the fast abdominal muscle of sculpin or cod (Fig. 1a,b). Slow muscle preparations were also

Fig 1. The effects of epinephrine (1 μM) on the contractile properties of fast muscle fibres from the sculpin. (a) Twitch response: twitch half activation time ($t_{\frac{1}{2}}a$) = 18.0 ms (control), 18.4 ms (epinephrine); twitch half relaxation time ($t_{\frac{1}{2}}r$) = 28.4 ms (control), 28.8 ms (epinephrine). (b) Sub-tetanic response (stimulation frequency = 20 Hz).

a)


Control
Epinephrine
2mN
20ms

b)


Control
Epinephrine
2.5mN
100ms

unaffected by isoprenaline (10^{-11} to 10^{-5}M). However, epinephrine (10^{-11} to 10^{-5}M) induced concentration dependent decreases in the half-time for twitch relaxation (Table 1), and in most cases was accompanied by a reduction in tension (Fig. 2a,i). In accordance with these observations there was a reduction in both tension and the degree of fusion of incomplete tetanic contractions (Fig. 2b). The dose dependent reductions in twitch relaxation were used to produce a cumulative dose response curve (Fig. 3). These effects were maximal at 1 μmol l^{-1}, with peak responses occurring within 8 mins (the time between each stimuli) and were readily reversed by washing out the drug. The maximal depression in tension ranged from 0% to 38.6% in cod and from 0% to 28.9% in sculpin. Maximal depression in twitch half relaxation ranged from 4.6% to 21.9% in cod and 8.1% to 12.6% in sculpin. Epinephrine had no effect on the rate of rise of twitch tension (Fig. 2a,i). These results are summarized in Table 1. In the sculpin the response of preparations obtained from both summer- and winter-acclimatized animals were the same. The response in tension proved too variable for an effective comparison; however the mean change in twitch half relaxation time was 9.7 ± 1.5 ms (n=6) for 15°C-acclimatized animals and 10.4 ± 2.1 ms (n=3) for 6°C-acclimatized animals (mean ± SE). The responses to epinephrine were competitively inhibited by the α-antagonist phentolamine (Fig. 2a,ii). Propranolol, a ß-antagonist, failed to inhibit the

Table 1. Effects of epinephrine (1 μM) on isometric contractile properties of slow muscle fibres. Values represent the mean ± SE.

Parameter	Species	
	Sculpin	Cod
% decrease in twitch half relaxation time.	9.7 ± 0.6 (n=9)	11.5 ± 3.2 (n=5)
% decrease in twitch tension.	7.7 ± 3.1 (n=9)	14.4 ± 10.0 (n=5)

Fig 2. The effects of epinephrine (1 μM) on the contractile properties of slow muscle fibres from the sculpin. (a)i. Twitch response: $t_{\frac{1}{2}}a$ = 45 ms (control and epinephrine); $t_{\frac{1}{2}}r$ = 195 ms (control), 175 ms (epinephrine). (a)ii. The effects of the α-antagonist, phentolamine (2 μM) on the twitch response of the epinephrine treated preparation from Figure 2a, i. $t_{\frac{1}{2}}a$ = 45 ms, $t_{\frac{1}{2}}r$ = 195 ms (phentolamine). (b) Sub-tetanic response (stimulation frequency = 10 Hz).

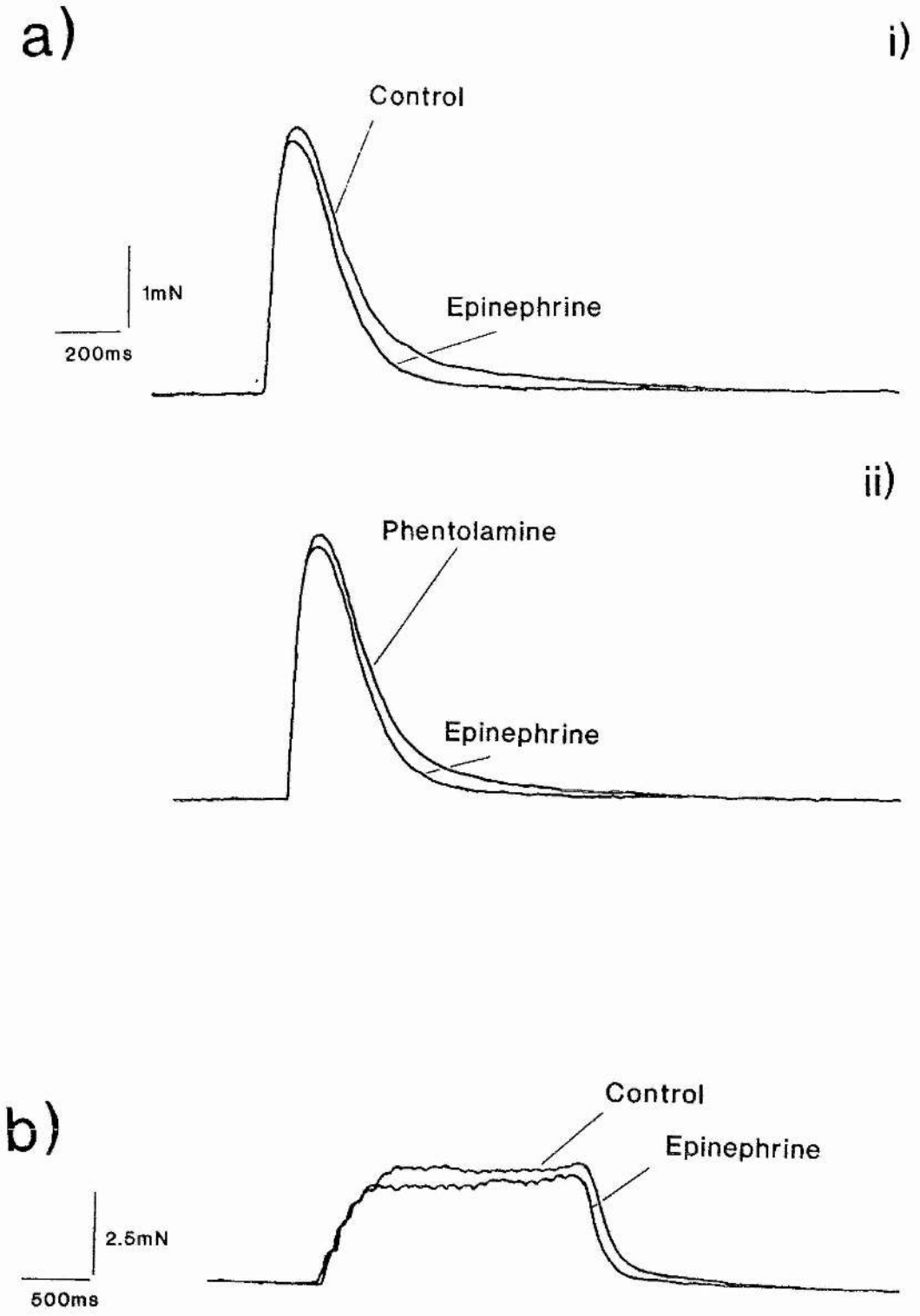
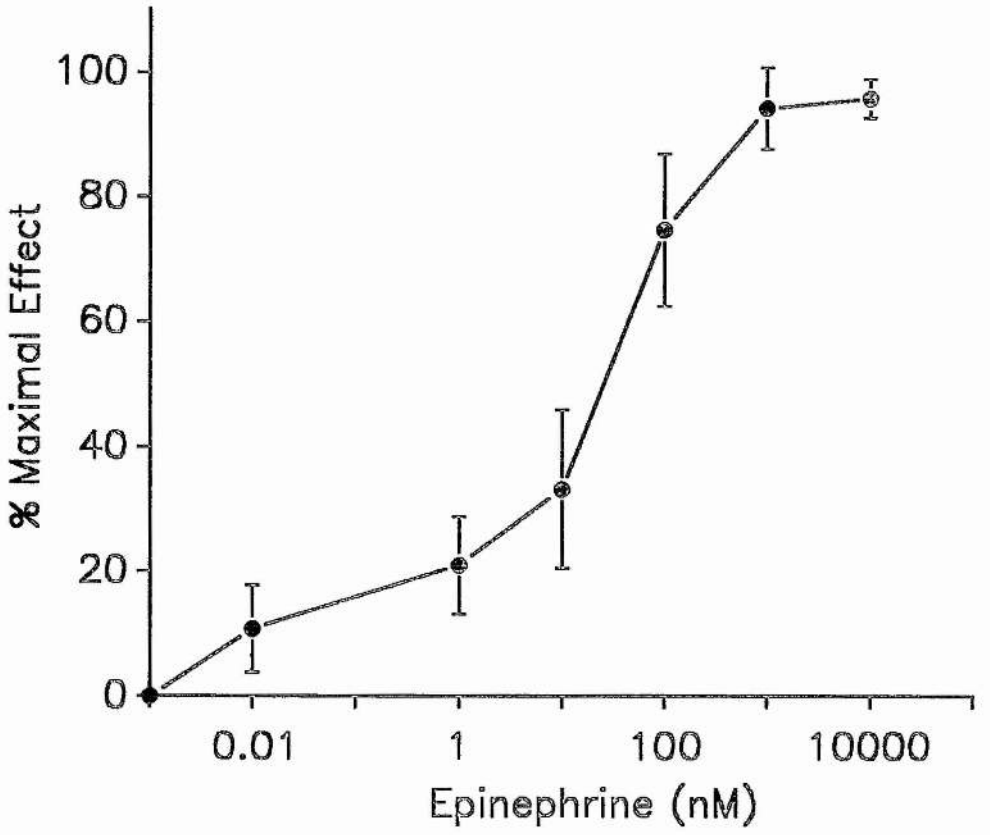

a)
i)
Control
1mN
200ms
Epinephrine
ii)
Phentolamine
Epinephrine
b)
Control
Epinephrine
2.5mN
500ms

Fig 3. Cumulative dose response curve for the effects of epinephrine (0.01-10,000 nmoll^{-1}) on isolated slow muscle preparations from the cod. The data represent the mean ± SE decrease in the twitch half relaxation time expressed as a percentage of the maximum response observed. The curve is based on five experiments. The concentration producing a 50% effect was 25 nmol l^{-1}, determined graphically.


100
80
60
40
20
0
% Maximal Effect
0.01
1
100
10000
Epinephrine (nM)

adrenergic response in either winter- or summer-acclimatized animals.

In contrast, forskolin produced a large increase in tension and twitch half relaxation time in slow fibres at a concentration of 10 μM (Fig. 4). The addition of an equivalent quantity of 95% ethanol (the solvent) prior to the application of the drug produced no measurable change in contractility.

Cyclic AMP concentrations

The mean concentration of cAMP in the control cod slow fibre preparations was 1.8 ± 0.4 pmol/ mg dry wt (mean ± SD, n=7). Following incubation with 1 μmol l^{-1} epinephrine for 20 min the mean concentration of cAMP increased significantly to 3.1 ± 1.0, a 72% increase (Fig. 5) ($P<0.01$).

Discussion

The actions of catecholamines on fish skeletal muscle differ from those reported for other vertebrate muscles in a number of important respects. For example, fast twitch fibres from the limb muscles of frog (Gonzalez-Serratos *et al.* 1981), cat (Merican *et al.* 1983) and guinea pig (Holmberg and Waldeck, 1977) respond to epinephrine and isoprenaline with an increase in the force of contraction and twitch duration. In contrast, fast twitch fibres in cod and sculpin were insensitive to even high doses of these drugs. Epinephrine caused a speeding of relaxation in fish slow fibres (Fig. 2a,i),

Fig 4. The effect of forskolin (10 μM) on the twitch response of an isolated slow muscle preparation of the sculpin; $t_{\frac{1}{2}}a$ = 40 ms (control and forskolin); $t_{\frac{1}{2}}r$ = 120 ms (control), 135 ms (forskolin). Maximum tension = 4.3 mN (control), 5.2 mN (forskolin).

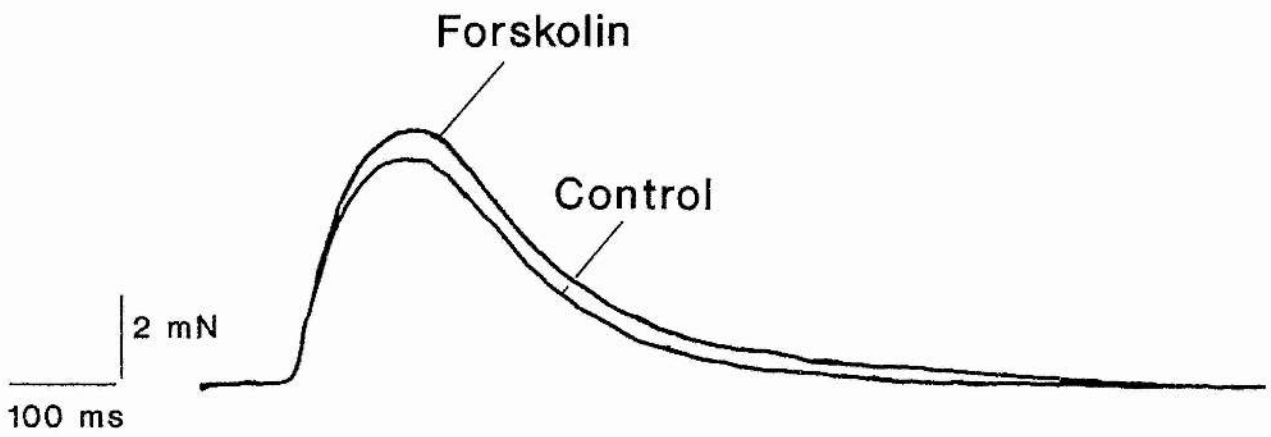
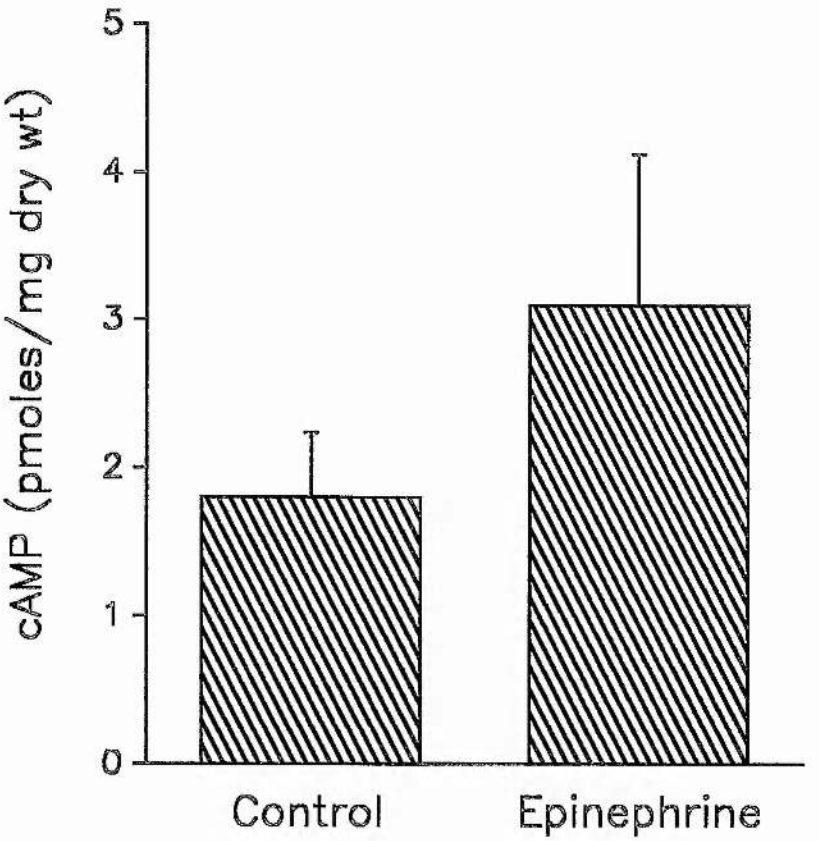

Forskolin
Control
2 mN
100 ms

Fig 5. Effect of epinephrine (1 μM) on the intracellular concentration of cAMP. Data represent the mean ± SD, n=7 for the control and n=8 for the preparations incubated at 5°C for 20 mins with epinephrine.


cAMP (pmoles/mg dry wt)
5
4
3
2
1
0
Control
Epinephrine

and an increase in cAMP concentrations (Fig. 5). Although similar results have been reported for mammalian slow fibres (Bowman, 1980), evidence was obtained that in fish muscle these actions are mediated via α-adrenoreceptors (Fig. 2a,ii) rather than β_2-adrenoreceptors. Alpha-adrenoreceptors which elevate cAMP levels have been previously characterized, for example, in mammalian sympathetic ganglia (Bowman and Rand, 1980). In frog heart, positive ionotropic responses to epinephrine are largely mediated by α-adrenoreceptors below 14°C and by ß-adrenoreceptors above 23°C (Kunos and Nickerson, 1976). In contrast, Peyraud-Waitzenegger et al. (1980) found that an alpha adrenergic bradycardia was present in the hearts of winter-acclimatized eels, perhaps reflecting a state of winter dormancy, whereas summer animals showed a tachycardia to the same intravenous dose of epinephrine. Cod and sculpin remain active throughout the year and experience a seasonal temperature range of 5-15°C. No evidence for seasonal differences in the response to catecholamines or in the type of receptor involved was found for fish skeletal muscle.

Two main mechanisms have been proposed to explain the role of cAMP in speeding the rate of relaxation in slow skeletal muscle. One possibility is that cAMP acts via its dependent protein kinase to phosphorylate proteins in the sarcoplasmic reticulum which increase Ca^{2+}-ATPase activity and consequently increase the rate of calcium sequestration (Schwartz et al. 1976; Kirchberger and

Tada, 1976; Jorgensen and Jones, 1986). A similar mechanism has long been known to operate in cardiac muscle (Tada et al. 1975; Kirchberger et al. 1974). The other idea is that tension changes are secondary to enhancement of membrane Na^+-K^+ ATPase activity by cAMP which act to increase the removal of Ca^{2+} from the cytosol (Tashiro, 1973; Clausen, 1981). The problem with both proposed mechanisms is that forskolin elevates the cAMP content of skeletal muscle fibres without producing the expected sympathomimetic responses in tension (Bowman et al. 1985; this study). It is, however, likely that adenylate cyclase is compartmentalized in slow skeletal as well as in cardiac muscle cells (Zahler, 1983). In this case the adenylate cyclase upon which the forskolin is effective may not be involved with Ca^{2+} sequestration. Sarcoplasmic reticulum purified from trout slow myotomal muscle was found to have a reduced content of the 100 kDa Ca^{2+}-ATPase protein relative to fast muscle and contained additional proteins of 48 and 31 kDa molecular mass (McArdle and Johnston, 1981). However, no evidence was obtained for the modulation of calcium transport by cAMP-dependent protein kinases (McArdle and Johnston, 1981). Further work is required to elucidate the mechanisms of action of epinephrine reported in the present study.

During swimming fish myotomal muscles undergo cyclic contractions and mechanical power output is a complex function of the amplitude, frequency and relative timing of nerve impulses in relation to muscle length changes

(Johnston and Altringham, 1988; Altringham and Johnston 1990a,b). In order to maximize power output at any given tail-beat frequency it is important to optimize the time period at which force levels remain high (Altringham and Johnston 1990a,b). At a particular cycle frequency there will be an optimal number of stimuli. If there are too few stimuli then force will decline too early in the shortening cycle and power output will be sub-optimal. Too many stimuli will result in incomplete relaxation such that work will have to be done against the fibres by the antagonistic set of muscles. It is theoretically possible, therefore, that epinephrine acts to increase swimming performance by subtle modulation of the active state of muscle fibres during each cycle. The effects of epinephrine would be expected to vary with the frequency of locomotory movements. In this connection, it is interesting to note that, in cod, infusion of catecholamines during low intensity exercise actually inhibits swimming, whereas the same level of catecholamines at higher exercise intensity enhances swimming performance (Butler et al. 1989).

CHAPTER 4

POWER OUTPUT OF FISH MUSCLE FIBRES PERFORMING OSCILLATORY WORK: EFFECTS OF ACUTE AND SEASONAL TEMPERATURE CHANGE

Introduction

The muscle fibres in fish myotomes undergo cyclical contractions of increasing frequency as swimming speed increases. Kinematic studies have shown that for steady swimming the strain fluctuations of muscle fibres are essentially sinusoidal (Hess and Videler, 1984; van Leeuwen et al. 1990). Altringham and Johnston (1990a,b) measured the power output of isolated fish muscle fibres under conditions which mimic their activity during swimming. The approach adopted, was first used to measure the power output of asynchronous insect flight muscles by Machin and Pringle (1959) and latter adapted for synchronous insect muscle by Josephson (1985). Briefly, isolated muscle fibres are subjected to sinusoidal length changes and phasically stimulated during the strain cycle whilst simultaneously recording fibre length and force. The area of the force-position loops produced during each cycle corresponds to the work done during shortening minus the work required to re-lengthen the muscle. At each cycle frequency the strain amplitude and the number and timing (phase) of electrical stimuli can be adjusted to maximize net work output.

Altringham and Johnston (1990a) found that in the marine teleost Myoxocephalus scorpius L., maximum mechanical power output (work per cycle times cycle frequency) was produced at a cycle frequency of 2 Hz for slow (5-8 W kg^{-1}) and 5-7 Hz for fast (25-35 W kg^{-1}) muscle fibres (at 3°C). Curtin and Woledge (1989) have also reported measurements of the power output of dogfish fast muscle fibres at 12°C using cyclical ramp length-changes.

Electromyographical studies have shown that fish progressively recruit slow then fast muscle fibres as swimming speed increases (Johnston et al. 1977; Rome et al. 1984). In the common carp (Cyprinus carpio L.), as water temperature decreases fast muscle fibres are recruited at lower swimming speeds, in order to compensate for the reduced mechanical power output of the slow muscle (Rome et al. 1985; Heap and Goldspink, 1986). After several weeks at low temperature however, both the speed at which fast muscle fibres are first recruited and swimming performance increase (Rome et al. 1985; Heap and Goldspink, 1986). These improvements in swimming performance are associated with a major remodelling of the skeletal muscles (reviewed in Johnston and Dunn, 1987). For example, cold acclimation in carp results in a decrease in twitch duration and an increase in maximum contraction velocity (V_{max}) and tension development at low temperature (Fleming et al. 1990; Johnston et al. 1990b). Adaptations in contractile properties with temperature acclimation are more pronounced for fast than slow muscle fibre types (Langfeld et al. 1990). The

molecular mechanisms underlying these adjustments in muscle contractile properties include changes in sarcoplasmic reticulum ATPase activity (Fleming et al. 1990) and alterations in myosin light chain (Crockford and Johnston, 1990: Langfeld et al. 1990) and myosin heavy chain composition (Gerlach et al. 1990; Hwang et al. 1990).

In the present study the effects of acute and seasonal temperature change on muscle power output during oscillatory work have been investigated. All experiments were carried out on fast muscle fibres isolated from the short-horned sculpin, Myoxocephalus scorpius. Locally, this species experiences sea temperatures ranging from 3-5^{o}C in winter to 12-16^{o}C during the summer.

Methods

Fish

Short-horned sculpin (Myoxocephalus scorpius L.), were caught in St. Andrews Bay during the winter (Jan-Feb) and summer (July-Aug) of 1990. Fish were maintained in sea-water aquaria for 2-21 days prior to experiments at ambient temperatures and photoperiods; 4-5^{o}C (8h light:16h dark) in the winter and 12-13^{o}C (16h light:8h dark) in the summer. To reduce variation resulting from scaling (Altringham and Johnston, 1990b), fish of a similar size were used. The standard lengths and weights of the fish studied were 270.4 ± 52.5g, 22.4 ± 2.0cm (n=7), for winter specimens and 257.4 ± 39.4g, 22.3 ±

1.5cm (n=7) for summer specimens (mean ± SD). A small number of winter-acclimatized fish were also used to determine the effects of varying stimulus phase and strain amplitude on muscle power output (272.8 ± 65g, 22.4 ± 2.1cm, n=4, mean ± SD).

Preparation of fibre bundles

Fast muscle fibre bundles were isolated from the abdominal myotomes and maintained in Ringer solution, as described previously (Chapter 2). Preparations consisted of 20-30 fibres and had a mean length 8.3 ± 1.1 mm (mean ± SD, n=31). Up to two preparations were dissected from each fish as reproducible results could be obtained from preparations over a two day period, if stored in fresh Ringer at $4^{o}C$.

Apparatus

The basic design of the live fibre chamber was essentially that described in Chapter 2. The cooling system for circulating Ringer, platinum electrodes, transducers (AE801) for tension measurement and arrangement of the servo-motor were identical. However the servo-motor was a more powerfull model (MFE R4-155). Particular attention towards the screening of the apparatus was made, including an earthed cage around the servo-motor and a standard Faraday cage.

The apparatus was designed to subject isolated muscle fibre preparations to sinusoidal length changes and periodic stimulation and to subsequently determine the

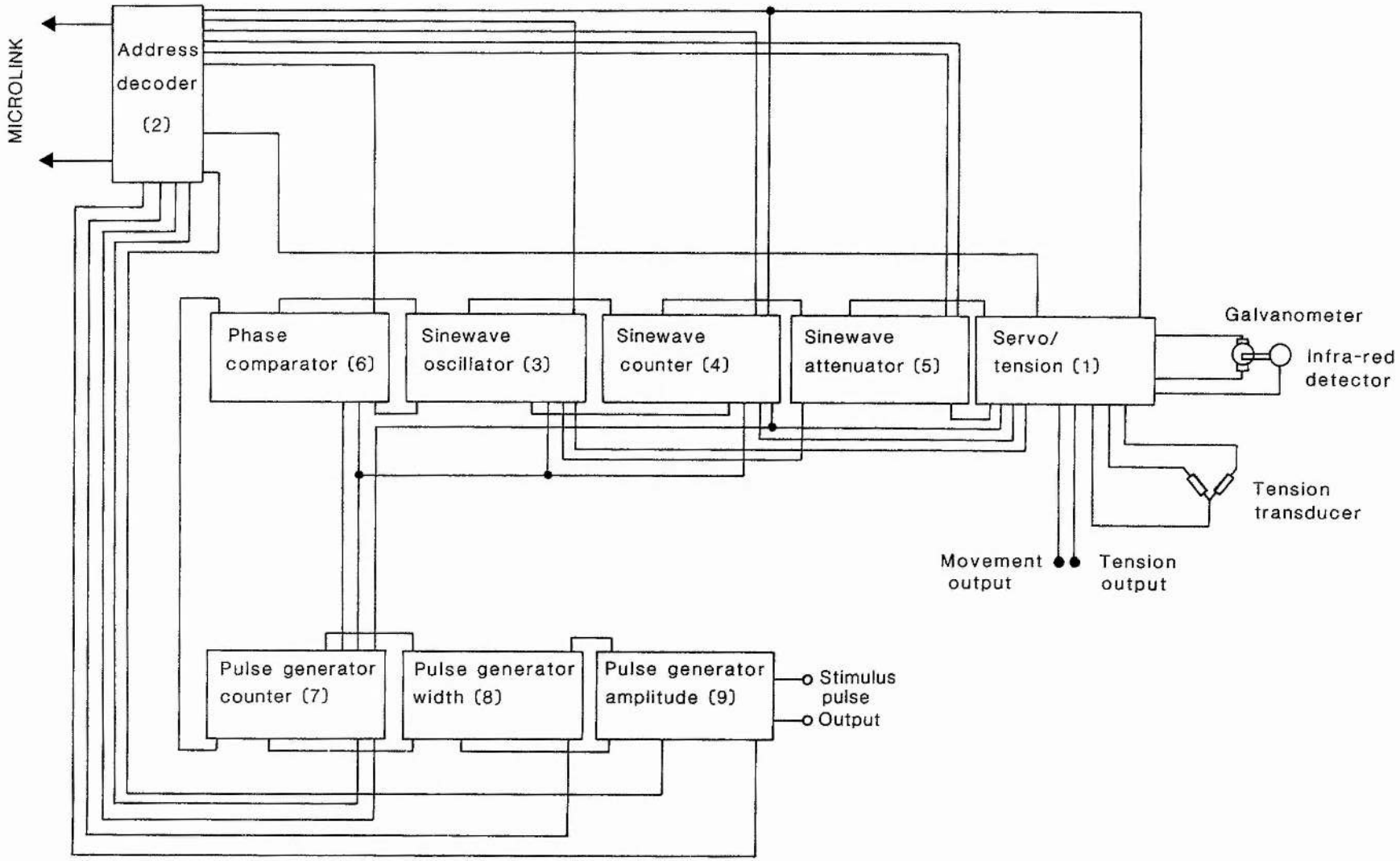
work done by the preparation. The magnitude and frequency of length changes and stimulation pulses were controlled. The timing of stimulation in relation to the start of the length change or " stimulus phase shift" was also controlled (Table 1). The apparatus also calculated the phase delay between peak length and peak force (ie. if peak length coincided with peak force then the phase delay was equal to 0^{o}).

The system was controlled and information stored by an Amstrad computer (PC1640 HD20) using in-house software ("GENLOOP") and Hewlett Packard plotter for hardcopy facilities. Details of the menu and output facilities of this software are displayed in Appendix 2. The control circuitry consisted of nine basic units (Fig. 1). Three units controlled the number (7), width (8) and amplitude (9) of the stimulus pulse(s) and another three controlled the frequency (3), number (4) and amplitude ("strain amplitude") (5) of the length change cycles. The phase comparator (6) adjusted the stimulus phase delay by integrating information from the units controlling length and stimulus. The "address-decoder" (2) set up each of these units with the appropriate parameters prior to an experimental run and received/transmitted information directly from the transducers. This information was communicated via the "servo-tension" unit (1) which also served to allow the calibration of the analogue output from the control circuitry using gain and offset controls (0.2mm/V and 5mN/V).

Table 1. The range of parameters controlling the characteristics of the stimulation and length changes involved in muscle fibres performing oscillatory work.

Parameter	Range
a)Stimulation	
Number	0-255
Frequency	1-200Hz
Width	100μs-25.5ms
Voltage	0-100V
b)Length change	
Amplitude	0-6553μm
Frequency	0-25Hz
Phase	2-360^{o}

Fig. 1. A block diagram of the control circuitry referred to in the text (designed by Mr M.Mc Candless, Department of Psychology, University of St Andrews).


MICROLINK
Address decoder (2)
Phase comparator (6)
Sinewave oscillator (3)
Sinewave counter (4)
Sinewave attenuator (5)
Servo/ tension (1)
Galvanometer
Infra-red detector
Tension transducer
Movement output
Tension output
Pulse generator counter (7)
Pulse generator width (8)
Pulse generator amplitude (9)
Stimulus pulse
Output

An IEEE-488 interface was installed into the computer for the integration with the control circuitry through a MICROLINK system. The MICROLINK was installed with "modules", specific to the requirements of this system. AN1 modules were used in conjunction with analogue to digital (A-D) converters to receive and process the analogue signals from tension and length transducers. The gain and offset controls of this module facilitated the calibration of the MICROLINK mainframe to the computer. This calibration was performed by inputting an analogue signal of known amplitude to the module, from a GRASS S48 stimulator. An 08-8DO ("digital output") module sent the coded information which setup the appropriate parameters in each of the units of the control circuitry, prior to an experimental run. Direct display of transducer output was facilitated by the computer and a Gould 1602 Digital Storage Oscilloscope.

Components and Software

Amstrad PC1640 (20MB hard disk)(Amstrad PLC, Essex),

Microlink, with 2xAN1 and 08-8DO modules (Biodata Ltd, Manchester),

IEEE-488 interface (GPIB) and software for IBM (Biodata Ltd, Manchester),

Control circuitry (McCandless, Department of Psychology, University of St Andrews),

"GENLOOP" software for Amstrad PC (Dr J. Eastwood, Department of Computing, University

of Aberystwyth).

Hewlett Packard Color-Pro plotter (7440A), (Hewlett-Packard Ltd, USA).

Experimental protocol

Muscle fibre preparations were transferred to the chamber, the sarcomere length was measured by laser diffraction and set to 2.3-2.4μm (Chapter 2) and resting length accurately measured using an eye-piece graticule. Using the appropriate menu of the GENLOOP software, the input sensitivities (ie. 0.2mm/V and 5mN/V) were set and the parameters controlling the characteristics of stimulation and length change entered (Appendix 2, menu F3).

Muscle fibres were subjected to sinusoidal length changes about *in situ* resting length and stimulated at a selected phase in the strain cycle. Stimulation was supramaximal, administered via two platinum electrodes lying on either side of the preparation (12V, 2ms duration, 50Hz at 4°C and 75Hz at 15°C). The stimulation frequencies used were those required to give a maximal fused tetanus. By plotting force against muscle length a series of force-position loops were obtained for each cycle, the area of which represented the net work done (Josephson, 1985). Anticlockwise components indicate positive work and clockwise components indicate negative work. Power output which is net work mulitiplied by cycle frequency is a complex function of the cycle frequency, strain amplitude, number of stimuli and

stimulus phase shift. Strain amplitude was expressed as a percentage of resting muscle fibre length. Stimulation phase was defined as the delay between the start of stimulation and the start of the length change cycle expressed in degrees (full cycle = 360^{o}). Eight cycles of work were performed during an experimental run. Maximum force and work output were relatively constant over 8 cycles (Figs. 2a and 7). Power output was calculated using the mean of eight cycles.

The estimation of cross sectional area

i)Preparation

A small pyramid of fish liver was mounted on moist filter paper, on a cryostat "chuck" and surrounded with the mounting compound, Tissue-Tek (Miles Scientific, Ilinois, USA). A small vertical hole was then made in the liver with a fine pair of tweezers. When an experiment had been successfully completed the muscle fibre preparation was pinned out by its foil clips to resting length on a thin strip of the dissection medium, Sylgard (ref. Chapter 2). The preparation was rapidly frozen by plunging into liquid iso-pentane, cooled to near freezing with liquid nitrogen (-159^{o}C). Using a cooled scalpel blade, the frozen fibres were cut free of the foil clips and inserted into the liver, which was subsequently plunged into the freezing iso-pentane.

Using a cryostat (Bright, Huntingdon, England), 13μm sections were cut, mounted on cover-slips and left to air dry for approximately 45 minutes.

ii)Staining

Sections were stained for actomyosin ATPase, using a technique described in Johnston et al (1974), based on an earlier method by Padykula and Herman (1955).

iii)Chemicals

Chemicals were obtained from Sigma (Poole, Dorset);

Adenosine 5'-triphosphate (from equine muscle), disodium salt, trihydrate.

Trizma base (Tris[hydroxymethyl]aminomethane) reagent grade

Cobalt chloride ($CoCl_2$), hexahydrate (pfs),

Calcium chloride ($CaCl_2$), dihydrate (pfs), approximately 99% pure.

221 Alkaline buffer solution (2-amino-2-methyl -1-propanol, 1.5M, pH 10.3 at 25°C),

and from BDH (Poole, Dorset);

Potassium chloride (KCL), (Analar, 99.8% pure)

Ammonium polysulphide solution (10.0% w/v).

(Standard distilled water was used for all solutions).

iv)Solutions

Tris-HCl pH 7.8;

Tris	12.1g
$CaCl_2$ (0.18M)	100ml
H_2O	900ml

(Adjusted to pH 7.8 with HCl)

Incubation solution pH 9.4;

221 Alkaline buffer solution (Sigma) 3.35ml

$CaCl_2$ (0.18M) 5.0ml

KCl 0.185g

ATP 0.076g

H_2O 40ml

(Adjusted pH to 9.4 using HCl and made up to 50ml with distilled water). This solution could be stored frozen for up to one week.

Alkaline washing solution pH 9.4 (made fresh);

221 Alkaline buffer solution (Sigma) 13.4ml

H_2O 160ml

(Adjusted pH to 9.4 using HCL and made up to 200ml using H_2O).

0.18M $CaCl_2$ Stock;

$CaCl_2$ 26.46g

H_2O 1litre

v)Procedure

Staining was carried out at room temperature. Air-dried sections were rinsed in tris-HCl (pH 7.8) and left in incubation solution for approximately 10 minutes. The sections were washed with 1% (w/v) $CaCl_2$ for about 1 minute and then transferred to 2% (w/v) $CoCl_2$ for 3 minutes. After washing once in distilled water, sections were washed three times with alkaline washing solution and transferred to 1% ammonium sulphide to precipitate cobalt sulphide. Sections were finally washed in running tap-water and then mounted on slides using glycerol gelatin (Sigma). Live muscle fibres appeared deeply and

uniformly stained, whilst damaged fibres were more "patchy" in appearence (Plate 1).

The profiles of the fibres were traced (x100) using a microscope drawing arm (Nikon, Labophot). The cross sectional area of the preparation was then determined using a planimeter interfaced to a Hewlett Packard 86B microcomputer.

From this measurement of fibre area and the length of the preparation, the weight of the preparation could be calculated by assuming a density of $1g/cm^3$. Values of work and power output were expressed in terms of joules or watts per kilogram wet weight. Measurements of tension were expressed in terms of kN/m^2.

Statistics

Results from winter- and summer-acclimatized fish were compared at a given cycle frequency and temperature using a standard t-test.

Results

Effects of varying strain amplitude

The cycle frequency, optimal strain amplitude, number of stimuli and stimulus phase shift required to maximize power output at 4^oC and 15^oC were determined in preliminary experiments. Whilst maintaining the other parameters required for maximum power output constant the effects of varying strain amplitude were determined. Typical results from a winter-acclimatized fish are shown

Plate 1. Photograph of a section taken of a muscle fibre preparation, mounted in liver and stained for myofibrillar ATPase. Note that live fibres (L) are uniformly stained whilst dead fibres (D) are more "patchy" in appearance.


L
D

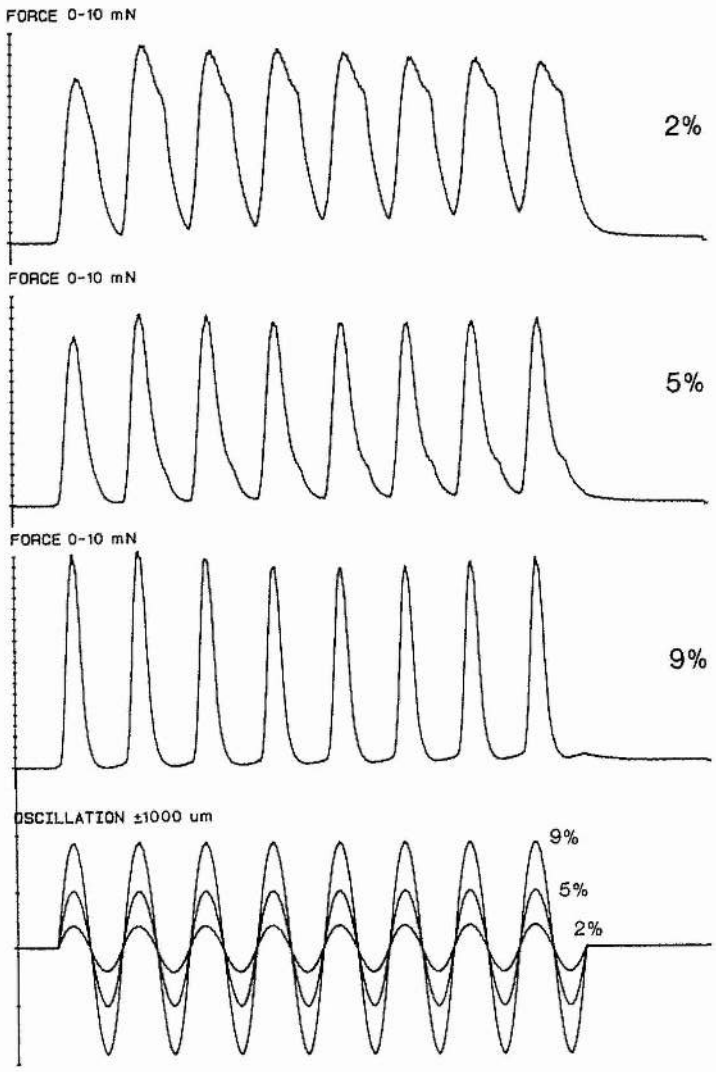
in Figure 3. The optimal strain amplitude occured at ± 5% resting muscle length and was independent of temperature. Changes in work output with variations in strain amplitude were a consequence of changes in the shape and position of work loops (Fig. 2b). The area of work loops is reduced at strain amplitudes of <±5% muscle length. Under these conditions net work is further reduced because fibres fail to relax completely between successive cycles, thereby increasing the work required to return the muscle to its resting length (Fig. 2). Work output is reduced for strain amplitudes of >±5% resting length since force generation is limited by the faster imposed relaxation rate (shortening-induced inactivation) (Fig. 2).

Effects of varying stimulus phase

The effects of varying stimulus phase shift were investigated for winter-acclimatized fish whilst maintaining the other parameters required to produce maximum power output constant. Maximum positive work loops were obtained over a narrow range of stimulus phase shift angles for which the fibre was given a small stretch prior to stimulation. At a phase shift of 30^{o} net positive work was greater than at 5^{o} or 60^{o}, due to optimum force enhancement by pre-stretch (Figs. 4 and 5). Under these conditions the additional work produced during the shortening part of the cycle, by virtue of the higher force, was greater than the extra negative work performed during stretch. At 210^{o} phase shift, negative

Fig. 2. (a) Force and length records of fibres from a winter-acclimatized fish performing oscillatory work at 4°C. Fibres were stimulated two times every cycle, with a stimulus phase shift of 25°, at a cycle frequency of 5Hz. Muscle strain amplitude was ± 2%, ± 5% and ± 9% resting length. (b) Force has been plotted against fibre length for the fourth cycle of oscillatory work to produce the corresponding work loops.

a) 5Hz/2 stimuli/25 phase



b)

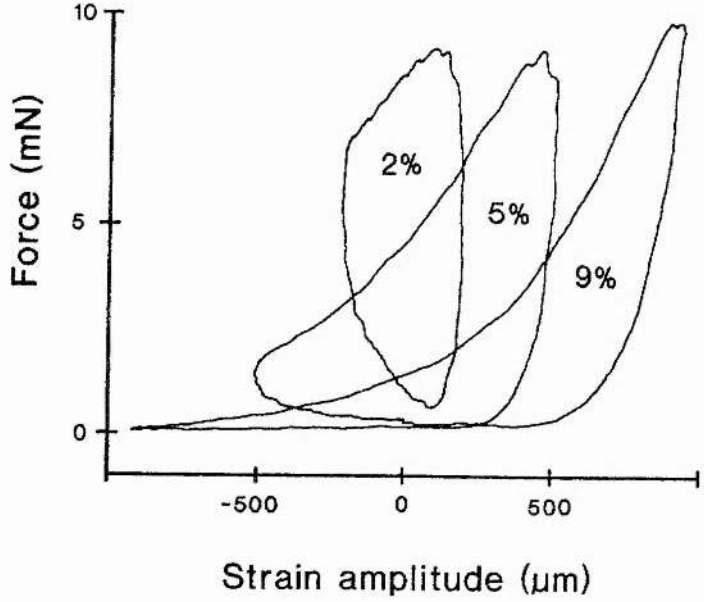


Fig. 3. The relationship between strain amplitude (% fibre length) and power output at 4°C and 15°C, for a representative preparation from a winter-acclimatized fish. Stimulation parameters and cycle frequency were adjusted to maximize power output at each temperature: 4°C; 3 stimuli per cycle, 30° stimulus phase shift, 5Hz cycle frequency, 15°C; 2 stimuli per cycle, 25° phase shift, 13Hz cycle frequency.

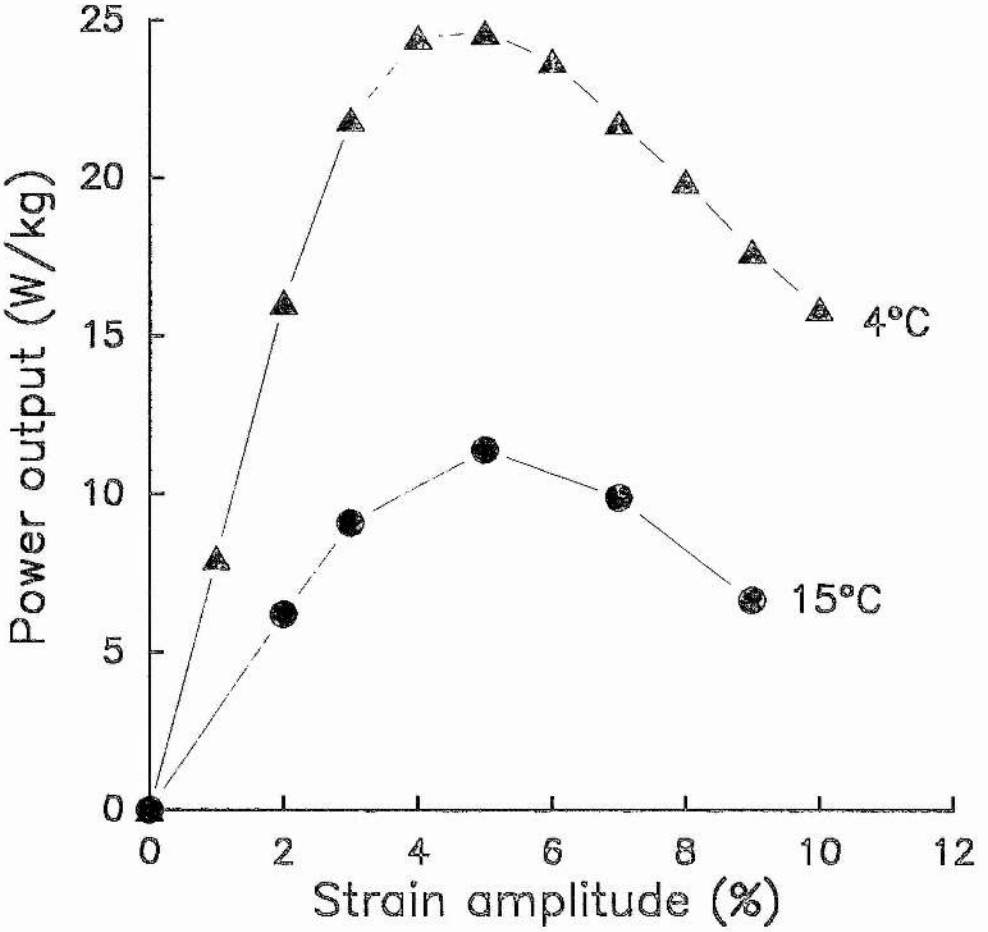

Power output (W/kg)
25
20
15
10
5
0
0
2
4
6
8
10
12
Strain amplitude (%)
4°C
15°C

Fig. 4. The relationship between power output and (a) stimulus phase shift, (b) the phase delay between peak length and peak force, for fibre bundles performing oscillatory work at 4°C, 5 Hz cycle frequency, ± 5% strain amplitude and stimulated 3 times per cycle (·· ····· ; resting length, ·· ·· ; muscle length). Data represent mean ± SE, obtained from four winter acclimatized fish of mean weight, 273 ± 90.7g and standard length, 22.4 ± 2.1cm (mean ± SD).

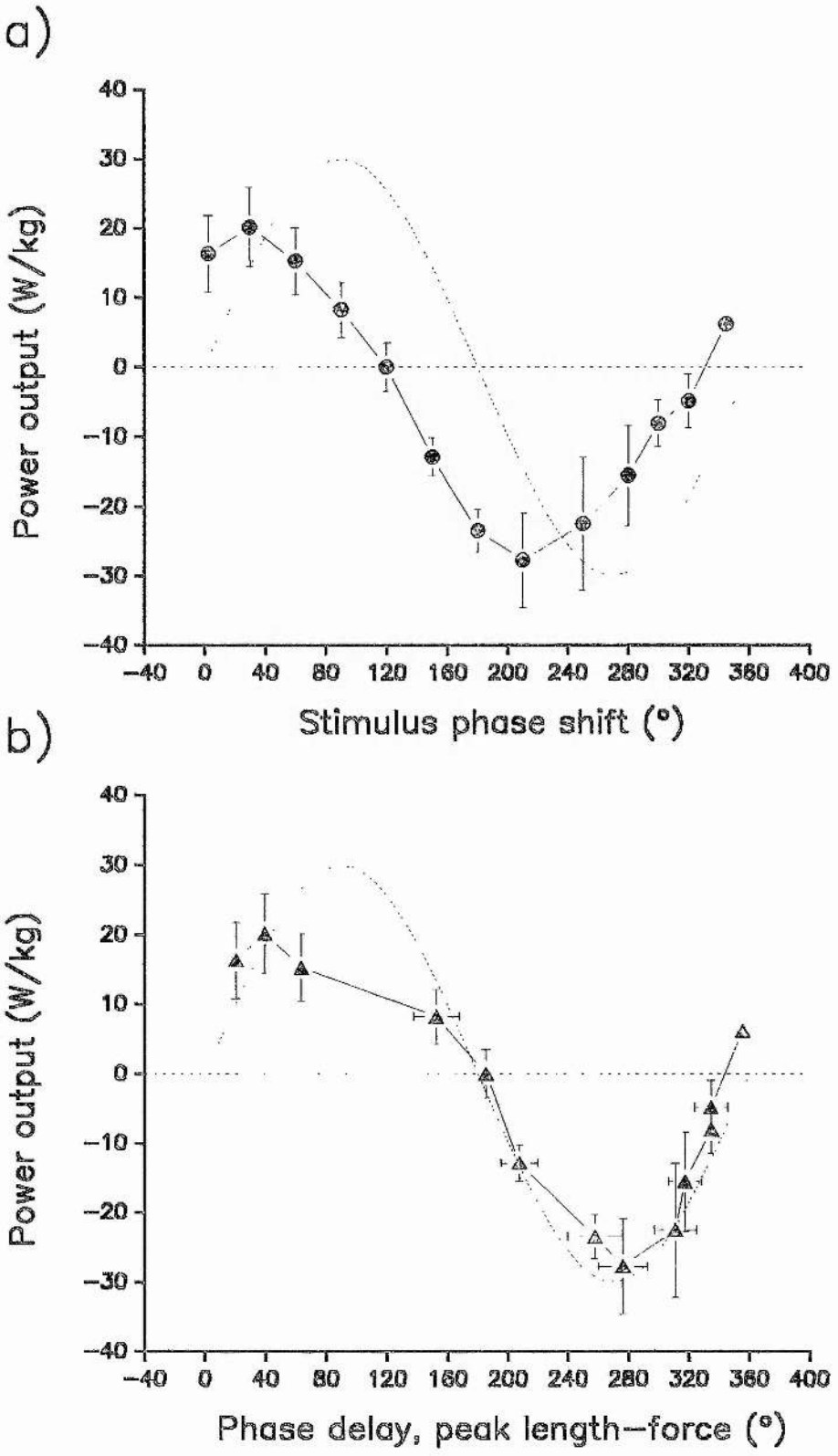
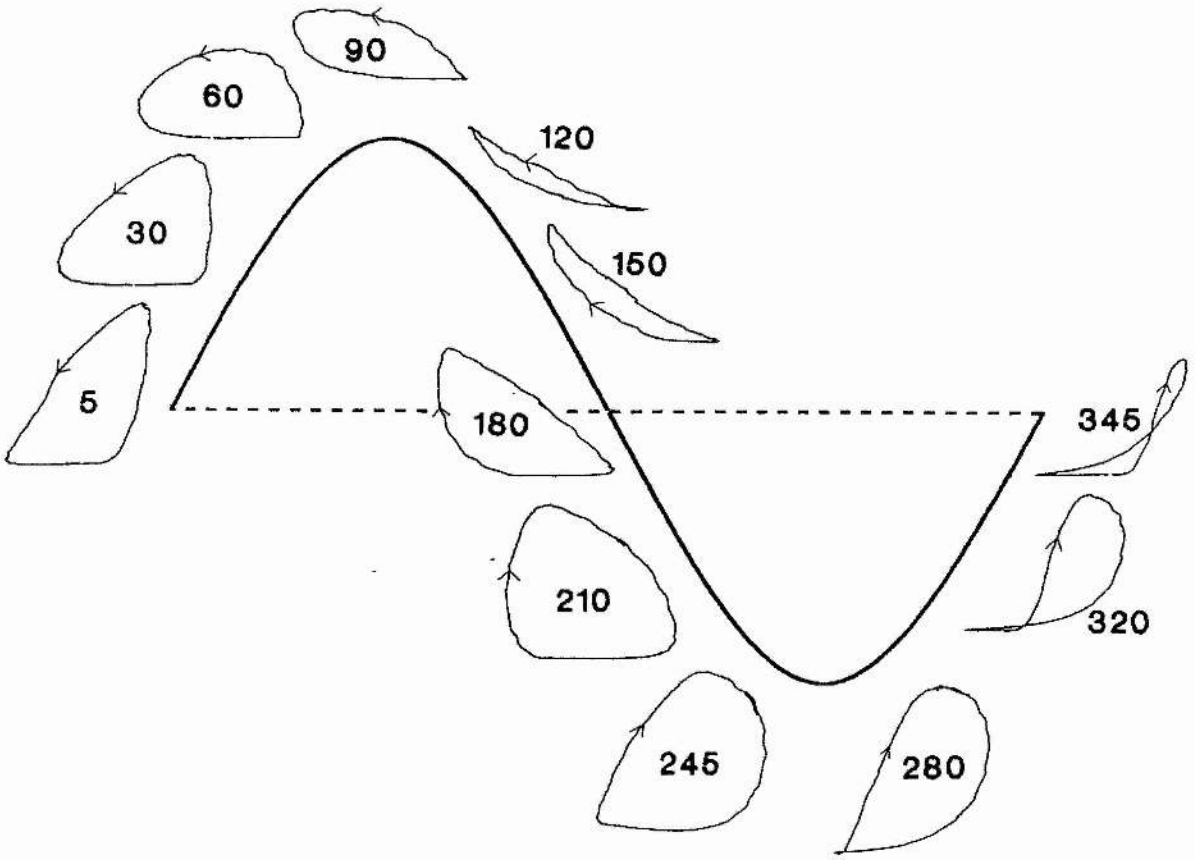

a)
Power output (W/kg)
40
30
20
10
0
−10
−20
−30
−40
−40 0 40 80 120 160 200 240 280 320 360 400
Stimulus phase shift (°)
b)
Power output (W/kg)
40
30
20
10
0
−10
−20
−30
−40
−40 0 40 80 120 160 200 240 280 320 360 400
Phase delay, peak length−force (°)

Fig. 5. The effect of stimulus phase shift on the work loops of a representative preparation from the data presented in Fig. 4. The numerical values represent the stimulus phase shift in degrees (^{o}). The dotted line represents resting length and the solid line corresponds to the muscle length change (± 5% resting length). Work loops are positioned according to the timing of stimulation in relation to the length change.


90
60
120
30
150
5
180
345
210
320
245
280

work output was maximal (clock-wise work loop) and exceeded the maximum positive work output at 30° phase shift by 20-30% (Fig. 4). Between 30° phase shift (maximum positive work) and 210° phase shift (maximum negative work), power output systematically varied with stimulus phase shift (Fig. 4) and work loops became complex with both positive and negative components (Fig. 5). When fibres were stimulated during shortening (90-180° phase), the delay between peak length and peak force increased dramatically (Fig. 4). This resulted in a non-linear relationship between stimulus phase shift and the phase delay between peak length and peak force (Fig. 6). Note that the phase delay between peak length and peak force is expressed in degrees, with a full cycle equal to 360°.

Effects of temperature

The effects of temperature on the work output produced during cyclical contractions varied between summer- and winter-acclimatized fish (Figs. 7 and 8a). Work output was maximized at a range of cycle frequencies by adjusting the number and phase shift of stimuli per cycle at a strain amplitude of ± 5% resting muscle length. For both acclimatization groups, work per cycle decreased with increasing cycle frequency and at very low cycle frequencies (< 3Hz) (Fig. 8a). The decline in work output with increasing cycle frequency was less pronounced at 15°C than at 4°C, thus the cycle frequency required to produce maximum power output increased from

Fig. 6. The relationship between stimulus phase shift and the phase delay between peak length and peak force for four preparations isolated from winter-acclimatized fish. The dotted line represents linearity. Other details are given in the legend to Fig. 4.

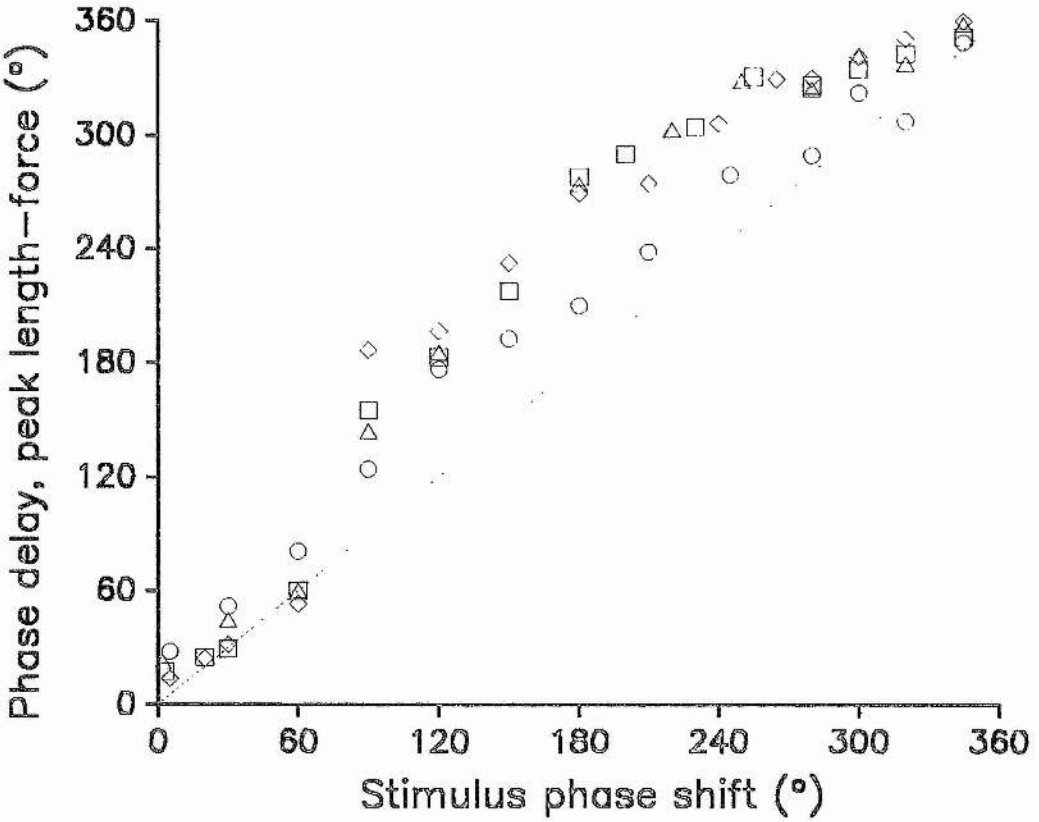

Phase delay, peak length–force (°)
360
300
240
180
120
60
0
0
60
120
180
240
300
360
Stimulus phase shift (°)

Fig. 7. Representative tension and length records with corresponding work loops, of fibre bundles isolated from winter- and summer-acclimatized fish. Each preparation was stimulated at 4°C and 15°C, under conditions required to yield optimum power output.

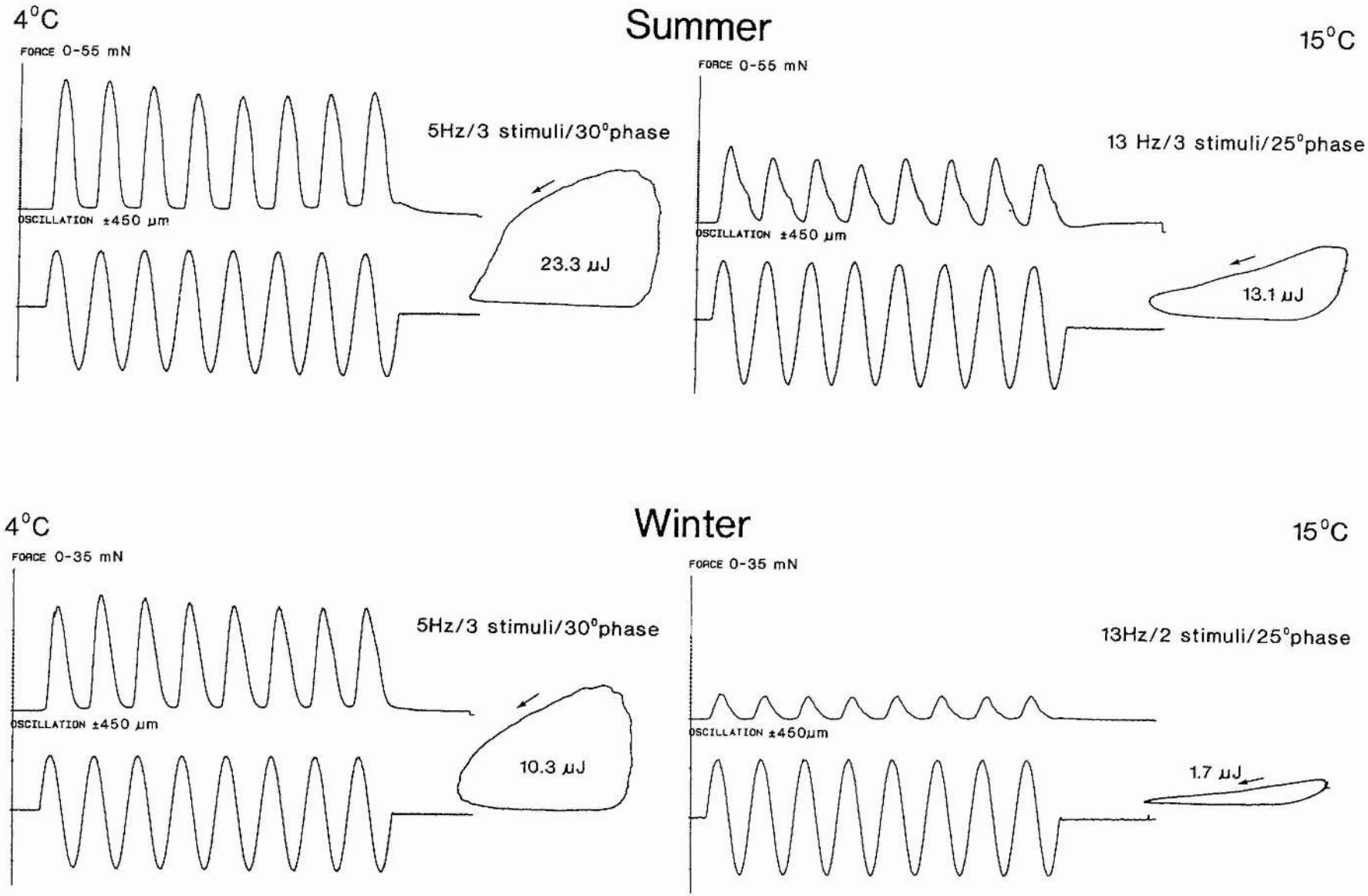
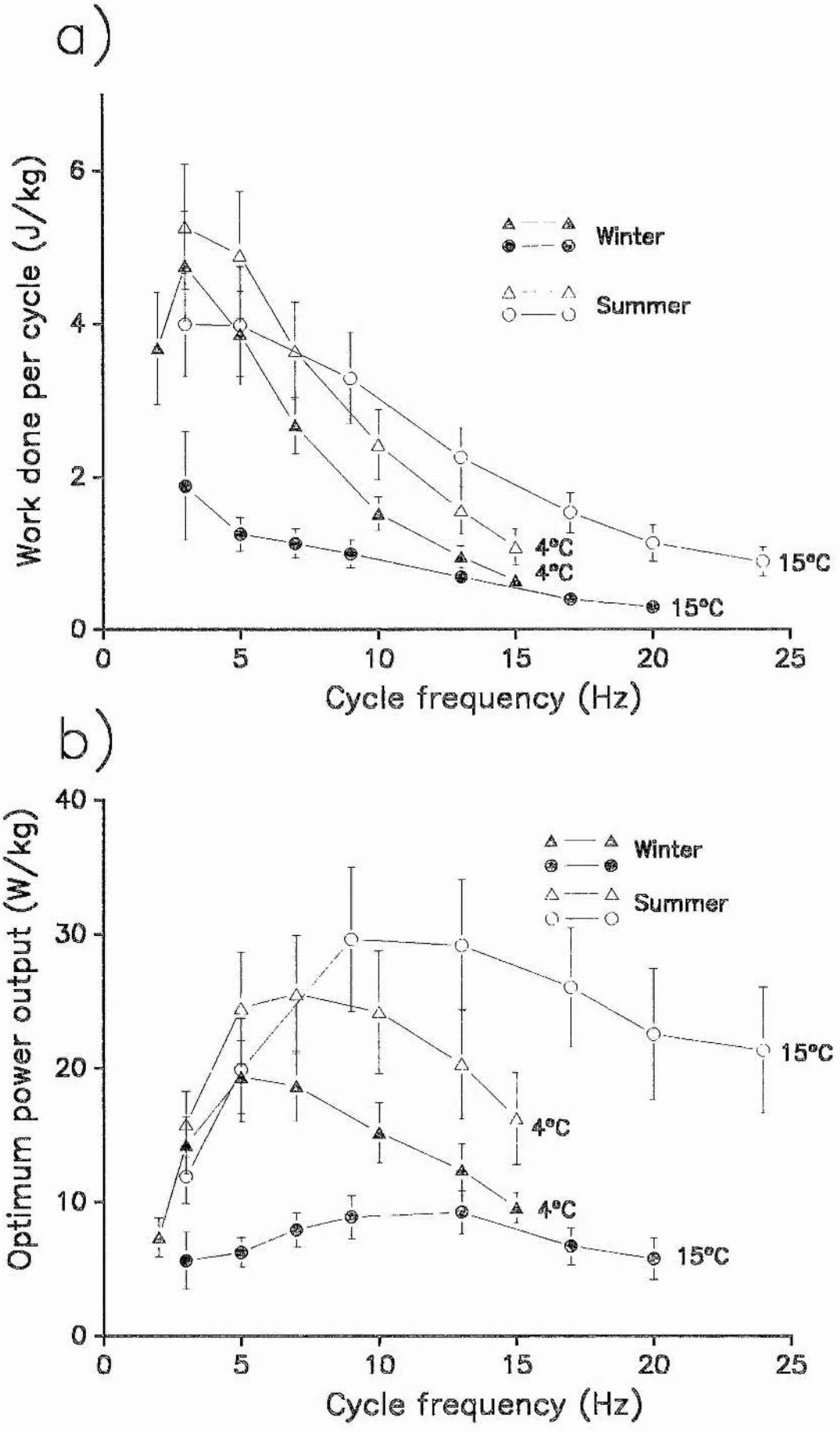

Summer
4°C
FORCE 0-55 mN
5Hz/3 stimuli/30°phase
OSCILLATION ±450 µm
23.3 µJ
15°C
FORCE 0-55 mN
13 Hz/3 stimuli/25°phase
OSCILLATION ±450 µm
13.1 µJ
Winter
4°C
FORCE 0-35 mN
5Hz/3 stimuli/30°phase
OSCILLATION ±450 µm
10.3 µJ
15°C
FORCE 0-35 mN
13Hz/2 stimuli/25°phase
OSCILLATION ±450µm
1.7 µJ

Fig. 8. The relationship between cycle frequency and (a) the work done per cycle (J/kg) and (b) power output (W/kg) for muscle fibre bundles from summer (n=11) and winter (n=10) acclimatized fish. Strain amplitude was ± 5% resting fibre length, cycle frequency and stimulation parameters were adjusted to maximize work output at a range of cycle frequencies. See the legend to Fig. 3 for further details (Data represent mean ± SE).

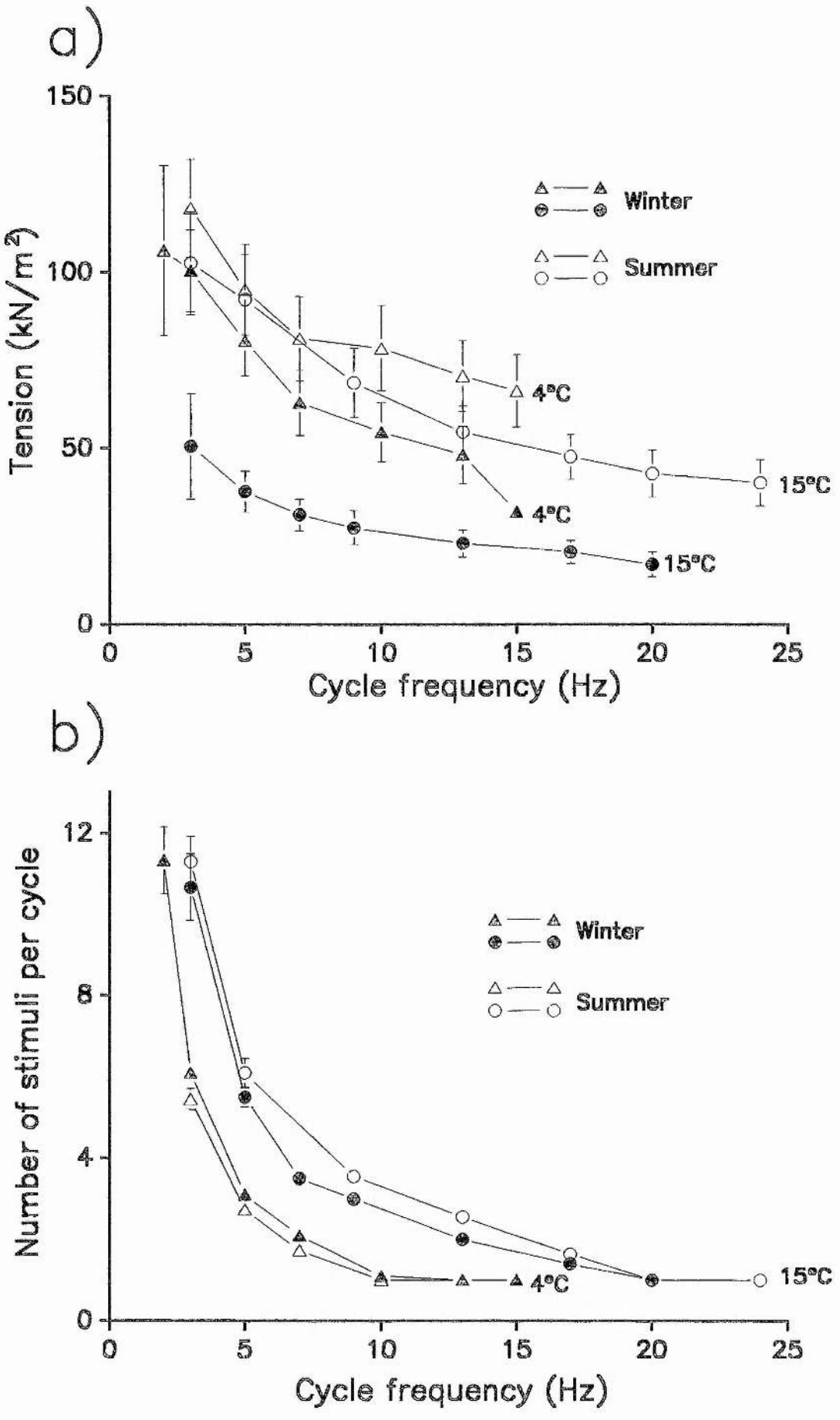

a)
Work done per cycle (J/kg)
0
2
4
6
0
5
10
15
20
25
Cycle frequency (Hz)
Winter
Summer
4°C
4°C
15°C
15°C
b)
Optimum power output (W/kg)
0
10
20
30
40
0
5
10
15
20
25
Cycle frequency (Hz)
Winter
Summer
15°C
4°C
4°C
15°C

around 5-7 Hz at 4°C to 9-13 Hz at 15°C (Fig. 8b). At 4°C, the maximum mechanical power output, 19-26 W kg^{-1}, was not significantly different in summer and winter acclimatized fish ($P>0.05$). However, at 15°C work per cycle and power output were significantly higher in summer- than winter-adapted fish ($P<0.05$), particularly above a cycle frequency of 5 Hz (Figs. 7, 8a and b). For example, at 13 Hz maximum power output was 9.3 ± 1.6 W kg^{-1} for winter fish and 29.2 ± 4.9 W kg^{-1} for summer fish (mean ± SD) ($P<0.01$) (Fig. 8b).

The maximum tension generated per cycle decreased by around half between 3 Hz and 8 Hz cycle frequency and thereafter remained relatively constant (Fig. 9a). At 15°C, maximum tension per cycle was higher at all cycle frequencies in summer- compared to winter-acclimatized fish ($P<0.05$ at 3 Hz, $P<0.01$ at 5-20 Hz) (Fig. 9a). Although maximum tension was consistently slightly lower at 4°C in winter than summer fish this difference was only statistically significant at 15 Hz ($P<0.01$) (Fig. 9a).

The number of stimuli per cycle required to maximize work output decreased with increasing cycle frequency and increasing temperature (Fig. 9b). Subtle changes in the number of stimuli required to maximize work per cycle were evident between summer- and winter-acclimatized fish (Fig. 9b). In general, summer-adapted fish required more stimuli at 15°C and fewer stimuli at 4°C (Fig. 9b). The stimulus phase shift required to maximize work decreased with increasing cycle frequency, particularly at 4°C

Fig. 9. The relationship between cycle frequency and (a) tension (kN/m^2) and (b) the number of stimuli per cycle for muscle fibres from summer (n=11) and winter (n=10) acclimatized fish. Strain amplitude was ± 5% resting length, cycle frequency and stimulation parameters were adjusted to maximize power output at a range of cycle frequencies. See the legend to Fig. 3 for details (Data represent mean ± SE).

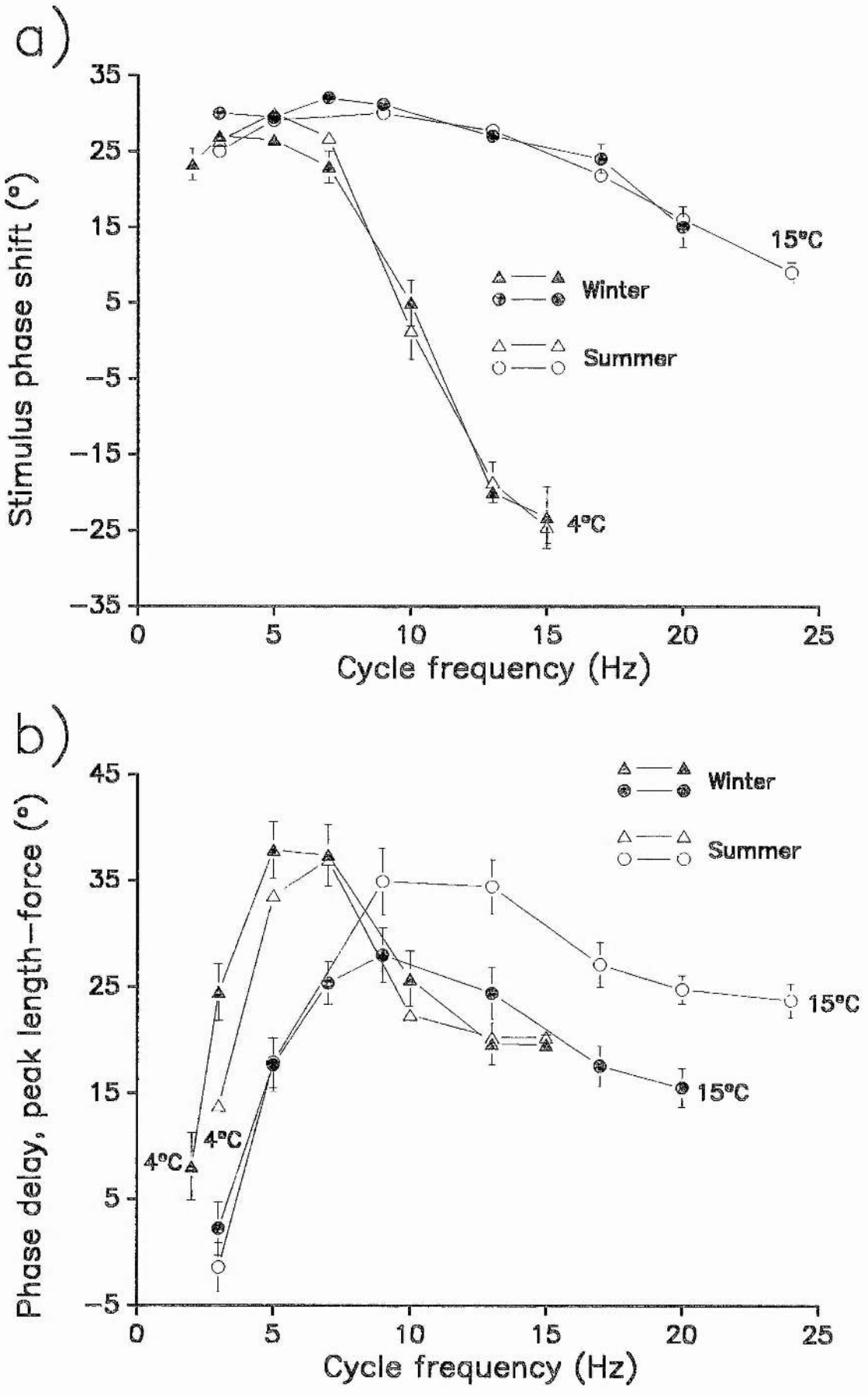

a)
150
100
50
0
Tension (kN/m²)
Winter
Summer
4°C
15°C
4°C
15°C
0
5
10
15
20
25
Cycle frequency (Hz)
b)
12
8
4
0
Number of stimuli per cycle
Winter
Summer
4°C
15°C
0
5
10
15
20
25
Cycle frequency (Hz)

(Fig. 10a). The optimal stimulus phase shift was independent of acclimatization temperature at all cycle frequencies (Fig. 10a). However, the phase delay between peak length and peak force was prolonged at 15°C and high cycle frequencies (9-20 Hz) and reduced at 4°C and low cycle frequencies (≤ 3 Hz), in summer- relative to winter-acclimatized fish ($P<0.01$) (Fig. 10b). The maintenance of higher force levels during the shortening part of the cycle contributes to the greater work output per cycle of muscle fibres in summer-acclimatized fish at 15°C (Fig. 7 and 8a).

Discussion

During swimming the sequential activation of myotomes on alternate sides of the body produces a wave of lateral bending from the head to the tail. The electromyographical wave proceeds faster than the mechanical wave of bending resulting in systematic phase differences in muscle force and length along the body (Hess and Videler, 1984; Williams et al. 1989). Several studies have modelled the strain fluctuations of muscle fibres in successive segments from a detailed study of the swimming movements and information on fibre recruitment patterns (Hess and Videler, 1984; van Leeuwen et al. 1990). These studies show that in anterior myotomes, force is maximal as the muscle fibres shorten through their resting lengths, such that power output is positive throughout most of the tail beat cycle (Hess and

Fig. 10. The relationship between cycle frequency and (a) stimulus phase shift and (b) the phase delay between peak length and peak force for muscle fibres from summer (n=11) and winter (n=10) acclimatized fish. Strain amplitude was ± 5% resting length, cycle frequency and stimulation parameters were adjusted to maximize work output at a range of cycle frequencies. See the legend to Fig. 3 for details (Data represent mean ± SE).


a)
Stimulus phase shift (°)
35
25
15
5
−5
−15
−25
−35
0
5
10
15
20
25
Cycle frequency (Hz)
15°C
Winter
Summer
4°C
b)
Phase delay, peak length–force (°)
45
35
25
15
5
−5
Winter
Summer
15°C
15°C
4°C
4°C
0
5
10
15
20
25
Cycle frequency (Hz)

Videler, 1984; van Leeuwen et al. 1990). Using the approach outlined in the present study, the stimulus phase shift can be adjusted to approximate these conditions (see also Altringham and Johnston, 1990a,b). Maximum positive work is obtained by giving muscle fibres a small initial stretch prior to shortening, corresponding to a stimulus phase shift of 30° (Fig. 4). In contrast, modelling studies show that more posterior myotomes are stretched whilst active producing significant amounts of negative work (Hess and Videler, 1984; van Leeuwen et al. 1990). Towards the end of the abdomen the amounts of positive and negative work almost balance each other, whereas in the caudal region net negative work is done (Hess and Videler, 1984; van Leeuwen et al. 1990). Increasing the stimulus phase shift in the present study, produced increasing amounts of negative work (Fig. 5). Thus, by adjusting the timing of stimuli in relation to the length change cycle it was possible to at least partially mimic the predicted work output of posterior myotomes (Hess and Videler, 1984; van Leeuwen et al. 1990). Maximum negative work output also exceeded maximum positive work output in line with the predictions of swimming models (see Fig. 7 in Hess and Videler, 1984). The stimulation of muscle fibres with a 90-150° phase shift, markedly increases the time to peak force (Fig. 6), due to shortening inactivation of the contractile apparatus (Josephson and Stokes, 1989).

Several features of the behaviour of isolated muscle fibres performing oscillatory work have a correspondence with muscle action during locomotion. For example, maximum tail-beat frequency increases with temperature (Webb, 1978; Batty et al. 1991) and this is paralleled by an increase in the cycle frequency required for maximum power output in isolated muscle fibres (Fig. 8b). Optimum muscle power output in vitro is produced at a strain amplitude of ± 5% resting length, over a range of temperatures (Fig. 3) and cycle frequencies (Altringham and Johnston 1990a). Similarly, tail-beat amplitude in rainbow trout (Salmo gaidneri) is relatively independent of temperature during fast-starts (Webb, 1978) and independent of tail-beat frequency over a range of swimming speeds (Webb et al. 1984). In contrast, the optimal strain amplitude for isolated flight muscle of the tobacco hawkmoth (Manduca sexta), increases with temperature (Stevenson and Josephson, 1990). However, this again parallels the in vivo situation since low amplitude wing movements are used to warm the thoracic temperature prior to flight (Stevenson and Josephson, 1990). Electromyography of mackeral swimming at 1-14 bodylengths s^{-1} show that the number of muscle action potentials per cycle decreases with increasing swimming speed (C.S. Wardle pers. comm). The functional significance of this observation may be that at higher cycle frequencies fewer stimuli per cycle are required to produce maximum power output (Fig. 9b). Since twitch duration increases at low temperature, small adjustments

in the number of stimuli per cycle and stimulus phase shift are required to allow complete relaxation between successive cycles and thereby maximize power output (Figs. 9b and 10a).

The short-horned sculpin is a cold-water species distributed in the seas around northern Europe and into the Arctic circle. The liver secretes a polypeptide antifreeze to enable it to survive freezing during the winter (Fletcher et al. 1989). A major finding of the present study is that the mechanical properties of muscle in this species are not fixed but can be modulated according to seasonal temperature changes. However, in contrast to the more widely studied carp, the major adjustments in mechanical properties occur at high temperatures (Fig. 7). At 15°C maximum muscle power output increased from $9Wkg^{-1}$ in winter to $30Wkg^{-1}$ in summer. The higher power output of muscles in summer-acclimatized fish is largely due to an increase in average force during the shortening part of the cycle, particularly at high cycle frequencies (Figs. 7 and 9a). This is reflected by an increase in the phase delay between peak length and peak force (Fig. 10b). Adaptations in power output can be explained by an increase in maximum tension generation (Fig. 9a) and/or a change in the shape of the force-velocity relationship. The twitch duration of carp fast muscle fibres is altered by temperature acclimation (Fleming et al. 1990). In the present study a small increase in the average number of stimuli was required to maximize power output at 15°C in

summer adapted fish, consistent with a decrease in twitch duration (Fig. 9b). Whatever the underlying mechanisms, the observed increase in muscle power output in summer-acclimatized fish will contribute to an improvement in swimming performance at high temperature. To my knowledge this is the first report of seasonal changes in the contractility of muscle fibres in a marine fish.

CHAPTER 5

TEMPERATURE AND THE ENERGY COST OF OSCILLATORY WORK IN TELEOST FAST MUSCLE FIBRES

Introduction

The mechanical constraints acting on muscle during contraction affect the energetic cost of generating force. For example, more heat is produced during shortening than during isometric contractions, a phenomenon known as the Fenn effect (Fenn, 1923, 1924; Hill, 1938). Cain and Davies (1962) found that the amount of chemical energy used was related to the work done, rather than being correlated with the magnitude of the length change. Dynamic changes in muscle fibre length also influence the magnitude of the measured energy changes (Heglund and Cavagna, 1987). For example, in rat muscle recovery oxygen consumption following shortening has been found to be reduced by a pre-stretch (Heglund and Cavagna, 1987). The energetics of contraction also varies with the time course of muscle activation, the rate of heat production being greater during the initial phase of an isometric tetanus (Abbott, 1951; Aubert, 1956).

During locomotion muscle fibres undergo cycles of shortening and lengthening and the state of activation varies continuously during the strain cycle (Josephson,

1985; Johnston and Altringham, 1988). Josephson (1985) has adapted the work loop technique developed by Machin and Pringle (1959) to measure power output of isolated muscles under conditions simulating locomotion (see also Chapter 4). Using a similar approach Altringham and Johnston (1990a,b) investigated the power output of fish muscle fibres at cycle frequencies appropriate to swimming. It was found that in cod (Gadus morhua L.) the cycle frequency for maximum power output of fast muscle fibres decreased from 12.5 Hz in 13cm fish to 5 Hz in 67 cm fish at 4°C (Altringham and Johnston, 1990b). Moon et al. (1990) extended these observations to measure the energy cost of cyclical contractions in cod muscle fibres treated with iodoacetate and nitrogen gas to block glycolysis and aerobic metabolism.

There have been relatively few studies on the influence of temperature on the energetics of muscle contraction in ectotherms (Johnston and Altringham, 1989; Rall and Woledge, 1990). Experiments with skinned muscle fibres in teleosts have shown that force generation has a lower Q_{10} than ATPase activity (Altringham and Johnston, 1986). Thus the economy of contraction, defined as force.time integral/ATPase activity, declines only gradually with increasing temperature (Q_{10}= 1.5) (Altringham and Johnston, 1986). The energetics of contraction in live muscle fibres is more complex, since free energy is not only utilized at the level of the cross-bridges, but also by the sarcoplasmic reticulum for calcium pumping (Kushmerick, 1983; Woledge et al. 1985).

It has been shown that in live frog muscle, isometric tetanic force is relatively independent of temperature whereas the cost of generating force has a Q_{10} of about 3.0 (Hill and Woledge, 1962; Rome and Kushmerick, 1983).

The short-horned sculpin (Myoxocephalus scorpius L.) is a cold water teleost widely distributed in shallow seas around Northern Europe. The mechanical properties of isolated muscles from this species have previously been investigated, including studies on the effects of temperature on the force-velocity relationship (Langfeld et al. 1989) and power output during oscillatory work (Chapter 4). During swimming an increase in temperature results in a higher tail-beat frequency (Webb, 1978; Batty et al. 1991), without a significant change in tail-beat amplitude (Webb, 1978). This is paralleled by the behaviour of isolated muscle fibres performing oscillatory work (Chapter 4). For example, the strain amplitude required for optimal oscillatory work is independent of temperature whereas the cycle frequency for maximum power output has a Q_{10} of around 2 (Chapter 4). The present study investigates the effects of temperature on the breakdown of phosphocreatine during oscillatory work.

Methods

Fish

Specimens of the short-horned sculpin (Myoxocephalus scorpius L.) were caught locally in the Firth of Forth in

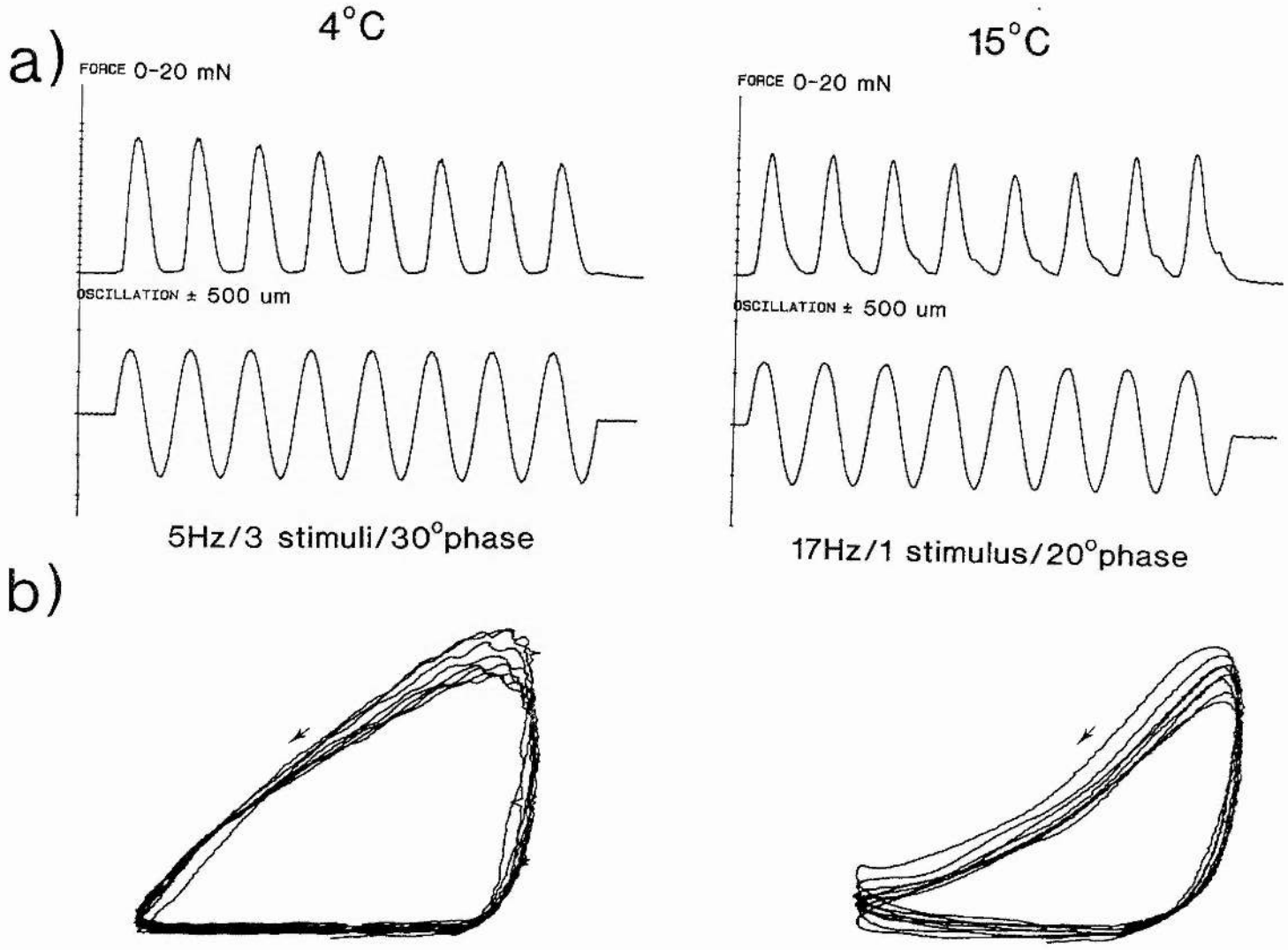
July/August 1989 and maintained in seawater aquaria at ambient temperature (14-15^{o}C). Fish of a narrow size range were chosen to reduce variation encountered due to scaling (Altringham and Johnston, 1990b). The standard length and weight of the fish used was, 20.6 ± 1.6 cm and 197.1 ± 42.8 g (mean ± SD, n=17).

Preparation of fibre bundles

Bundles of 20-30 fibres were isolated from abdominal myotomes as described in detail in Chapter 2. Cinematographic and modelling studies of fish swimming have shown that muscle length changes are essentially sinusoidal (Hess and Videler, 1984; van Leeuwen *et al.* 1990). Fibres bundles were therefore subjected to sinusoidal length changes about *in situ* resting length and phasically stimulated during the strain cycle. The work done was calculated from the area of the loop obtained by plotting force against length (Fig. 1) (Josephson, 1985). Power output which is the net work per cycle multiplied by frequency, is a complex function of cycle frequency, strain amplitude and the number/timing (phase shift) of stimuli relative to the start of the length change cycle. For a number of preparations these parameters were adjusted to optimize power output over a range of cycle frequencies at 4^{o}C and 15^{o}C (refer to Chapter 4).

For measurements of the energy cost of contraction, up to ten preparations were dissected from each specimen. Preparations were first pared down to fibre bundles 2-3

Fig 1. (a) Force and length records of fibres performing oscillatory work at 4°C and 15°C. Fibres were stimulated 2 times per cycle, with a stimulus phase shift of 25°, 5 Hz cycle frequency at 4°C and 1 stimulus per cycle, 20° stimulus phase shift, 17 Hz cycle frequency at 15°C. (b) Fibre length has been plotted against force to form work loops, the area of which represent work done.


a)
4°C
FORCE 0-20 mN
OSCILLATION ± 500 um
5Hz/3 stimuli/30°phase
15°C
FORCE 0-20 mN
OSCILLATION ± 500 um
17Hz/1 stimulus/20°phase
b)

times their eventual size and stored in recirculating, aerated Ringer at 4°C. The fibre bundles were gradually pared down to around 20 fibres with frequent Ringer changes and foil clips were attached.

Preparations were incubated for a minimum of 1 hour in normal aerated Ringer and for 1-2 hours in Ringer containing 0.5 mM iodoacetic acid (IAA) bubbled with nitrogen gas. Half the preparations were treated at 4°C and the other half at 15°C. The Ringer was changed frequently at 15°C, as vigorous aeration resulted in calcium deposition. IAA/N_2 serves to block glycolysis and aerobic metabolism, thus ensuring that the only free energy available for contraction was from ATP and phosphocreatine (Carlson and Siger, 1959).

Experimental protocol

Apart from at least one control at each temperature, preparations were rapidly transferred to the chamber of the oscillatory work apparatus described in Chapter 4. The apparatus was supplied with circulating Ringer at incubation temperature containing 0.5 mM IAA and the reservoir was bubbled with N_2 gas. Muscle fibre length was adjusted to give an optimal twitch, which corresponded to a sarcomere length of 2.3-2.4μm as measured by laser diffraction (Chapter 2). The fibres were then left to equilibrate for at least 5 minutes.

Preliminary findings suggested that depletion of high energy phosphates per unit of work was greater at higher temperature. Thus, IAA treated fibres were subjected to

16, 32 and 64 cycles of oscillatory work at 4°C or 8, 16 and 32 cycles at 15°C under conditions required to maximize power output. At the end of the last cycle, the chamber was rapidly emptied of Ringer by aspiration and the preparation freeze-quenched with a jet of isopentane (from a pasteur pipette) cooled in liquid N_2 to near its freezing point (-159°C). The foil hooks were severed and the preparation transferred to an eppendorf tube (1.5 ml) filled with freezing iso-pentane. Control preparations were frozen without stimulation and subsequently analysed in the same way.

Biochemical analysis

The preparations were freeze-dried for 12 hours, allowed to reach room temperature under vacuum and weighed to the nearest 1µg using a micro-balance (Mettler). Dried samples were homogenised in 250-300 volumes of 0.3 M perchloric acid for 15 minutes on ice, in a Duall 20 tissue grinder (Kontes, Vineland, New Jersey). Following removal of a 50 µl sample for glycogen analysis, a known volume of homogenate was removed to an eppendorf tube (1.5 ml). Measured with a micro-pipette, small volumes of 1M KOH were used to neutralize the homogenate to the acid side of pH 7, (using wide-range pH indicator paper) and centrifuged for two minutes at 13000 rpm (MSE, microcentaur). The supernatent was stored on ice and immediately assayed for high energy phosphates.

HPLC; assay of metabolites

A rapid isocratic high performance liquid chromatography (HPLC) method was used to assay adenine compounds (AMP, ADP, ATP), inosine monophosphate (IMP), free creatine ($Cr_{(free)}$), total creatine ($Cr_{(total)}$) and phosphocreatine (PCr). The technique was pioneered for the analysis of myocardial tissue (Sellevold et al. 1986) but has also proved successfull with fast teleost muscle (Moon et al. 1990).

The HPLC system consisted of a Gilson, Holochrome UV Detector, Model 602 Data Module (Data Master), Model 303 Pump, Model 802 Manometric Module and Model 811 Mixer. The system was controlled with an Apple II computer (with NEC PC8023BE-C printer) and Gilson 704 HPLC System Manager software. The column used, ODS-YMC (5µm packing), 150 x 4.6 mm and 30 mm guard column (Capital HPLC specialists, Edinburgh, Scotland) was equivalent to the Supelcosil used by Sellevold et al. 1986.

i)Reagents

All reagents used were of the highest degree of purity, obtained from Sigma (London);-

Adenosine 5'-Monophosphate (from Yeast), sodium salt, hydrate,
Adenosine 5'-Diphosphate (from eqiune muscle), sodium salt, hydrate,
Adenosine 5'-Triphosphate (from eqiune muscle), disodium salt, trihydrate,
Inosine 5'-Monophosphate (from yeast),

disodium salt, hydrate,
Phosphocreatine (enzymatically prepared),
disodium salt, pentahydrate,
Creatine (obtained thru. pfs), hydrate,
Tetrabutylammonium hydrogen sulphate
(TABHS),
Potassium dihydrogen phosphate (KH_2PO_4),
monobasic, anhydrous 99+%,

and from BDH (Poole, England);-

Perchloric acid 60% (HiPerSolv),
Acetonitrile (Methyl cyanide)(HiPerSolv).

De-ionised water was used for all solutions (Milli Q, Millipore Bedford, MA, USA). Solutions were de-gassed and filtered through a 0.22 µm filter (Millipore type GS), prior to elution.

ii)Experimental protocol

Standard solutions were prepared in the extraction medium ie. 0.3M perchloric acid neutralized with 1M KOH and spun down. Columns were calibrated daily with six standard concentrations and were stable for 300-500 injections.

Injections of standards/muscle extracts (20µl) were isocratically eluted at 1 ml/min, with a mobile phase of following composition (in $mmoles.l^{-1}$); KH_2PO_4, 215; TABHS, 2.3; acetonitrile, 3.5% (v/v). Each peak, determined at 206 nm, was baseline integrated and standard curves produced from peak area. Duplicate

muscle samples were run, peak areas compared to the appropriate standard curves and an average concentration calculated (in $\mu moles.g^{-1}$ dry weight). Conversion from dry weight to wet weight (x7.69) was determined from differences between wet and dry masses of ten preparations.

Detector sensitivity to pyruvate and lactate eluted at physiological concentrations has been found to be low, relative to the nucleotides and could therefore be ignored (Moon et al. 1990).

Glycogen analysis

The glycogen sample was assayed as glucose following amyloglucosidase hydrolysis (Bergmeyer, 1974).

i) Solutions and reagents

Acetate buffer: 9.75g sodium acetate (Sigma) per litre, adjusted to pH 4.6-4.9 with glacial acetic acid (BDH).

Amyloglucosidase solution: 10 units amyloglucosidase (Sigma) per 0.4 ml of acetate buffer.

Glucose mix: for 50 ml, 605 mg Trizma (Sigma), 16.5 mg NAD (from yeast, Sigma), 12.3 mg $MgSO_4$ (BDH, Analar) and 13.8 mg ATP (Sigma); adjust to pH 8.1 with 1M HCL and add 1 unit G6PDH (from Leuconostoc mesenteroides, Sigma) and 2.8 units hexokinase (from yeast, Sigma) per ml of mix.

ii)Experimental protocol

50 µl of homogenate was neutralized with 20 µl of 1M $KHCO_3$ prior to the addition of 0.4 ml amyloglucosidase solution. This was incubated for 2 hours at 45°C, with shaking after 1 hour. The addition of 25 µl of PCA terminated the incubation and 30 µl of 1M $KHCO_3$ neutralised the sample (a vortex being employed to thoroughly mix the reagents). The amyloglucosidase was found to be contaminated with starch; a PCA blank was thus carried through the procedure to correct for the errors involved.

To estimate glucose, 150 µl of the centrifuged sample (13000 rpm) was added to a solution containing 0.85 ml of glucose mix, stirred thoroughly (vortex) and incubated at room temperature for at least 40 minutes. The optical density (OD) was then measured at 340 nm.

iii)Calculation

if;

dOD = OD(sample) - OD(PCA blank)

then;

µmoles glucosyl units/g tissue

= dOD/6.22 x TV(A)/0.15 x TV(E)/0.05 x 1000/wt (mg)

where;

TV(A) = assay volume (ml)

(ie. 0.05 + 0.02 + 0.4 + 0.025 + 0.03 ml)

TV(E) = total extract volume (ml).

Statistical analysis

The mean values of measured parameters at different cycle numbers were compared using a single-factor analysis of variance (oneway ANOVA). For metabolite concentrations this was followed by a Dunnett's test to isolate the specific differences between control and experimental means (Steel and Torrie, 1960; Zar, 1974). A standard t-test was used to compare these parameters at $4^{o}C$ and $15^{o}C$.

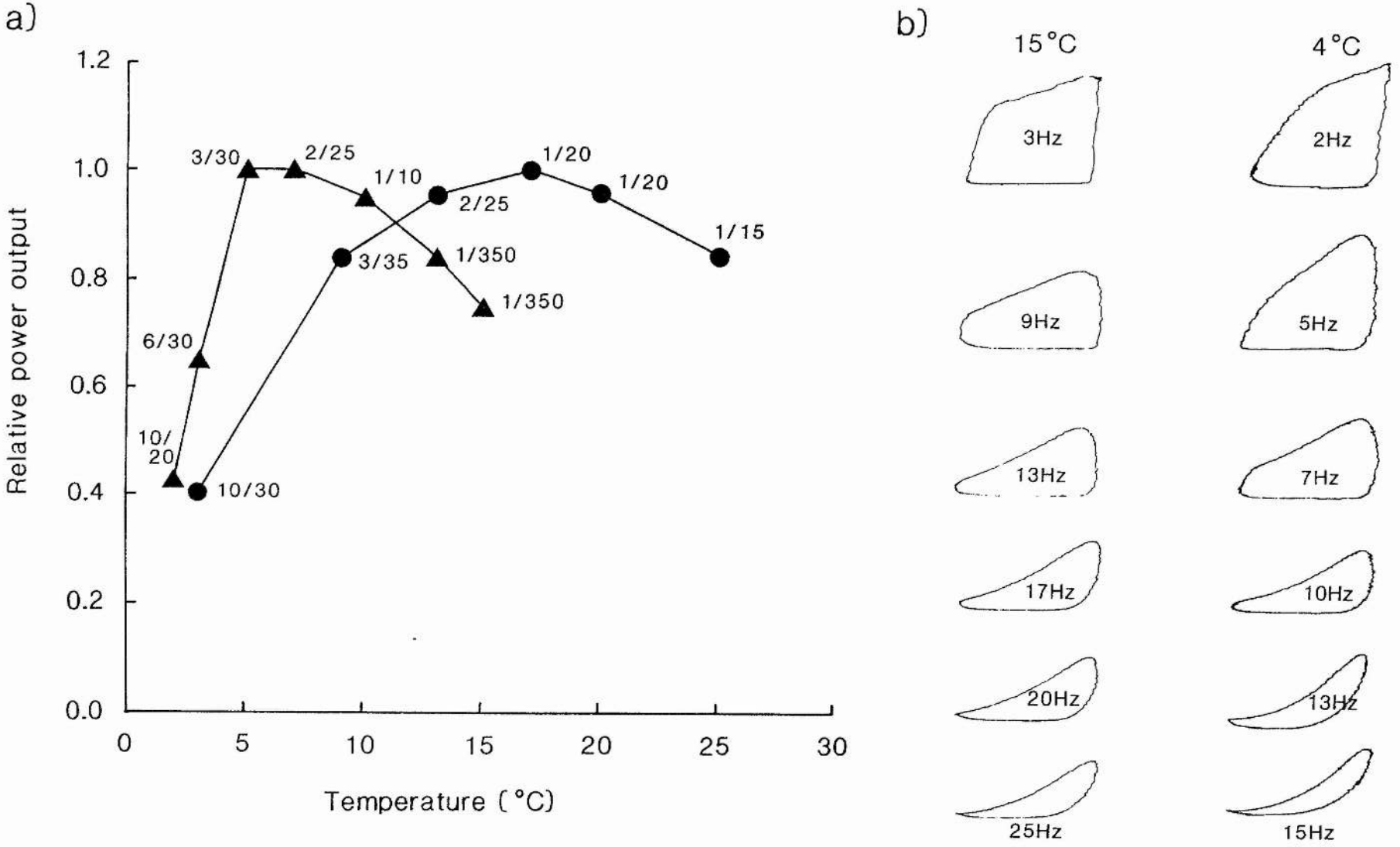
The mean values of calculated parameters (energy charge and muscle economy) were compared using the non-parametric Mann-Whitney test.

Results

Optimization of muscle power output

For 4 untreated preparations, the stimulation parameters for the production of maximum power output were determined over a range of cycle frequencies at $4^{o}C$ and $15^{o}C$. The results from two representative preparations are shown in Figure 2 with the corresponding work loops. At $4^{o}C$, power output was found to be optimal at a cycle frequency of 5 Hz, stimulating 2-3 times per cycle (2ms duration, 50Hz, 12v), at a stimulus phase shift of 30^{o}. In contrast, at $15^{o}C$ optimum power output was produced by, 1-2 stimuli per cycle (2ms duration, 75Hz) with a 20^{o} stimulus phase shift at 17Hz cycle frequency. Preliminary experiments indicated that

Fig. 2. (a) Optimum power output as a function of cycle frequency for a representative preparations at 4°C (▲) and 15°C (●); values were normalized to the maximum. The number of stimuli per cycle, stimulus phase shift and strain amplitude (± 5% resting length) were adjusted to maximize power output (x/y, number of stimuli/phase shift of stimulation). (b) Force has been plotted against fibre length for the fourth cycle of oscillatory work to produce the corresponding work loops.


a)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Relative power output
0
5
10
15
20
25
30
Temperature (°C)
3/30
2/25
1/10
1/350
1/350
6/30
10/
20
10/30
1/20
2/25
3/35
1/20
1/15
b)
15°C
4°C
3Hz
2Hz
9Hz
5Hz
13Hz
7Hz
17Hz
10Hz
20Hz
13Hz
25Hz
15Hz

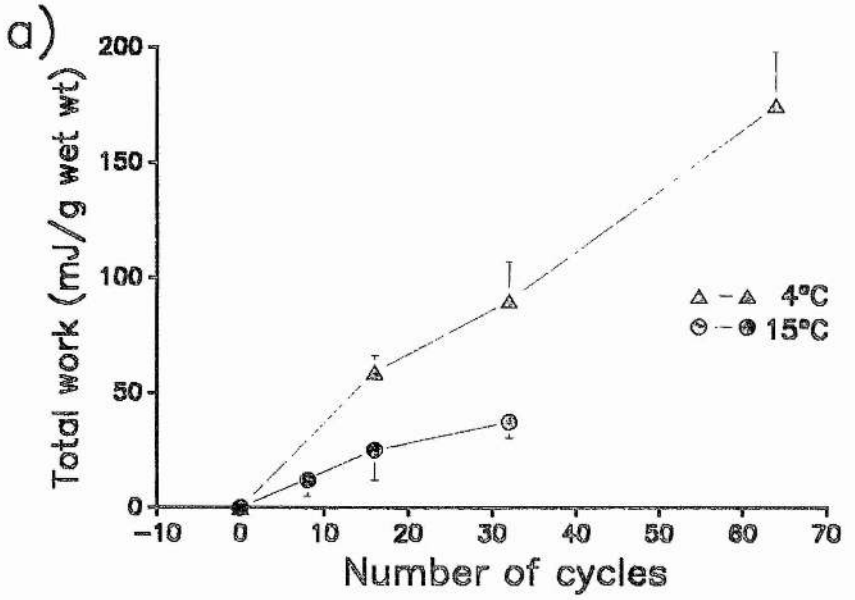
treatment with IAA and N_2 resulted in subtle changes in the contraction kinetics of muscle fibres. To maximize power output at the optimal cycle frequency in IAA/N_2-treated preparations, the number of stimuli per cycle at 4°C and 15°C had to be reduced to 1. Despite the changes, these results are in good agreement with the previous experiments using non-poisoned muscles from the same species (Chapter 4).

In iodoacetate treated preparations the total work done at each temperature was highly correlated with cycle number ($P<0.001$, ANOVA) and was significantly greater at 4°C compared to 15°C after 16 and 32 cycles of work ($P<0.01$) (Fig. 3a). Power output was consistently higher at 15°C than 4°C although the differences were not significant (Fig. 3b). In contrast to cod fast muscle fibres (Moon et al. 1990), work done per cycle generally remained relatively constant up to 30-40 cycles at 4 and 15°C (Figs. 4 and 5), although tended to decline beyond this (Fig. 4). Sculpin fast fibres also demonstrated the "muscle tone" described by Moon et al. (1990) where resting tension rose during cyclical work to a typical plateau of 0-5% maximum tension.

Energetics of contraction

As observed with similar experiments on cod (Moon et al. 1990), the concentration of glycogen was unaffected by work done and temperature (Table 1). The inhibition of glycolysis by IAA/N_2 was therefore effective (Carlson and Siger, 1959).

Fig. 3. The relationship between cycle number and (a) total work done and (b) power output, for IAA/N_2 treated preparations performing oscillatory work at 4°C and 15°C. Strain amplitude was ± 5% resting length, fibres were given 1 stimulus per cycle, with a 30° stimulus phase shift at 4°C and 20° stimulus phase shift at 15°C. Data represent mean ± SE (n; refer to Table 3).


a)
Total work (mJ/g wet wt)
200
150
100
50
0
−10
0
10
20
30
40
50
60
70
Number of cycles
4°C
15°C

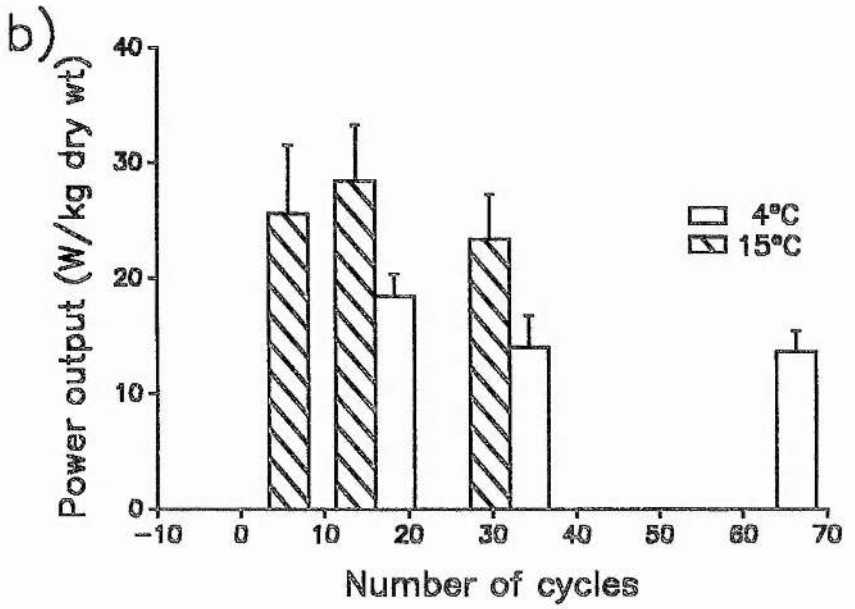
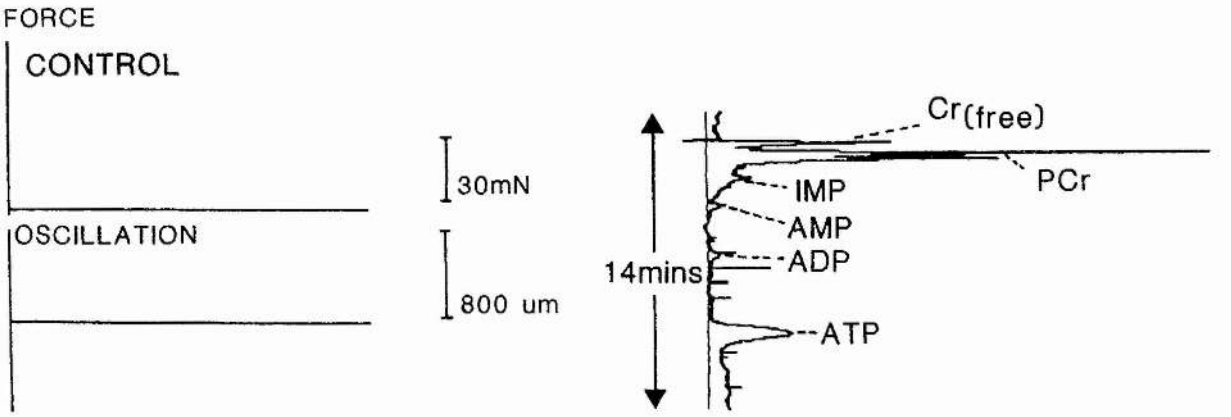
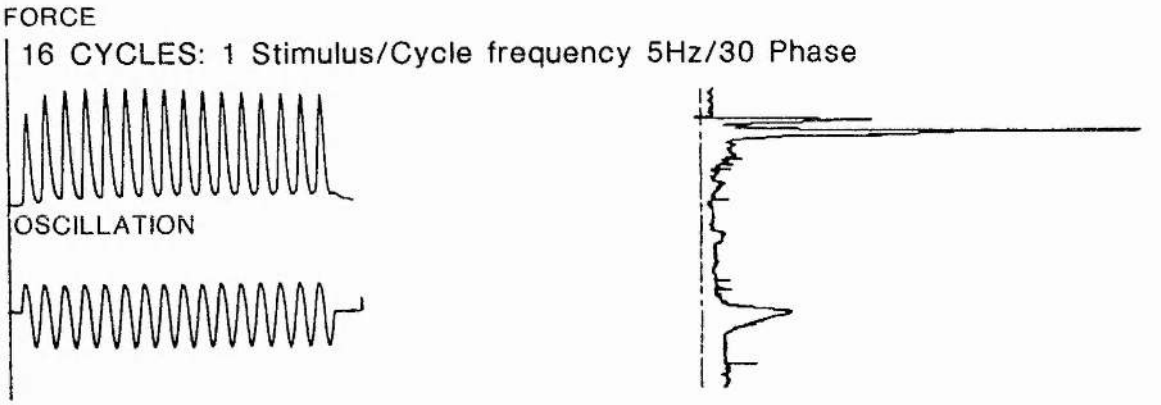
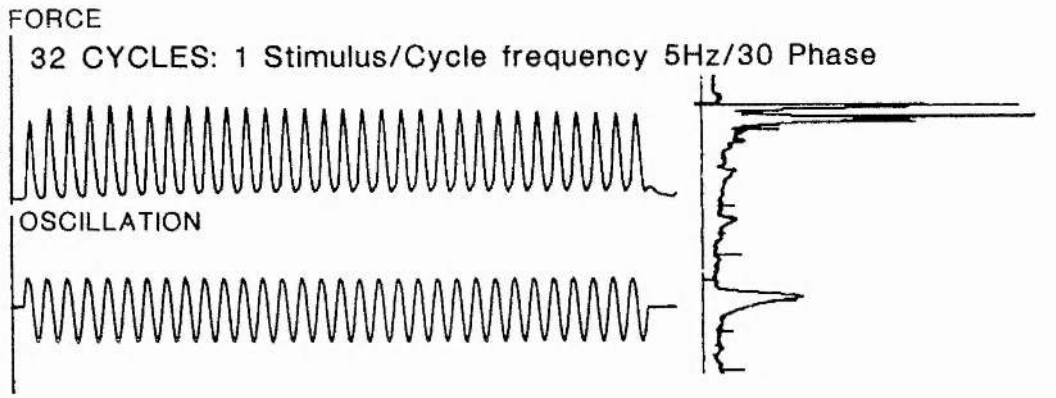

b)
Power output (W/kg dry wt)
40
30
20
10
0
−10
0
10
20
30
40
50
60
70
Number of cycles
4°C
15°C

Fig. 4. Representative force and length records with the corresponding HPLC chromatograms from IAA/N_2 treated preparations dissected from a single specimen performing 0, 16, 32 and 64 cycles of oscillatory work at 4°C. Strain amplitude was ± 5% resting length, cycle frequency and stimulation parameters were those required to produce maximum power output. See legend to Fig. 3 for details.


FORCE
CONTROL
OSCILLATION
30mN
800 um
14mins
Cr(free)
PCr
IMP
AMP
ADP
ATP


FORCE
16 CYCLES: 1 Stimulus/Cycle frequency 5Hz/30 Phase
OSCILLATION


FORCE
32 CYCLES: 1 Stimulus/Cycle frequency 5Hz/30 Phase
OSCILLATION

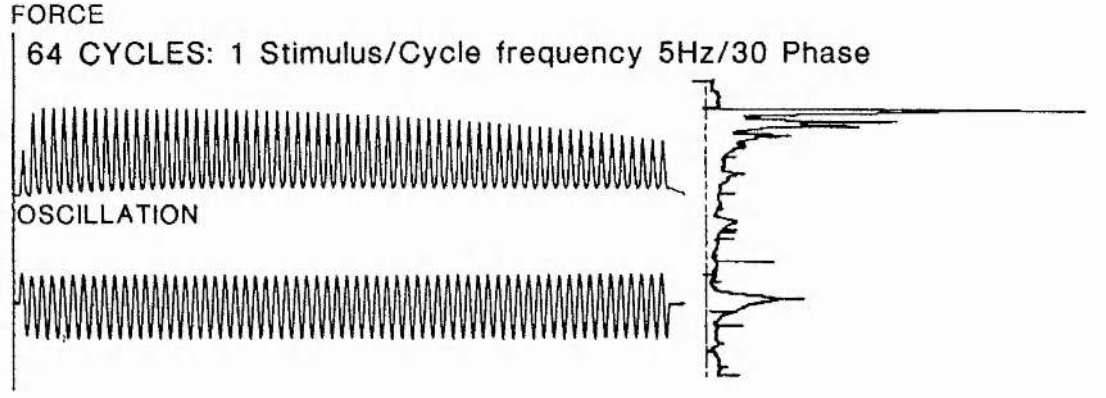
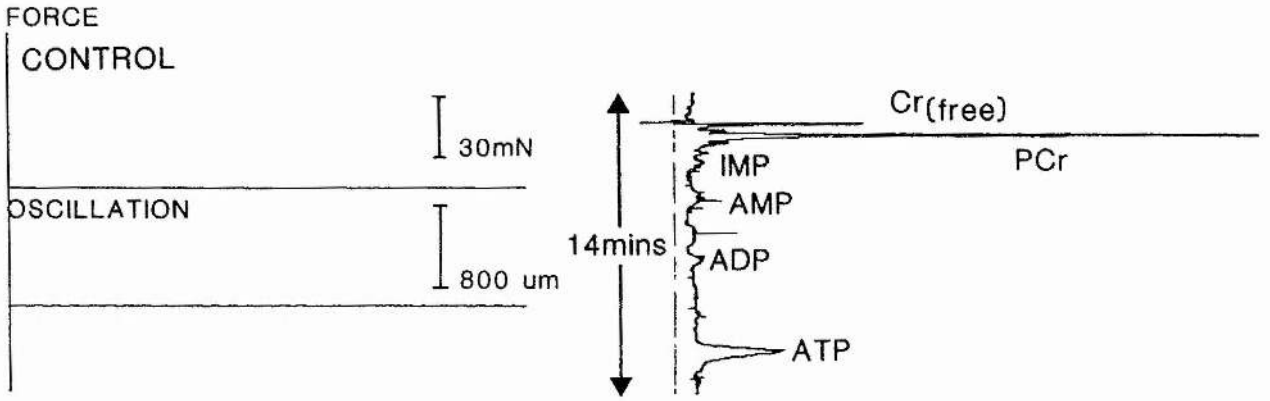
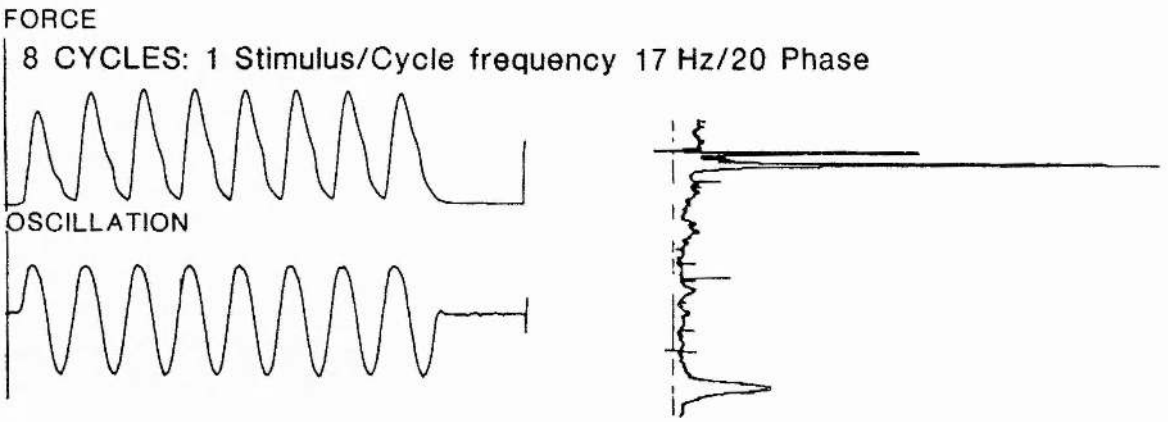
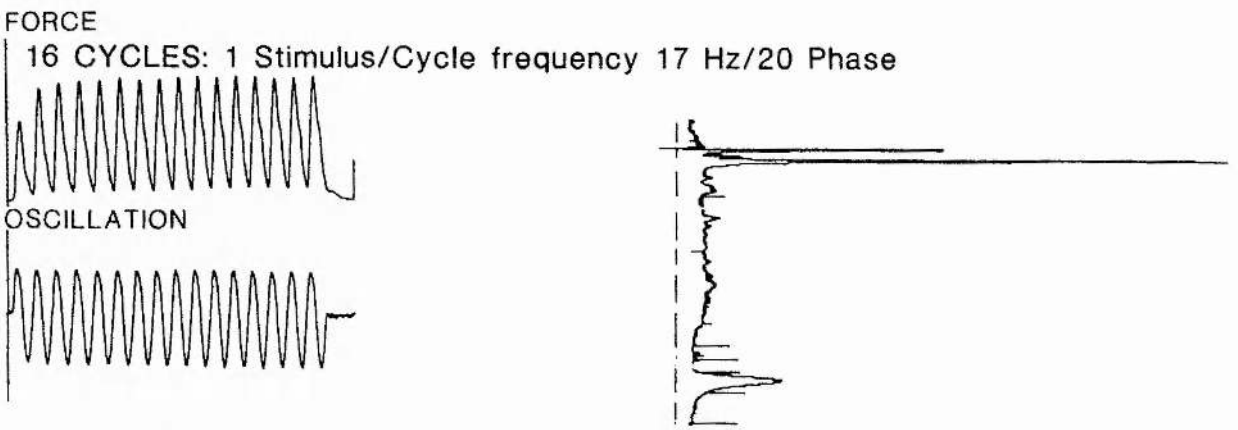

FORCE
64 CYCLES: 1 Stimulus/Cycle frequency 5Hz/30 Phase
OSCILLATION

Fig. 5. Representative force and length records with the corresponding HPLC chromatograms from IAA/N_2 treated preparations dissected from a single specimen performing 0, 8, 16 and 32 cycles of oscillatory work at 15°C. Strain amplitude was ± 5% resting length, cycle frequency and stimulation parameters were those required to produce maximum power output. See legend to Fig. 3 for details.


FORCE
CONTROL
30mN
OSCILLATION
800 um
14mins
Cr(free)
PCr
IMP
AMP
ADP
ATP


FORCE
8 CYCLES: 1 Stimulus/Cycle frequency 17 Hz/20 Phase
OSCILLATION


FORCE
16 CYCLES: 1 Stimulus/Cycle frequency 17 Hz/20 Phase
OSCILLATION

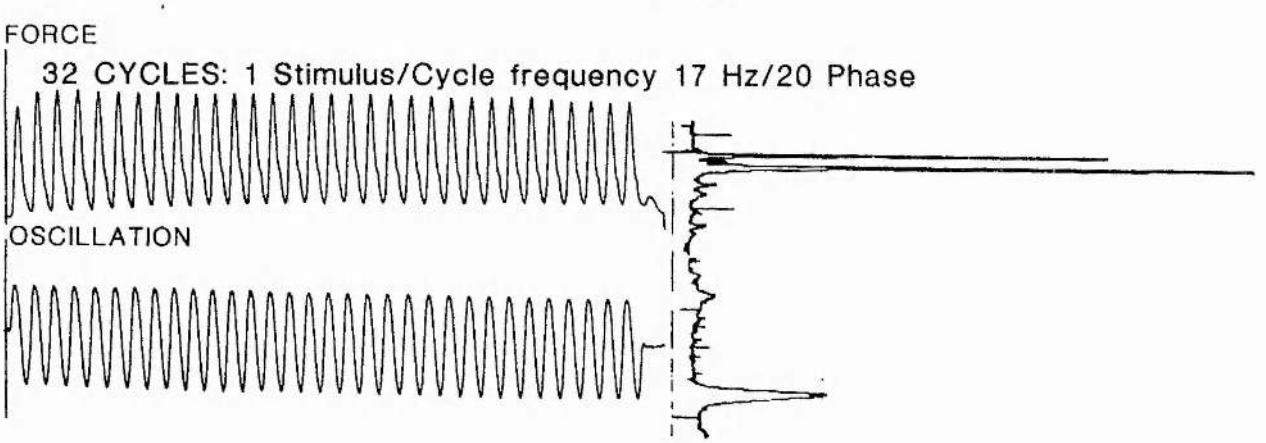

FORCE
32 CYCLES: 1 Stimulus/Cycle frequency 17 Hz/20 Phase
OSCILLATION

Table 1. The glycogen content of sculpin fast muscle fibres performing cycles of oscillatory work at 4°C and 15°C. (Values represent mean concentration in $\mu moles.g^{-1}$ dry weight ± SE (n)).

Cycle Number	Glycogen Content	
	4°C	15°C
Control (0)	127.3 ± 17.8 (10)	147.2 ± 30.9 (9)
8	-	100.7 ± 18.4 (6)
16	153.9 ± 51.0 (7)	114.7 ± 17.4 (9)
32	100.7 ± 28.3 (5)	114.5 ± 21.5 (8)
64	113.3 ± 22.8 (5)	-

Concentrations of adenylate compounds did not change significantly with cycle number or temperature following comparable cycles of oscillatory work (Figs. 4 and 5). Thus, energy charge values remained high and were not significantly altered by either temperature or work done (Table 2). However, at 4°C, AMP and ATP tended to decrease over 64 cycles of work whilst ADP increased in concentration (Fig. 6a). Similar trends in ATP and ADP concentrations were observed at 15°C, but were less noticeable because of the narrower range of cycles studied (Fig. 6b). The concentration of IMP did not change with temperature or for up to 32 cycles of work, but was highly significantly increased after 64 cycles at 4°C ($P<0.01$, Dunnett) (Fig. 6a).

The concentrations of ATP and ADP are similar to those found in the literature for fast muscle of other teleosts (Johnston et al. 1983a; van Waarde et al. 1990; Pearson et al. 1990), although the concentrations of AMP reported using the HPLC technique are greater (see also Moon et al. 1990).

At 4°C and 15°C approximately 70% of total creatine was phosphorylated (Fig. 7). This compares with similar results obtained using an in vivo ^{13}P-NMR technique (van Waarde et al. 1990). Phosphocreatine breakdown during the time lag in freezing tissues was therefore apparently minimal.

Using all the data there was a work dependant increase in the concentration of free creatine ($Cr_{(free)}$) at both temperatures (Figs. 4 and 5), although this was only

Table 2. Energy charge values for sculpin fast muscle fibres performing cycles of oscillatory work at 4°C and 15°C. Values are calculated as ([ATP] + 0.5[ADP])/([AMP] + [ADP] + [ATP]) and represent the mean ± SE (n).

Cycle Number	4°C	15°C
Control (0)	0.85 ± 0.03 (11)	0.88 ± 0.01 (10)
8	-	0.87 ± 0.03 (7)
16	0.82 ± 0.04 (7)	0.90 ± 0.01 (9)
32	0.81 ± 0.06 (7)	0.84 ± 0.04 (8)
64	0.83 ± 0.01 (5)	-

Fig. 6. The concentrations of adenylate compounds (ATP, ADP, AMP) and IMP as a function of cycle number in IAA/N_2 treated preparations performing oscillatory work at (a) 4°C and (b) 15°C. Strain amplitude was ± 5% resting length, cycle frequency and stimulation parameters were those required to produce maximum power output (see legend to Fig. 3 for details). Values represent the mean ± SE (n; refer to Table 3).

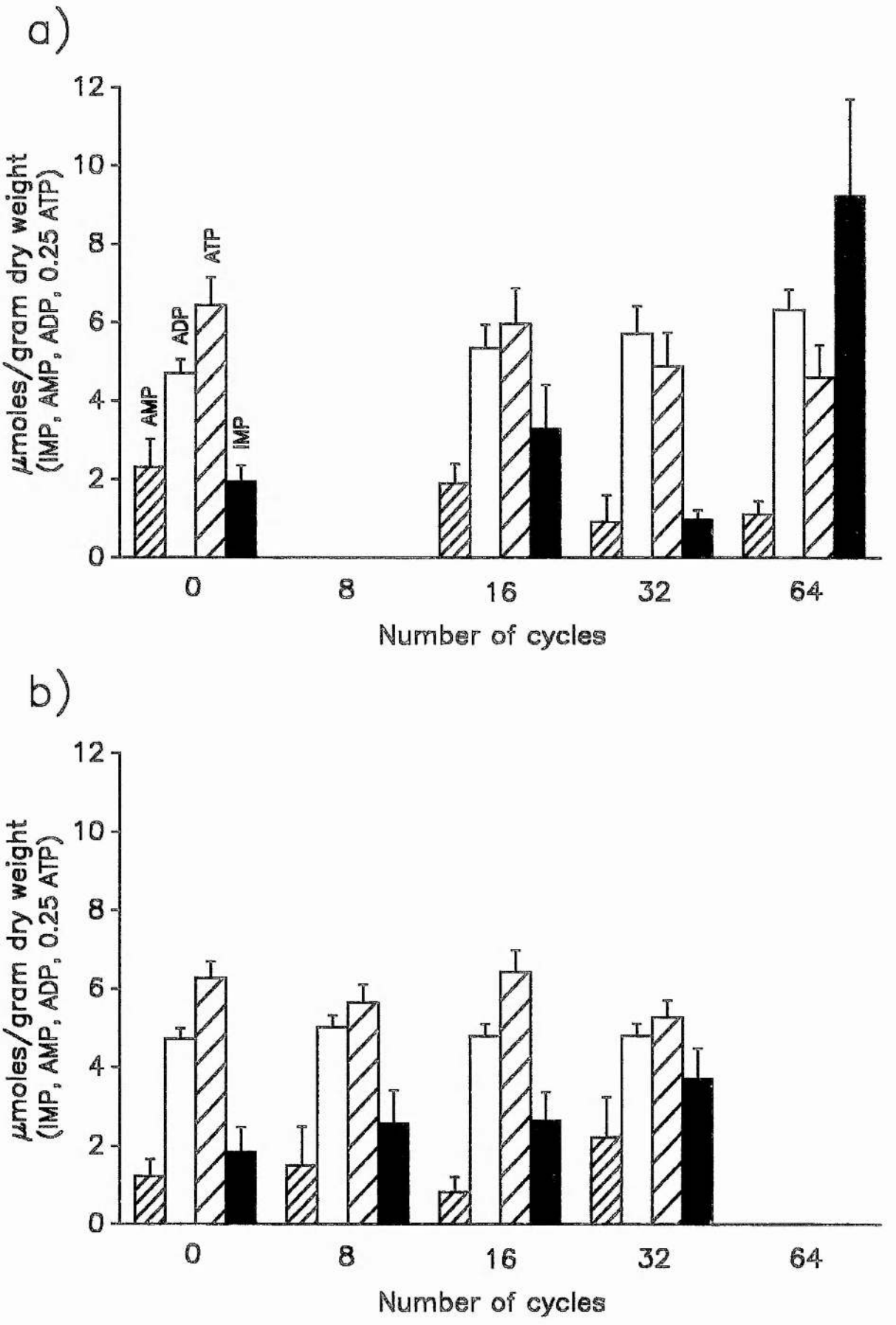
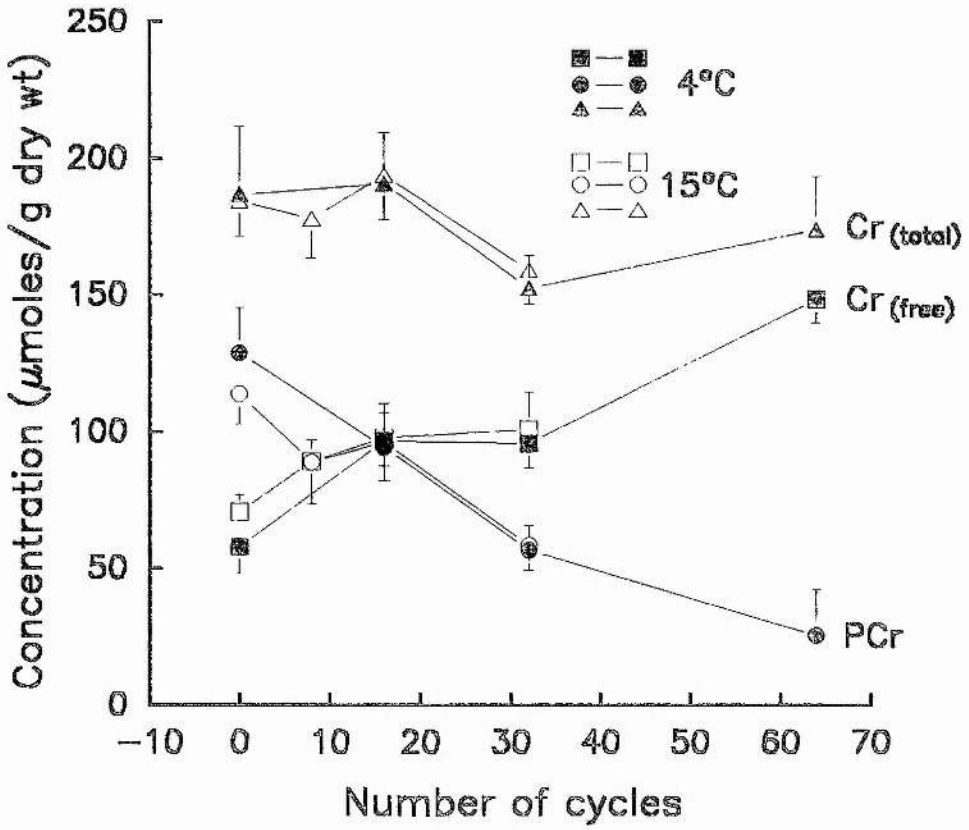

a)
µmoles/gram dry weight
(IMP, AMP, ADP, 0.25 ATP)
AMP
ADP
ATP
IMP
0
2
4
6
8
10
12
0
8
16
32
64
Number of cycles
b)
µmoles/gram dry weight
(IMP, AMP, ADP, 0.25 ATP)
0
2
4
6
8
10
12
0
8
16
32
64
Number of cycles

Fig. 7. Concentrations of free creatine ($Cr_{(free)}$), phosphocreatine (PCr) and total creatine ($Cr_{(total)}$), as a function of the number of oscillatory work cycles for IAA/at 4°C and 15°C. Strain amplitude was ± 5% resting length, cycle frequency and stimulation parameters were those required to produce maximum power output (see legend to Fig. 3 for details). Data represent the mean ± SE (n; refer to Table 3).


250
200
150
100
50
0
Concentration (μmoles/g dry wt)
4°C
15°C
Cr (total)
Cr (free)
PCr
−10
0
10
20
30
40
50
60
70
Number of cycles

significant at 4°C ($P<0.001$, ANOVA) (Fig. 7). Compared to control preparations $Cr_{(free)}$ concentration was significantly reduced after 16-32 cycles of work at 4°C ($P<0.01$, Dunnett) and 15°C ($P<0.05$, Dunnett). The concentration of phosphocreatine (PCr) was significantly correlated with cycle number at 4°C ($P<0.001$, ANOVA) and 15°C ($P<0.025$, ANOVA). Concentrations of PCr in control preparations were significantly different from all experimental groups at 4°C ($P=0.01-0.05$, Dunnett), but only after 32 cycles of work at 15°C ($P<0.01$, Dunnett). At both temperatures the concentration of total creatine ($Ct_{(total)}$) was unaffected by stimulation. The net result of these changes was a significant correlation between $Cr_{(free)}$/PCr and work done (Fig. 8).

Economy was calculated as work done/μmole phosphocreatine breakdown, since the concentration of ATP did not significantly change (Table 3). There were relatively large variations in values of economy due to the summation of errors in mechanical and chemical measurements (see also Moon et al. 1990). However, economy was consistently higher at 4°C compared to 15°C (Table 3) and was relatively independent of cycle number after the first 8 cycles.

Discussion

The energy for contraction was almost entirely derived from phosphocreatine breakdown for up to 32 cycles of oscillatory work (Figs. 7 and 8). After 64 cycles there

Fig. 8. The relationship between the ratio $Cr_{(free)}$/PCr and (a) total number of stimuli (cycles) and (b) total work done in IAA/N_2 treated preparations performing 0-32 cycles oscillatory work at 4°C and 15°C. Lines are drawn through comparable points at 4°C and 15°C, ie. following 0, 16 and 32 cycles of work (data at 64 cycles is not shown as $Cr_{(free)}$/PCr is >300). Strain amplitude was ± 5% resting length, cycle frequency and stimulation parameters were those required to produce maximum power output (see legend to Fig. 3 for details). Data represent the mean ± SE (n; refer to Table 3).

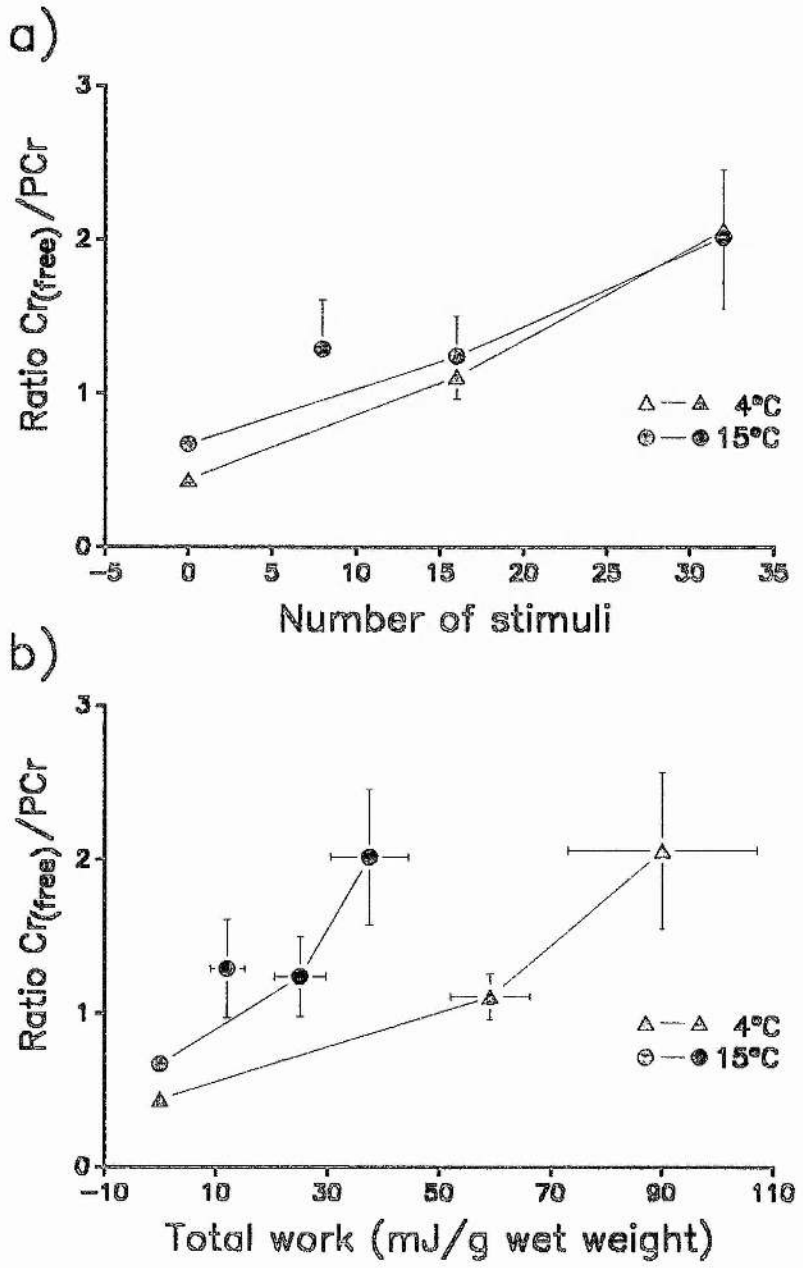

a)
Ratio Cr(free)/PCr
3
2
1
0
−5
0
5
10
15
20
25
30
35
Number of stimuli
4°C
15°C
b)
Ratio Cr(free)/PCr
3
2
1
0
−10
10
30
50
70
90
110
Total work (mJ/g wet weight)
4°C
15°C

Table 3. The economy and efficiency of contraction of sculpin fast muscle fibres at $4^{o}C$ and $15^{o}C$ performing cycles of oscillatory work. Economy was calculated as the total work done per µmole creatine phosphate utilization. Efficiency (%) was calculated by assuming the Gibb's force free energy change for the hydrolysis of phosphocreatine as being -55 $kJ.mol^{-1}$, at pH 7, pMg 3 (Woledge, 1989). Data represent mean ± SE (n).

Cycle	Economy (mJ/μmol Cp)		Efficiency (%)	
Number	4°C	15°C	4°C	15°C
8	-	4.2 ± 1.5 (6)	-	7.6 ± 2.9 (6)
16	12.5 ± 1.9 (7)	9.6 ± 2.3 (9)	22.7 ± 3.4 (7)	17.5 ± 4.2 (9)
32	12.0 ± 3.0 (7)	6.9 ± 1.2 (8)	21.9 ± 5.4 (7)	12.6 ± 2.1 (8)
64	12.5 ± 1.9 (5)	-	22.7 ± 3.4 (5)	-

was a significant increase in IMP concentration (Fig. 6) although energy charge remained unchanged (Table 2). This is the result of an active 5'-AMP deaminase in fish fast muscle which enables the adenylate kinase reaction (2ADP = ATP + AMP) to go to completion when phosphocreatine levels are severely depleted (Driedzic and Hochachka, 1976; Moon et al. 1990).

The energy cost of contraction is often expressed in terms of "economy" (positive work/unit energy expenditure) since the exact definition of "efficiency" involves more than the simple ratio of energies (Woledge, 1989). The term "efficiency" is generally defined as the ratio of the work done to the free energy of the driving reaction (Woledge et al. 1985). By assuming a value of -55 kJ mol^{-1} for the Gibb's force free energy change for the hydrolysis of phosphocreatine, Woledge (1989) calculated the efficiency of isotonic shortening from data in the literature. The values obtained were 30% for dogfish fast myotomal muscle at $12^{o}C$, 42% for frog sartorius and 77% for tortoise rectus femoris muscle at $0^{o}C$. It was suggested that during evolution high power outputs may have been acheived at the expense of a reduction in efficiency (Woledge, 1989). The muscle efficiency and power outputs measured in the present study are comparable to those obtained for cod fast muscle fibres performing oscillatory work at $4^{o}C$ (Moon et al. 1990). Moon et al. (1990) found that the economy of contraction was reduced over the initial cycles of oscillatory work. In the present study the breakdown of

phosphocreatine per unit of work was also higher after 8 than 16 cycles, although this difference was not significant (Table 3; Figs. 7 and 8). It is known that energy utilization is higher during the initial stages of an isometric tetanus (Aubert, 1956; Heglund and Cavagna, 1987). Therefore it seems likely that one or more of the reaction steps involved in contraction require some form of "priming" before maximal economy is acheived.

In sculpin fast muscle fibres treated with IAA and nitrogen gas, maximum power output was acheived with 1 stimulus per cycle at 4°C and 15°C. Heglund and Cavagna (1987) showed in frog muscle that for a given number of stimuli the energy utilized during isometric tetani was independant of temperature (0-20°C). Similarly in the present study, for a given number of stimuli/cycles the breakdown of phosphocreatine was not significantly different at 4°C and 15°C (Figs. 7 and 8a).

In conclusion, the cycle frequency for optimum power output during oscillatory work increases with increasing temperature (Fig. 2). This is paralleled by a similar temperature dependent increase in tail-beat frequency at maximum swimming speed (Webb, 1978; Batty et al. 1991). Although breakdown of phosphocreatine was temperature independent, work production per cycle decreased from 2.7-3.7 mJ/g at 4°C to 1.2-1.6 mJ/g at 15°C and the rate of work production (power output) increased from 14-18 to 23-27 W/kg (Figs. 3 and 8). In this way efficiency was found to decrease from 22-23% at 4°C to 8-18% at 15°C (Q_{10} = 1.3-1.7) (Table 3). Thus it seems likely that as

tail-beat frequency increases with temperature, efficiency is partially sacrificed in return for a higher work rate.

CHAPTER 6

GENERAL DISCUSSION

The study of the mechanical and energetic properties of isolated muscle has significantly increased our understanding of the function of muscle during locomotion. Previously, estimates of muscle power output *in vitro* have been calculated from measurements of the force-velocity relationship (Rome, 1990). However, during swimming, muscle fibres are neither subjected to tetanic stimulation or shortened under a constant load. However, with the development of the work-loop technique for isolated muscle fibres (Josephson, 1985), it is now possible to measure muscle power output under conditions simulating normal locomotory activity in fish (Chapters 4 and 5).

The present study has primarily investigated the effects of temperature on the performance of fast muscle fibres which are recruited for burst swimming (Bone, 1966). Although information on burst swimming activity is limited, it would be reasonable to expect that during maximum acceleration performance all muscle fibres would be recruited (Hirano and Rome, 1984). In this way, it is likely that the properties of the muscle fibres alone set the limits of locomotory performance (Webb, 1978; Rome, 1990). Thus, through its influence on muscle

contractility (Chapters 2-5), temperature is one of the most important factors controlling maximum swimming performance.

Evolutionary trends

Temperature adaptation

Locomotor capacity has been shown to possess several attributes for evolutionary adaptation including variability, repeatability, heritability and differential survivorship (Bennett, 1990). In fish, burst swimming ability has been linked to predator avoidance (Taylor and McPhail, 1985) and is presumably subject to high selective pressures (Johnston *et al*. 1990b). The maximal swimming speeds of some fish species from different thermal environments have been reported to be similar (Blaxter and Dickson, 1959; Beamish, 1978). However, kinematic studies of Antarctic fish have shown only limited compensation of larval and adult fish swimming performance (Archer and Johnston, 1989; Johnston *et al* 1990b) although it is clear that Antarctic fish are able to maintain locomotory activity at temperatures which would kill other fish species (Wohlschlag, 1964). These observations are supported by the direct measurement of fast muscle contractility, the partial compensation of muscle power output largely involving adaptations in maximum force generation and the curvature of the force-velocity relation with only limited contribution from time dependent contractile properties (Macdonald *et al*.

1987; Chapter 2). Similar patterns of adaptation in muscle contractility are also common to other phylogenetic groups (see Rall and Woledge, 1990; Rome, 1990). As discussed in Chapter 1, certain factors or constraints interfere with the ability of natural selection to direct adaptive processes (Pogson, 1990). In this connection it would appear that there is an underlying pattern to the constraints imposed on the adaptation of muscle contraction to temperature change; collectively, the mechanisms controlling rate functions of contractility are presumably more "constrained" than those involved in force-generation.

The influence of temperature on locomotory activity is not limited to the effects on the muscle fibres themselves. At extremes of temperature a reduction in power output may result from the failure to recruit muscle fibres, the most likely site of action being the neuromuscular junction (Rome, 1990; Chapter 2). For example, heat block of neuromuscular transmission has been observed in Antarctic fish (Macdonald et al. 1987).

Polyneuronal innervation

The selective pressure for the evolution of polyneuronal innervation in teleosts is high, since there is evidence that it has evolved on eight separate occasions (Ono, 1983). It has been suggested that the differing responses of teleost fish slow muscle to neuromuscular blockers from different species may depend on the density of multi-terminal innervation (Granzier et

al. 1983; Altringham and Johnston, 1988a; Rome and Sosnicki, 1990). From the studies reported in this thesis it is clear that these differences are related to the ancestral pattern of innervation ie. focal or polyneuronal (Appendix 1). However, the significance of polyneuronal innervation is still unclear (Altringham and Johnston, 1988b; Archer et al. 1990).

In multiply innervated muscle fibres from the frog (Xenopus laevis), the quantal release of transmitter is independent of temperature and the proportion of fibres responding to sub-threshold end plate potentials increases from 6% to 42% between 30°C and 5°C (Adams, 1989). It has therefore been suggested that multi-terminal innervation may be of functional significance to ectotherms experiencing large body temperature variations (Adams, 1989; Archer et al. 1990). Thus, the evolution of polyneuronal innervation in teleosts may have had a significant role in the exploitation of a wide variety of habitats by teleost species, in which temperatures range from -1.9 to 44°C.

Effects of acute and seasonal temperature change

Modelling studies: the effects of temperature

Wardle (1975) proposed that maximum tail-beat frequency in fish is limited by the twitch contraction time of fast muscle fibres. Thus at low temperature, the increase in the duration of an isometric twitch (Chapter 2) is associated with an increase in the duration of

propulsive strokes (Webb, 1978) and subsequent reduction in maximum velocity and acceleration rate during burst swimming activity (Rulifson, 1977; Webb, 1978; Fuiman, 1986). However, there is evidence to suggest that fast muscle contraction *in vivo* may be prolonged beyond the duration of a single twitch in order to complete a tail-beat cycle at maximum sprint speeds (Webb, 1978; Johnsrude and Webb, 1985). For example, in rainbow trout, Webb (1980) found the time to complete a tail-beat cycle in fast starts was longer than the minimum contraction times reported by Wardle (1975). Clearly this is an over-simplification, maximum swimming performance will be a pay-off between increased tail-beat frequency and the ability to maintain work output at increased shortening velocity. It is in this way that muscle power output *in vivo* has been modelled *in vitro* during oscillatory work (Johnston and Altringham, 1990a,b; Chapter 4). Interestingly both the cycle frequency for optimum power output in isolated muscle fibres (Chapter 4) and tail-beat frequency in herring larvae, *Clupea harengus* L. (Batty *et al*. 1991), have a similar temperature dependence ($Q_{10} \approx 2$). Thus, as observed with force-velocity measurements in carp (Rome *et al*. 1988), white muscle *in vivo* shortens at a velocity where mechanical power output is maximal.

To maintain maximal performance it appears that energetic efficiency is at least partially sacrificed at high temperature (Chapter 5). Thus, despite the increase in maximum swimming performance at high temperature

(Rulifson, 1977; Webb, 1978; Fuiman, 1986), one might predict that the duration of burst swimming activity would be limited at high temperature by the increased rate of depletion of energy reserves.

Thermal acclimatization

Studies with skinned muscle fibres of the carp have shown that cold acclimation increases force production, contraction velocity (Johnston et al. 1985) and the economy of isometric contraction at low temperature (Altringham and Johnston, 1985). In contrast, for the cold-temperate sculpin it is at high temperature that the most significant modifications in muscle contractile properties occur with thermal acclimatization (Chapter 4). It is probable that maximum acceleration performance is largely set by the properties of the muscle fibres alone (Webb, 1978; Rome, 1990) and that to swim at a given speed, fish must generate nearly the same mechanical power irrespective of temperature (Rome, 1990). Thus the observed changes in maximum power outputs of sculpin fast myotomal muscle with thermal acclimatization (Chapter 4) will almost certainly be reflected in the burst swimming performance of these fish. Similarly, it is likely that the thermal dependence of the energetic costs of contraction in sculpin fast fibres are also altered by acclimatization. The energy expenditure per stimulus in sculpin fast fibres from summer-acclimatized fish is independent of temperature (Chapter 5). However, even if energy costs

per stimulus remain independent of temperature following winter acclimatization, work output would still be reduced at high temperature in winter-adapted fish (Chapter 4). Thus economy, expressed as the work done per unit of phosphocreatine utilized, would be significantly reduced at high temperature in winter compared to summer-acclimatized fish.

Clearly, the interpretation of the thermal dependence of muscle function *in vivo* and *in vitro* is incomplete without information on the thermal history of the animals under investigation.

Mechanisms of adaptation

The adaptive shift in mechanical and energetic properties of muscle in response to seasonal changes, are related to a major remodelling of the contractile machineary (Johnston and Dunn, 1987; Johnston *et al.* 1990; Chapter 1). For example, in freshwater fish cold acclimation results in an increase in the proportion of aerobic muscle fibre types (Johnston and Luckling, 1978; Jones and Sidell, 1982), the volume density of mitochondria and in the activities of aerobic and fatty acid oxidative enzymes at low temperature (Johnston and Maitland, 1980; Johnston *et al.* 1985). Acclimation to low temperature also results in an increase in sarcoplasmic reticulum ATPase (Fleming *et al.* 1990) and myofibrillar ATPase activities (Johnston *et al.* 1975) and altered myosin light chain and heavy chain compositions (see Chapter 4).

Neuromuscular transmission is also affected by thermal acclimation in anurans (Jensen, 1972) and fish (Harper et al. 1989). For example, in carp the temperature sensitivity of the minature end plate potentials (Mepp's) in skeletal muscle from cold-acclimated fish is akin to that of toad (ie. linear), whereas that for the Mepp's of warm acclimated fish is similar that reported for warm blooded vertebrates and thus may indicate some adaptive alteration for optimal performance at high temperature in the warm-acclimated group (Harper et al. 1989). Since it is known that isolated sculpin muscle fibres are activated through the neuromuscular junction (Appendix 1), the observed changes in the contractile performance of sculpin fast fibres at high temperature in response to seasonal change (Chapter 4) may at least in part result from changes at the neuromuscular junction.

Conclusions

The results presented in this thesis are the first to demonstrate seasonal changes in the contractile properties of muscle from a marine teleost (Chapter 4). In this instance, adaptation simply involves adjustments acheived through the plasticity of the existing phenotype rather than through a process of natural selection (Chapter 1). Of course the capacity to display this phenomenon may be genetic (Wells, 1990), the selective advantage being conferred through the independence of burst swimming performance and therefore survivorship

(Taylor and McPhail, 1985) from seasonal temperature changes.

Future directions

Johnsrude and Webb (1985) made measurements of power (42.4 W/kg) and force (11.8 kN/m^2), in situ during fast-start swimming in rainbow trout. By comparison with the somewhat larger estimates obtained for isometric contractions, Johnsrude and Webb (1985) suggested that losses in the mechanical linkages between fibres and propulsive surfaces were up to 50% for power and possibly greater for force transmission. In contrast, the measurements obtained in situ are comparable to estimates of muscle power output and force generation for fast teleost muscle under conditions simulating locomotion (Altringham and Johnston, 1990a,b; Chapters 4 and 5). Thus, it is likely that such losses are probably less significant than previously thought, although estimates of power from isolated muscle performing oscillatory work and from in situ contractions in the same species are clearly needed.

Despite the recent advances in biomechanics discussed above, information on the kinematics and energetics of burst swimming activity is still relatively limited. It is also clear that hormones can play an important role in the modification of muscle contractility and thus much work is still needed to investigate their effects and

thereby complete the model of muscle function during swimming (Chapter 3). Future models of muscle function *in vivo* may also be able to incorporate variations imposed by the complex orientations of muscle fibres in fish (Alexander, 1969).

Recent studies on teleost, fast muscle (Altringham and Johnston, 1990a, b; Chapter 4) have modelled power output based on the kinematic analysis of continuous swimming (Hess and Videler, 1984; van Leeuwen *et al*. 1990). Although the behaviour of fast muscle during the later stages of burst swimming activity is probably approximated by the kinematic models of continuous swimming (Lighthill, 1970; Wu, 1971), fast muscle fibres behave differently for the first tail-beats during intermittent swimming (van Leeuwen *et al*. 1990). For example, in the first stroke, muscle length changes are not sinusoidal and positive work is done by the fast fibres over a greater length of the body than that done by the red fibres during continuous swimming (van Leeuwen *et al*. 1990). Before attacking, piscivorous predators commonly approach to within 5-15cm of their prey and an attack is completed in about 100-200ms (see Webb, 1978). Thus, improvements in swimming performance over the initial tail-beat cycles are likely to have the most adaptive significance.

With further electromyographical and kinematic studies, it would be of great interest to provide a more accurate description of burst swimming activities in fish. Future technical developments may facilitate the

modelling of fast-muscle power output during different stages of burst swimming activity.

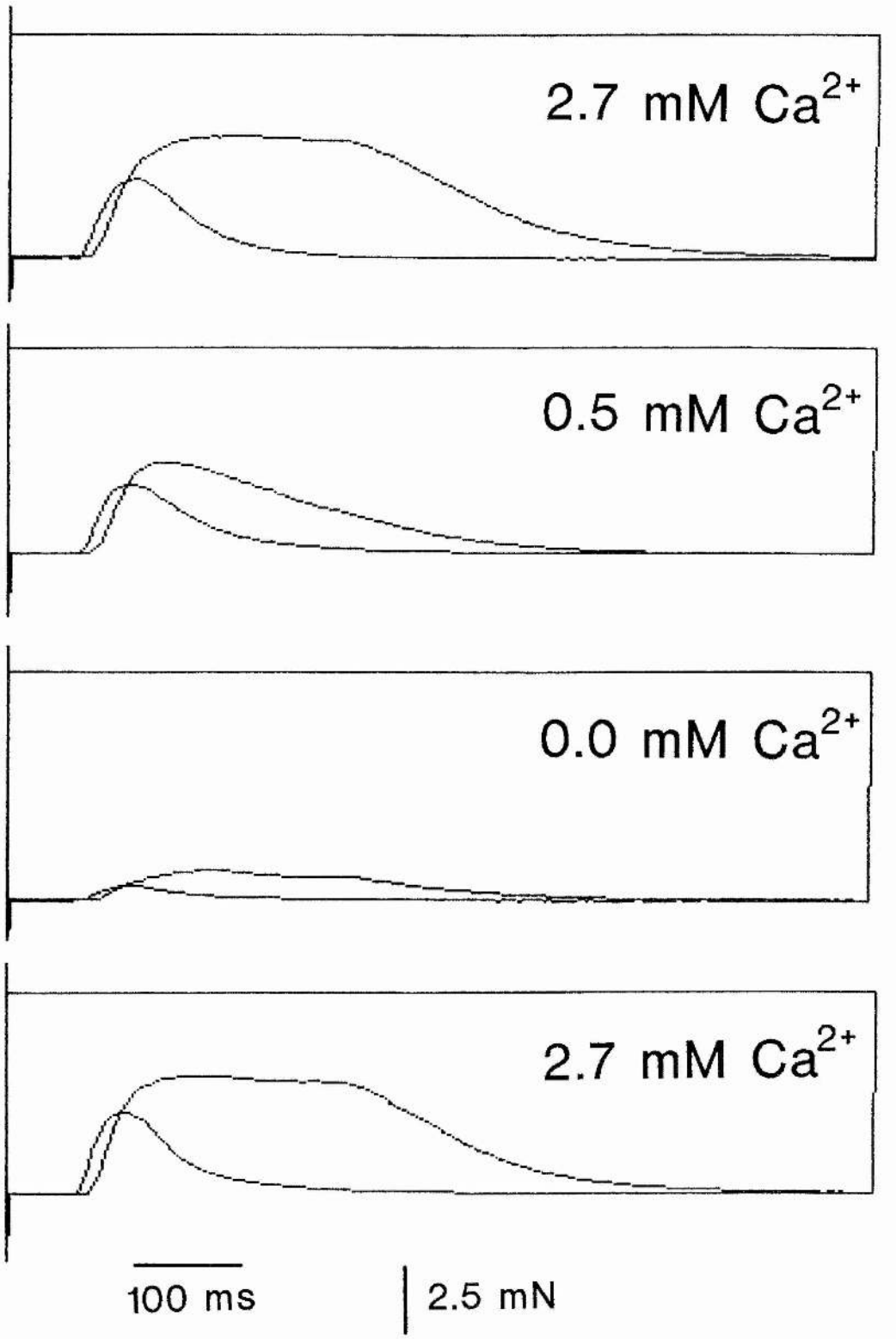
APPENDIX 1

The effects of Ca^{2+} and neuromuscular blockers on the activation of fish muscle fibres.

The fast muscles of most vertebrates are focally innervated whilst those of advanced teleosts have a distributed or polyneuronal innervation (Bone, 1964). Small bundles of fast fibres were isolated from the abdominal myotomes of various species of fish (ref. Chapter 2). Preparations were supramaximally stimulated to elicit isometric twitch and tetanic contractions at $4^{o}C$.

In preparations from the short-horned sculpin (Myoxocephalus scorpius L.), which has the polyneuronal pattern of innervation, reducing the calcium concentration in the Ringer solution resulted in a significant decline in twitch tension (40.7 ± 12.5% at 0mM, 24.3 ± 5.6% at 0.5mM) and tetanic tension (50.1 ± 12.3% at 0mM, 28.4 ± 10.9% at 0.5mM) (mean ± SD) (Fig. 1). Time dependent contractile parameters were unaffected by changes in external calcium concentration. Effects were fully reversible on returning to normal Ringer (Fig. 1). This contrasts with findings for other vertebrate muscle in which the inflow of calcium ions from the extracellular medium is not necessary for contraction, although it may have a modulating role (see Dulhunty and Gage, 1988).

Fig. 1. The effects of calcium concentration on the contraction of a fast myotomal preparation isolated from Myoxocephalus scorpius. Fibres were stimulated to elicit maximal twitch and tetanic contractions at 4°C, in Ringer containing 2.7mM, 0.5mM and then 0.0mM Ca^{2+}, before returning to normal Ringer (2.7mM Ca^{2+}).

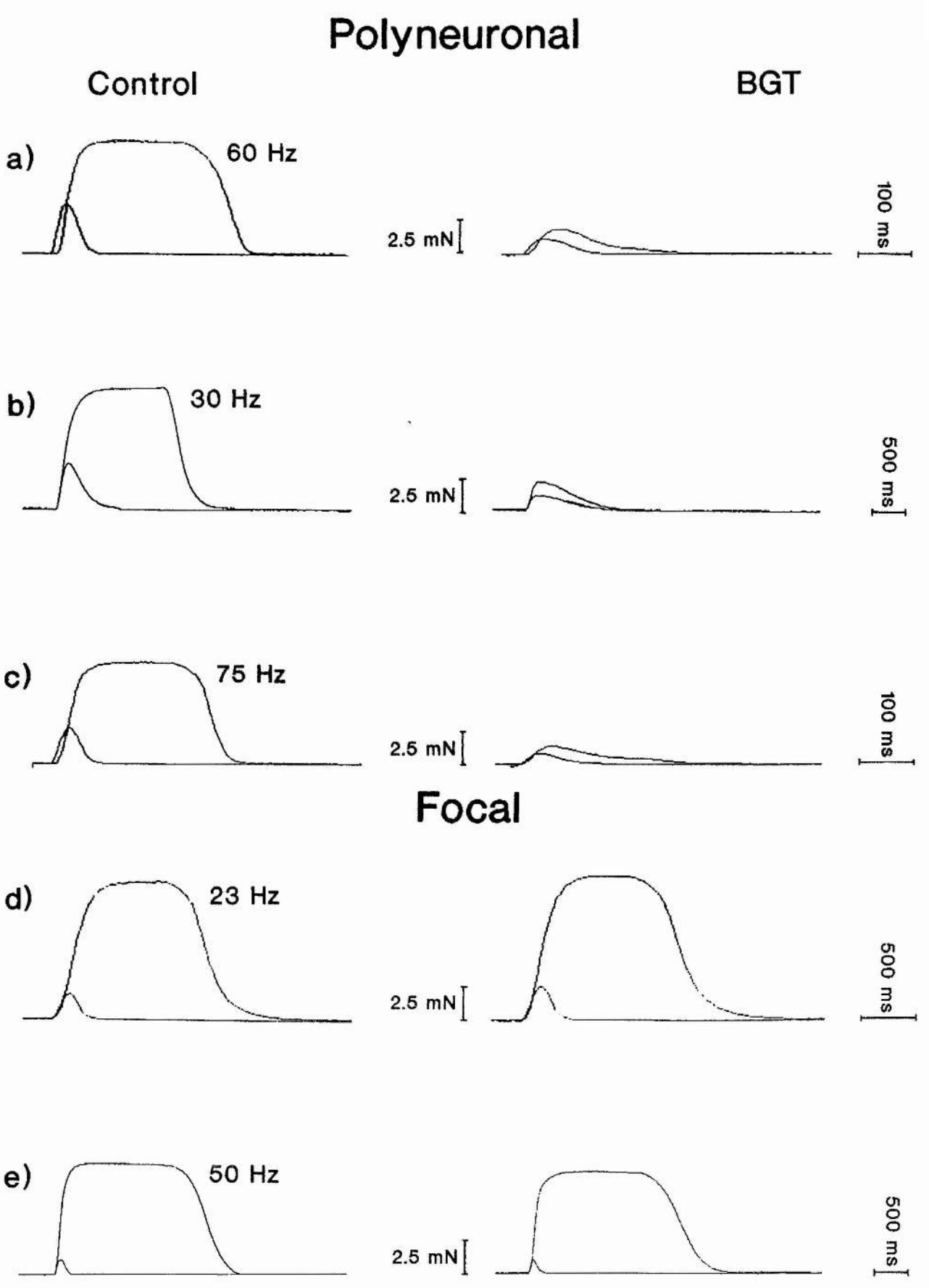

2.7 mM Ca^{2+}
0.5 mM Ca^{2+}
0.0 mM Ca^{2+}
2.7 mM Ca^{2+}
100 ms
2.5 mN

Fast myotomal fibres from the polyneuronally innervated sculpin (M. scorpius) and dragonet (Callionymus lyra L.) and the focally innervated conger eel (Conger conger) and seawater acclimated eels (Anguilla anguilla L.) were treated with the neuromuscular blockers α-bungarotoxin (BGT) at 10^{-6}M and d-tubocurarine (TBC) at $2x10^{-5}$ to $2x10^{-4}$ g/ml (obtained from Sigma). Slow muscle fibres from M. scorpius were also treated (refer to Chapter 3 for dissection). α-BGT was applied by adding a small quantity (approx. 40µl) of a 1mg/ml stock solution directly to the fibre chamber with the Ringer stationary, to produce the appropriate concentration (10^{-6}M). Preparations were incubated this way for 3-4 minutes on 2-3 occasions allowing the Ringer to be recirculated each time. TBC was simply incorporated into 250 ml of normal Ringer with which the preparation was washed.

α-BGT rendered polyneuronally innervated fibres virtually inexcitable in M. scorpius (fast and slow muscle) and dragonet, C. lyra (fast muscle) (Fig. 2). In contrast, α-BGT (10^{-6}M) had no measurable effect on the contraction of isolated fast fibres of the focally innervated teleosts, A. anguilla and C. conger (Fig. 2).

The neuromuscular blocker, d-tubocurarine ($2x10^{-5}$-10^{-4} g/ml) did not always affect tension in polyneuronally innervated muscle fibres. TBC has been used for many years as a neuromuscular blocker in the field of muscle mechanics. However, it is clear from this investigation that it is relatively ineffective compared to α-BGT. The

Fig. 2. Representative twitch and tetanic tension records from myotomal muscle preparations of polyneuronally and focally innervated teleost fish species in normal Ringer and following treatment with 10^{-6}M α-BGT: Polyneuronal; (a) short-horned sculpin (Myoxocephalus scorpius L.) fast muscle, (b) short-horned sculpin slow muscle, (c) dragonet (Callionymus lyra L.) fast muscle, (d) eel (Anguilla anguilla L.) fast muscle, (e) conger eel (Conger conger L.) fast muscle. The number to the right of tension records represents the stimulation frequency.

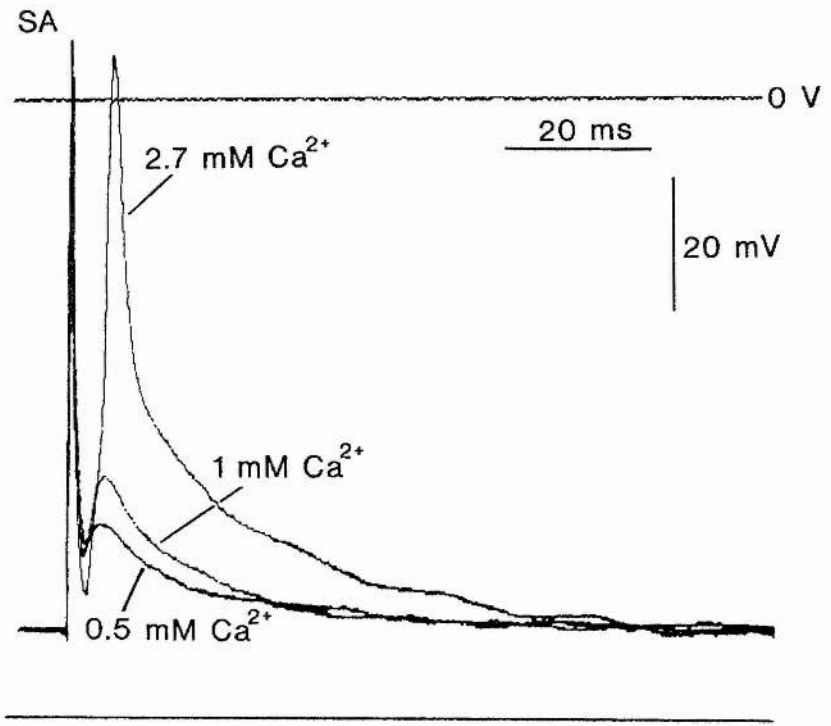

Polyneuronal
Control
BGT
a)
60 Hz
2.5 mN
100 ms
b)
30 Hz
2.5 mN
500 ms
c)
75 Hz
2.5 mN
100 ms
Focal
d)
23 Hz
2.5 mN
500 ms
e)
50 Hz
2.5 mN
500 ms

basic assumption that neuromuscular transmission is not involved in the activation of isolated muscle fibres must therefore be re-assessed.

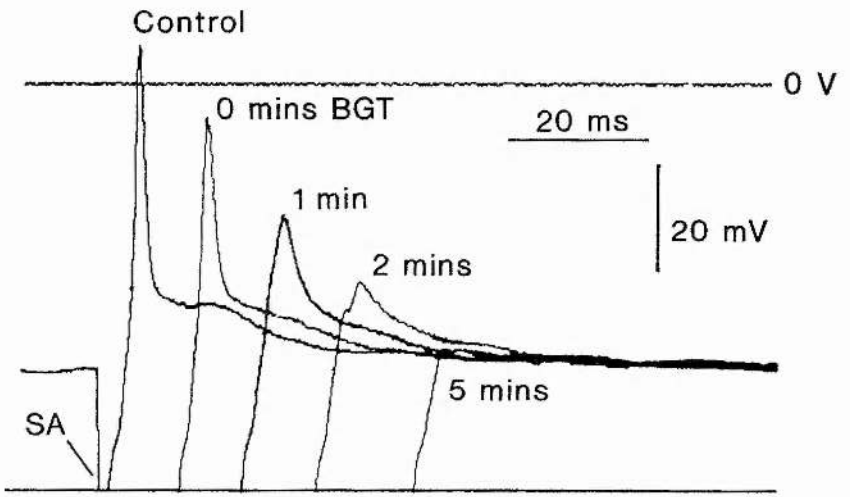
Some preliminary experiments were also performed to examine the electrophysiology of isolated preparations and determine the effects of reduced Ca^{2+} and α-BGT on membrane excitability. Fast muscle fibre bundles isolated from M. scorpius were mounted in the chamber as described for mechanical experiments (Chapter 2). Intracellular recordings were made using 20-30 MΩ electrodes filled with 3 mol l^{-1} potassium acetate floating on fine chlorided silver wire. Responses were recorded using a Simmonds amplifier (in house built) connected to the Gould Oscilloscope (Altringham and Johnston, 1988b). Preparations were stimulated as described for mechanical experiments, using single 1 ms pulses at 50v. This resulted in a large stimulus artefact but was clearly distinguishable from the membrane response. Recordings were made in the prescence of reduced Ca^{2+} (0.5mM, 1mM) and α-BGT (10^{-6}M). Both low calcium and α-BGT abolished muscle action potentials (Fig. 3). It is concluded that end plate transmission and therefore calcium are essential for the electrical excitability of these preparations.

Fig. 3. The effects of (a) reduced calcium and (b) the neuromuscular blocker, α-bungarotoxin (BGT, 10^{-6}M), on the action potentials recorded in fast muscle fibres from Myoxocephalus scorpius. Fibres were stimulated to elicit a maximal twitch contraction, SA representing the stimulus artefact.

a)


SA
0 V
20 ms
2.7 mM Ca2+
20 mV
1 mM Ca2+
0.5 mM Ca2+

b)


Control
0 V
0 mins BGT
20 ms
1 min
20 mV
2 mins
5 mins
SA

APPENDIX 2

An introduction into the "Genloop" Software

START-UP

```
C:\>GENLOOP
ENTER MAX SPEED OF ELECTRONICS (25 OR 50) :
25
ENTER THE  # POINTS PER LOOP (100, 192 OR 512) :
512
```

WORK LOOP CONTROL PROGRAM

version 3.02

J.C.EASTWOOD

JULY 1989

MULTI POINT 25/50Hz VERSION
BECAUSE OF IEEE LIMITATION
CHECK MAX. OSCILLATION FREQ

MENU

F1 - INITIATE EXPERIMENTAL RUN
F2 - PRINT WORK DONE TO PRINTER
F3 - SETUP EXPT. PARAMETERS
F4 - SAVE DATA
F5 - RETRIEVE DATA
F6 - PLOT RAW DATA
F7 - SETUP I/P #1
sF7 - OVERLAY LOOPS
F8 - SETUP I/P #2
sF8 - SETUP AUTO PLOT SCALES
F9 - AUTO PLOT
sF9 - EXIT TO SYSTEM

512 POINT MODE
25Hz ELECTRONICS

F2

```
 FILE = 12JUL25
OSCIL -   5.00Hz.  STIM -    3 @   50.0Hz   PH.   30DEG.


LOOP    1   NET WORK  20.744700uJ   PHASE 37.968750DEG
LOOP    2   NET WORK  21.045160uJ   PHASE 28.125000DEG
LOOP    3   NET WORK  20.928180uJ   PHASE 37.265630DEG
LOOP    4   NET WORK  20.663680uJ   PHASE 55.546880DEG
LOOP    5   NET WORK  20.527780uJ   PHASE 57.656250DEG
LOOP    6   NET WORK  20.447510uJ   PHASE 39.375000DEG
LOOP    7   NET WORK  19.930520uJ   PHASE 54.140630DEG
LOOP    8   NET WORK  19.294400uJ   PHASE 48.515630DEG
TOTAL WORK PERFORMED =       163.581900micro JOULES
```

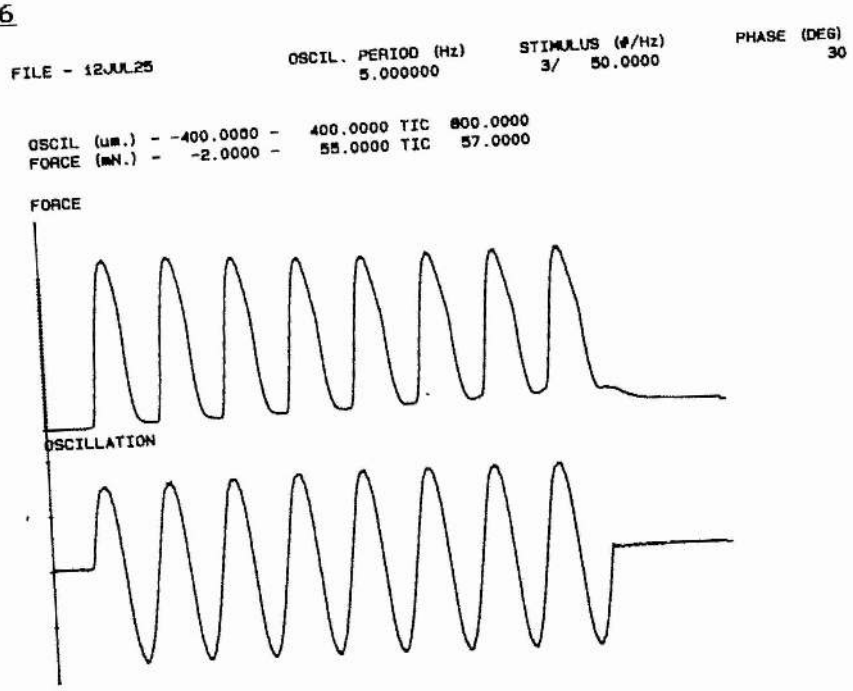
F3

```
1 - FREQUENCY OF STRETCHES
2 - # STRETCH CYCLES
3 - AMPLITUDE OF STRETCHES
4 - PHASE DELAY
5 - STIMULATOR PARAMETERS
E - ALL SET
L - LOAD PARAMETER FILE
S - SAVE PARAMETER FILE
P - I/P SENSITIVITIES
# - SET # OF POINTS CURRENTLY          512
**********************************
SET PARAMETERS ARE:
          0 STRETCH(ES) @     0.000000E+00Hz.
AMP    0.000000E+00 WITH             0 DEG SHIFT
STIMULATION            0 TIMES @     0.000000E+00Hz.
PULSE WIDTH     0.000000E+00MSEC @     0.000000E+00V
 TENSION      0.000000E+00V TRANS      0.000000E+00N/V
POSITION      0.000000E+00V TRANS     0.000000E+00m/V
SAMPLE CLOCK RANGES
  1uSEC        10.000000-      100.000000Hz
 10uSEC         1.000000-       10.000000Hz
100uSEC     1.000000E-01-        1.000000Hz
  1mSEC     1.000000E-02-    1.000000E-01Hz
ENTER CHOICE
```

F4 and F5

```
ENTER O/P NAME (8 CHARS MAX)
A:SCULPIN
```

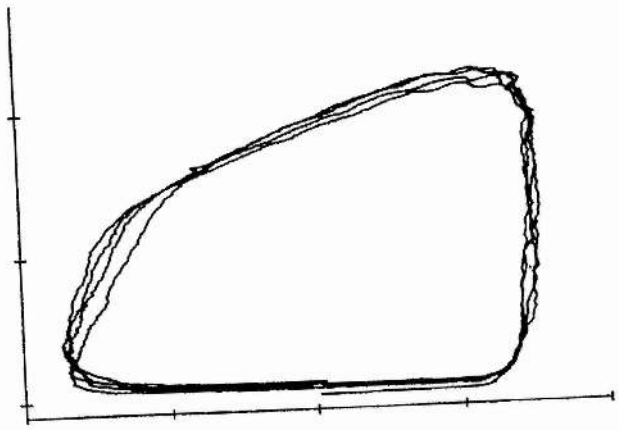
F6



sF7

This facility is used during the calibration of the tension transducer.

F7



sF8

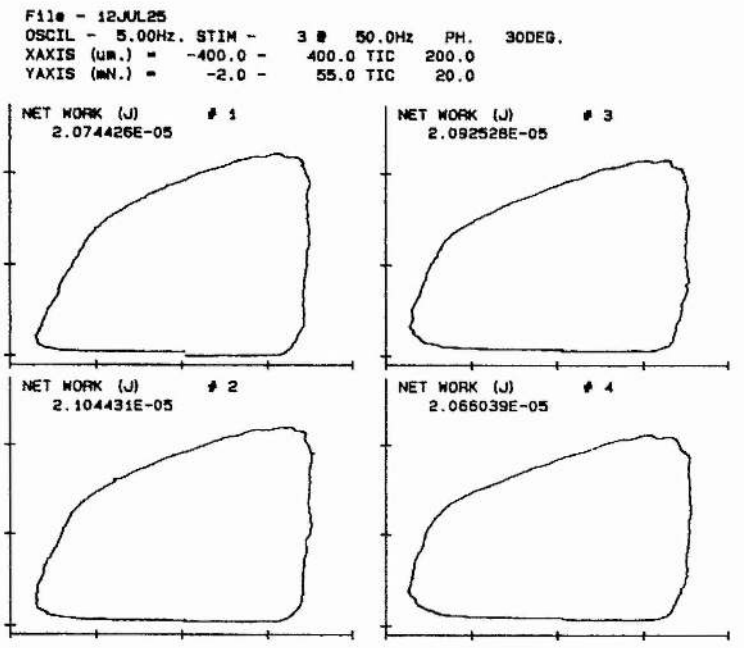
This facility is used during the calibration of the servo-controlled length transducer.

F8

```
XAXIS =      -800.000000 -       800.000000um
XTIC  =       200.000000
YAXIS =        -2.000000 -        20.000000mN
YTIC  =         5.000000

 IF SCALES O.K. PRESS P
 OTHERWISE ANY KEY
ENTER XMIN, XMAX, XTIC :
-500
500
250
ENTER YMIN, YMAX, YTIC :
-2
40
20
```

F9



sF9-EXIT

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