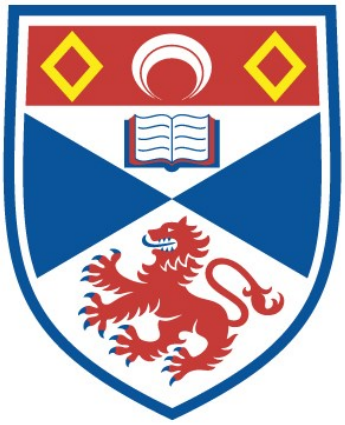
PHENOTYPIC AND EVOLUTIONARY VARIATION IN FISH MYOFIBRILLAR PROTEINS

Nicholas James Cole

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



1998

PHENOTYPIC AND EVOLUTIONARY VARIATION IN FISH MYOFIBRILLAR PROTEINS

Submitted for the degree of Doctor of Philosophy to the University of St. Andrews 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 School of Environmental and Evolutionary Biology, United College of St. Salvator and St. Leonard, University of St. Andrews, under the direction of Prof. I. A. Johnston.

Certificate

I hereby certificate that Nicholas James Cole has spent eleven terms engaged in research work under my direction, and that he has fulfilled the conditions of General Ordinance No. 2 (Resolution of the University Court No. 1, 1967) and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

Contents

SUMMARY

Chapter 1

General Introduction

The general introduction initially presents the major landmarks in muscle research of the last 3 millennia. The proteins of the contractile apparatus and their role in muscle contraction are described. There is then a description of how contractile proteins are known to alter through the expression of isoforms. Finally, a description of fish muscle and its fibre types is given, followed by the aims of the thesis.

Chapter 2

Materials and Methods

An account is given of the materials and methods used throughout this thesis. This includes myofibril preparation and electrophoretic techniques. The techniques described are one and two dimensional poly-acrylamide gel electrophoresis (PAGE), iso-electric focusing (IEF), peptide mapping, and one and two dimensional alkali-urea PAGE. The methods used to fix, stain and store the gels are then given, followed by the protocol used for Western blotting. Finally, the analysis of proteins bands is described.

Chapter 3

Temperature and the plasticity of myofibrillar proteins during ontogeny in the Atlantic herring (*Clupea harengus* L.)

Many aspects of development are influenced by temperature. The aim of this study was to investigate the effect of rearing temperature on the development and myofibrillar protein expression during ontogeny in the Atlantic herring (*Clupea harengus* L.). The development of herring embryos reared at 5, 8, 12 and 15 °C was examined. The rate of development was highly temperature dependant, increasing with increasing rearing temperature. The myofibrillar proteins expressed in embryos, larvae and juvenile herring during ontogeny were characterised. Embryonic isoforms of myosin light chain 2 (LC2), troponin I and troponin T were identified in the presumptive white muscle using two-dimensional gel electrophoresis. Expression of the embryonic isoforms was gradually switched off during the larval stages. The size range over which embryonic isoforms were present was inversely related to the rearing temperature. The expression of myofibrillar proteins was independently regulated and influenced by the rearing temperature, resulting in phenotypic variation in the pattern of isoform expression in the swimming muscles. Therefore the early thermal experience of fish embryos and larvae could influence their survival and recruitment into the adult population.

Chapter 4

The effect of body size on the myofibrillar protein composition of fast muscle fibres in the short horn sculpin (*Myoxocephalus scorpius* L.).

The contractile properties of muscle vary with body length in fish. The aim of this study was determine if the proteins altered with body size in the short-horn sculpin (*Myoxocephalus scorpius* L.). Furthermore, could changes in protein expression be related to differences in contractile properties. The myofibrillar proteins from the white myotomal muscle of different sized fish were characterised and the myofibrillar ATPase activity measured. ATPase activity was found to decrease with increasing body length, scaling to $aL^{-0.28}$. One dimensional SDS PAGE, 2D IEF PAGE and peptide mapping revealed no differences in the expression of myosin sub-units, tropomyosin, troponin T or troponin C in fish of 6 to 30 cm total length (TL). Troponin I (TnI) was the only protein to be expressed in a size-dependent manner. Three isoforms of TnI were present in fish less than 20 cm TL. Only one isoform was present in fish greater than 28 cm TL. This study was conducted to accompany an investigation of the contractile properties of the muscle. This revealed maximum shortening velocity and isometric twitch and tetanus kinetics to decrease with increasing fish length. It appears most likely that the altered expression of TnI with body size contributes to these differences in contractile properties, since TnI is an integral part of the troponin complex.

Chapter 5

The myofibrillar proteins of Antarctic and sub-Antarctic fish.

The myosins of Antarctic fish are specialised for function at low temperature. The aim of this study was to determine if the myofibrillar proteins present in Antarctic fish were highly conserved for function in this stable, low temperature environment. The variation in protein structure from myotomal fast muscles and the *m. adductor profundis* between five Antarctic fish species from two genera was compared with five sub-Antarctic species from four genera. The myofibrillar proteins of both the Antarctic and sub-Antarctic species showed a high degree of similarity between fish within the same genera. However, the isoforms present were considerably different between genera in both the Antarctic and sub-Antarctic species. Furthermore, the extent of the variation in protein isoforms between genera of the Antarctic fish was similar to that of the sub-Antarctic fish. This suggests that divergence in the tertiary structure of myosins from these species has occurred and that the Antarctic fish myofibrillar proteins are not highly conserved.

Chapter 6

General Discussion

The major findings of the thesis are discussed in relation to the expression of myofibrillar proteins, with reference to further studies.

Chapter 1

GENERAL INTRODUCTION

'The movement of animals may be compared with those of automated puppets which are set going on the occasion of a tiny movement...Animals have parts of a similar kind, their organs, the sinewy tendons to wit and the bones; the bones are like the wooden levers in the automation; the tendons are like the strings, for when these are tightened or released movement begins,' (Aristotle, 384-322 B.C. as cited by Needham, 1971).

We now know that movements such as running, walking, swimming and flying all depend on muscle contraction. Muscles are specialised for particular functions, not all of which are involved simply in motion. The hummingbird flight muscles are adapted for speed, whereas the brain heater organ in marlin and swordfish is used to generate heat, and the electric organ of some fish species is used to capture prey. Perhaps the more familiar examples of human muscle diversity would be the fatigue resistant muscles of a marathon runner and the powerful muscles of a weight lifter.

The major landmarks in muscle research, as with any research, have coincided with technological advances such as the electrical light source, the technology to produce high quality objectives for microscopes, X-rays, and the electron microscope. However, muscle research was begun a considerable time before these technological advances.

1.1 The First Millennia

The earliest record known to mention of the causes of animal movement are from Indian Vedic writings of the 14 century B.C. (Needham, 1971). These writings recognised four elements (fire, water, earth and air) from which everything was made. The breath was composed of the element air (vãyu), which the body divided into 7 parts. One of these parts (Vyana) ran through the limbs and caused their movement. Aristotle (384-322 B. C.) thought that the heart was the 'organ of movement'. The expansion and contraction of the heart communicated with the rest of the body through vessels full of blood containing *pneuma* (air) (see Needham, 1971). Erasistratus (early 3rd century B.C.) of the Alexandrian school, recognised the muscles as the organs of contraction, and the connection of the muscles to the brain by hollow tubular nerves. He theorised that contraction was the result of air, altered by the brain, travelling through hollow arteries and nerves to the muscles which initiated contraction (see Needham, 1971).

1.2 The Second Millennia

The beginnings of myology as a science are attributed to the Pergamene physician Galen (129 to 201 A.D.), who wrote the greatest works of western antiquity on animal anatomy and physiology. He demonstrated that muscles had only two possibilities, contraction and relaxation. The fundamental change Galen made in the physiological scheme of Erasistratus, was his demonstration that the arteries contained blood not air. Galen's theories of muscle contraction reigned unchallenged for the next 1300 years until the Renaissance.

1.3 The Present Millennia

Vesalius published *De Humani Corporis Fabrica* in 1543. In this volume, he described the contractile power as residing in the actual muscle substance. During the 16th century the 'new chemistry' of Boyle and Mayow, in addition to the science of mechanics described by Galileo, and mathematics typified by Newton and Descartes, lead to an intellectual era. These new ideas greatly inspired physiologists, resulting in much observation, experimentation and understanding of muscle contraction during the latter half of the 16th century by Croone, Borelli, Willis, Stensen and Meyow. Together these workers determined that something in the nerves produced contraction, that muscle contained tiny fibrils and cross striations, and that the contraction of muscle did not involve a change in the muscle volume (reviewed by Needham, 1971).

Workers on the physiology of muscle during the late 18th and 19th centuries were much occupied by the application of chemistry to living muscle, forming the foundations of modern muscle biochemistry. During this time there was little progress in determining muscle structure because there were no optical improvements in microscopes. Achromatic objectives became available for microscopes in 1850, as did electrical illumination. In 1857, Brück pioneered the use of polarised light, and immersion objectives were first produced by Abbe and Zeiss in 1866, all advances which led to a resurgence of microscopic examination of muscle.

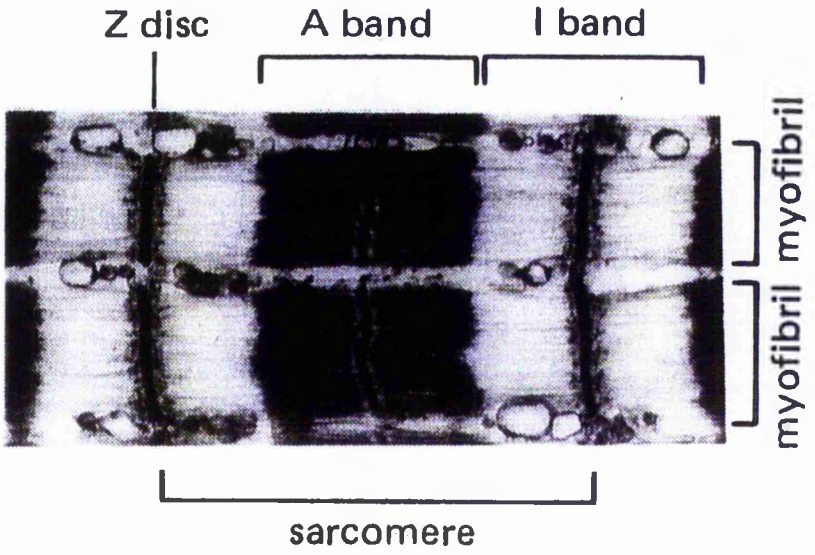
Today it is known that skeletal muscles are composed of long thin muscle fibres which are large cells formed by the fusion of many separate cells. Each fibre is surrounded by the sarcolemma, which is an electrically excitable membrane. Within each muscle fibre are many myofibrils. These long cylindrical elements run the entire length of the fibre. When viewed under the light microscope myofibrils have a banded or striated appearance

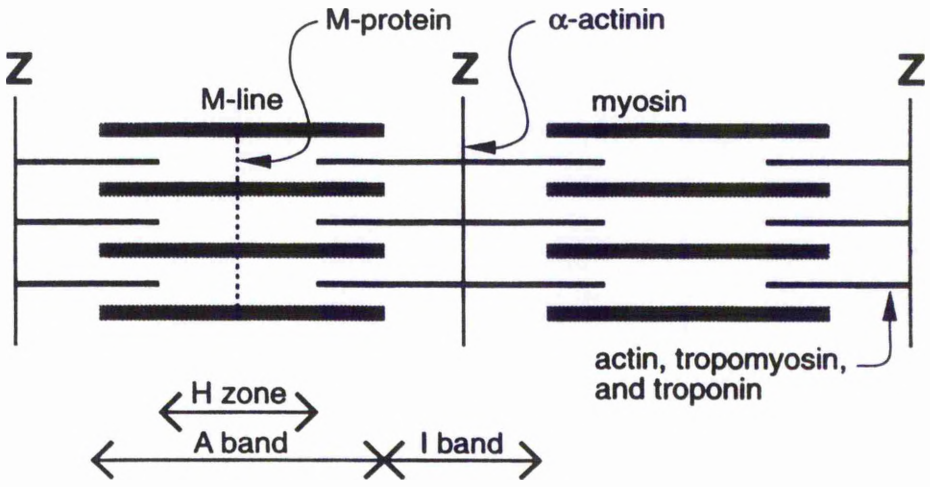
(Fig. 1.1). Each of the repeating units of this banding pattern is called a sarcomere. Sarcomeres consist of alternate dark (A) bands and light (I) bands (Fig. 1.1). The central region of the A band is less dense than the rest and is termed the H zone (Fig. 1.2). A dark M line is found in the middle of the H zone (Fig. 1.2). The centre of the I band has a very dense narrow band, known as the Z-line or Z-disc (Fig. 1.1). The Z line separates one sarcomere from another (Fig. 1.1). Sarcomeres repeat approximately every 2.3 μm (23 000 Å). Each sarcomere contains two sets of parallel and partly overlapping protein thick and thin filaments (Fig. 1.2). Thick filaments are about 1.6 μm long and 15 nm in diameter, and extend from one end of the A band to the other (Fig. 1.2). The thin filaments are about 1 μm long and 8 nm in diameter, and extend across the I bands and part way into the A bands (Fig. 1.2). Cross sections of the A band where the filaments overlap show that the filaments are arranged in a regular hexagonal lattice. The thin filaments are surrounded by 3 thick filaments and the thick filaments are surrounded by 6 regularly arranged thin filaments.

In the 1800s, several contradicting theories of muscle contraction were proposed from microscopic examinations of relaxed and contracted muscle, each observation being based on changes in lengths of both the A and I bands. The "inbibition theory" of Engleman in 1873, proposed that contraction was caused by the transport of water. Gad (1893), Fletcher and Hopkins (1907) and Bernstein (1901) proposed surface tension theories. A dehydration theory was proposed by Meyerhot in 1926, in which the removal of water accounted for contraction. Wohlish (1925) thought that contraction involved a system of invisible ultrafibrils with elastic and semipermeable walls, which he called the "colloid osmotic theory." Finally Meyer proposed the "charge change theory" in 1929 (see Needham, 1971).

Figure 1.1. Low magnification view of two adjacent myofibrils skeletal muscle fibre (from rabbit psoas muscle) showing the regular pattern of cross striations. The sarcomere is clearly defined by the surrounding Z disc. See text for details. (Photograph taken from Alberts *et al.*, 1994).

Figure 1.2. Schematic diagram of two sarcomeres showing origin of the I and A bands seen in low magnification micrograph of a skeletal muscle cell. Various biochemical components are identified as described in the text. (Diagram taken from Hochachka, 1994).


Z disc
A band
I band
myofibril
myofibril
sarcomere


M-protein
α-actinin
Z
Z
Z
M-line
myosin
actin, tropomyosin,
and troponin
H zone
A band
I band

The invention and use of the electron microscope (EM) helped lay these theories to rest by the 1940s. The first EM study of muscle was performed by Hall, Jakus and Schmitt in 1946. This confirmed many of the observations of classical histology and showed that the filaments extended in straight lines in both relaxed and contracted muscle. In 1949, Draper and Hodge correlated sarcomere length with contraction state. Observations made with the EM would eventually aid the formation of the "sliding filament model" of contraction. Further evidence to invalidate the old theories, which also contributed to the sliding filament model, involved the discovery of the complex nature of the muscle proteins, myosin and actin.

1.4 Muscle Proteins

Myosin was first prepared from frog muscle by Kühne in 1864. In 1880 Huxley showed that the A band was made up from this myosin substance, since after its extraction from muscle fibres, the A band would no longer polarise light. In 1887, Halliburton distinguished two components of mammalian muscle which he called paramyosin and myosinogen which were actually actin and actomyosin (see Needham, 1971). A new era in muscle biochemistry was opened up when, in 1939, Engelhart and Lyubimora discovered the ATPase activity of myosin. In 1943, Straub, who was working in the lab of Szent-Györgi prepared and named actin. Szent-Györgi introduced the terms G and F actin in 1944. The discovery of actin allowed a much closer look at muscle structure.

Szent-Györgi (1953), demonstrated that the myosin molecule could be digested into four molecules of light meromyosin (LMM) and two molecules of heavy meromyosin (HMM). In addition, in 1962, Mueller and Perry, showed that further trypsin digestion of HMM produced subfragment 1 (S1) and a rodlike subfragment 2 (S2). S1 contained the ATPase activity and the actin binding site. Weeds (1967) has been credited with the discovery of

the myosin light chains. He found three light chain components in myosin S1, two of which had similar amino acid structure. Two years later in 1969, Weeds distinguished between light chains 1 and 3, and light chain 2 by extracting the former with alkali buffers and the latter with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB).

1.5 The Sliding Filament Model

In the 1950s the sliding filament model of contraction emerged, which remains the accepted hypothesis for the mechanism of muscle contraction today. The first hints were provided by the low angle X-ray diffraction patterns obtained by H. E. Huxley; he used living and glycerol extracted muscle and noted that stretching did not alter the axial pattern. In 1952, his Ph.D. thesis concluded that a double array of filaments was present, some consisting of myosin, some actin. He also suggested that links were formed between actin and myosin in the absence of ATP .

In 1953 Huxley used a thin sectioning technique and for the first time the two sets of filaments were seen in both cross and longitudinal sections. The cross sections at different points along the sarcomere showed either thick or thin filaments or both in a regular arrangement. It was now suggested that extensibility depended upon the two sets of filaments sliding past each other. Hanson and Huxley (1953) confirmed that the thick filament was composed of myosin and the thin filament actin. In 1954 Huxley and Hanson used phase contrast microscopy and EM to show that during contraction, the I bands shorten and the A band stays the same length but increases in density and the I band and the H zone shorten in unison. Around the same time, in 1953, A. F. Huxley and Niedergecke were studying the striation changes in living muscle fibres using improved interference microscopy. They found that stretch and isotonic contraction changed the widths of the I band but not the A band. During isometric contraction there

was no change in either band. Measurements of sarcomere spacing showed that each sarcomere shortened proportionally as the muscle contracts. In 1957 H. E. Huxley brought definitive evidence for a double array of filaments in striated muscle by cutting sections less than 150 Å thick parallel to the filament lattice. Electromicrographs showed the arrangement of cross bridges linking actin and myosin. This led to the conclusion that the contraction was caused by the thick filaments sliding past the thin filaments with no change in length. Other evidence leading to this conclusion was that the internal packing of subunit molecules in each filament remained unchanged and the mechanical tension produced by the muscle varied in proportion to the amount of overlap between the thick and thin filaments. The thick filaments posses numerous tiny side arms, or cross-bridges that extend across the 13 nm gap to make contact with the adjacent thin filaments. During contraction the thick and thin filaments are pulled past each other by the cross bridges acting cyclically.

Since these pioneering studies, many more advances have discovered many more proteins involved in the contraction process and have resolved the structure and mechanism of muscle contraction down to the level of the molecule. The latest 'tool' used in the unlocking of the contraction mechanism is *in vitro* motility assays (Lowey *et al.,* 1993; Uyeda and Spudich, 1993). We now have considerable in depth knowledge of the composition of the thin and thick filament proteins as well as the supporting structural components.

1.6 The Thin Filament

The thin filament contains actin, tropomyosin and troponin as described below (also see figure 1.3).

Actin

Actin makes up 25 % of the myofibril. In low concentrations it is a globular shaped monomer of 42 kDa called G-actin. In higher concentrations G-actin forms filamentous fibres called F-actin which closely resemble thin filaments. Actin filaments consist of two strands of globular actin proteins about 4 nm in diameter twisted into a helix with 13.5 molecules per turn (Fig. 1.3).

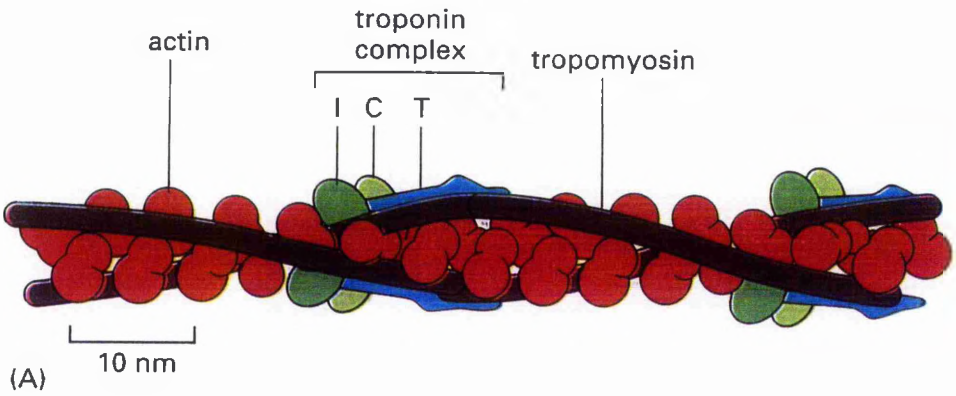
Tropomyosin

Tropomyosin molecules form coiled-coil dimers of 284-residue α-helices, which are associated with each strand of the actin helix in such a manner that each tropomyosin coiled-coil makes contact with seven actin monomers, as well as with neighbouring tropomyosins through head-to-tail contacts (Fig. 1.3). Tropomyosin forms a continuous filament along each strand of the actin double helix (Schiaffino and Reggiani, 1996) (Fig. 1.3). Tropomyosin has been shown to be essential for the Ca^{2+} sensitivity of actomyosin Mg^{2+} ATPase activity, in the presence of the troponin complex and is not required for the ATPase activity of purified actin and myosin (Lehman and Szent-Gyorgi, 1972).

The troponin complex

Troponin is a complex of the three proteins troponin I (TnI), troponin T (TnT), and troponin C (TnC) (Fig. 1.3). The three troponin subunits are present in an equimolar stochiometry (Yates and Greaser, 1983; Leavis and Gergely, 1984). Troponin I added to TnT and actomyosin inhibits the binding of myosin to actin even in the presence of calcium. Further addition of TnC completes the troponin complex and the binding of myosin and actin is inhibited in the absence of calcium. Neighbouring troponin complexes do not contact each other, nor more than half of the seven actin monomers within each regulatory unit (Fig. 1.3). Even so, Ca^{2+} binding to the thin

Figure 1.3. Schematic diagram of approximately 100 nm of a thin filament. The positions of tropomyosin and the troponin complex along the actin filament are shown. (Taken from Alberts *et al.*, 1994).


actin
troponin
complex
I C T
tropomyosin
10 nm
(A)

filaments is co-operative (Grabarek *et al.* 1983) and troponin is presumed to inhibit all seven monomers' interactions with myosin in the absence of Ca^{2+}. The troponin complex can be dissociated by denaturants and reassembled to yield a functional complex (Greaser and Gergely, 1971). Each troponin complex makes contact with tropomyosin along the COOH-terminal third of the coiled-coil including the head-to-tail overlap region. The troponin complex also interacts directly with actin.

Troponin-T

TnT is a key protein in the regulation of skeletal muscle contraction by calcium (Greaser and Gergely, 1973). TnT is a structurally asymmetric protein of molecular mass approximately 31,000 Daltons. It is the tropomyosin binding component of the troponin complex and binds troponin-C in the presence of calcium (Ebashi *et al.* 1972).

Troponin-C

TnC is the Ca^{2+} binding subunit of the troponin complex and has an approximate molecular mass of 18,000 Daltons. It has two globular domains connected by a long central helix (Herzberg and James, 1988; Satyshur *et al.*, 1988). Each domain contains two Ca^{2+} binding sites, which are numbered I to IV according to their order in the primary structure. Sites I and II are in the NH_2-terminal domain and bind calcium specifically with a relatively lower affinity than sites III and IV which are in the COOH terminal domain. Sites III and IV are occupied by Mg^{2+} under physiological conditions in relaxed muscle (Zot and Potter, 1987; Potter and Gergely, 1975; Leavis *et al., 1978*).

Troponin-I

TnI is a basic globular protein containing approximately 180 amino acids (Mr 21,000). TnI binds to actin, tropomyosin, TnT, and TnC (Ohtsuki *et al., 1986*; Zot and Potter, 1987). It is involved in the inhibition of the magnesium stimulated actomyosin ATPase reaction (Perry, 1979), and also inhibits the ATPase activity of desensitised actomyosin (Wilkinson *et al.*, 1984).

The Ca^{2+} dependence of contraction (Fig. 1.4)

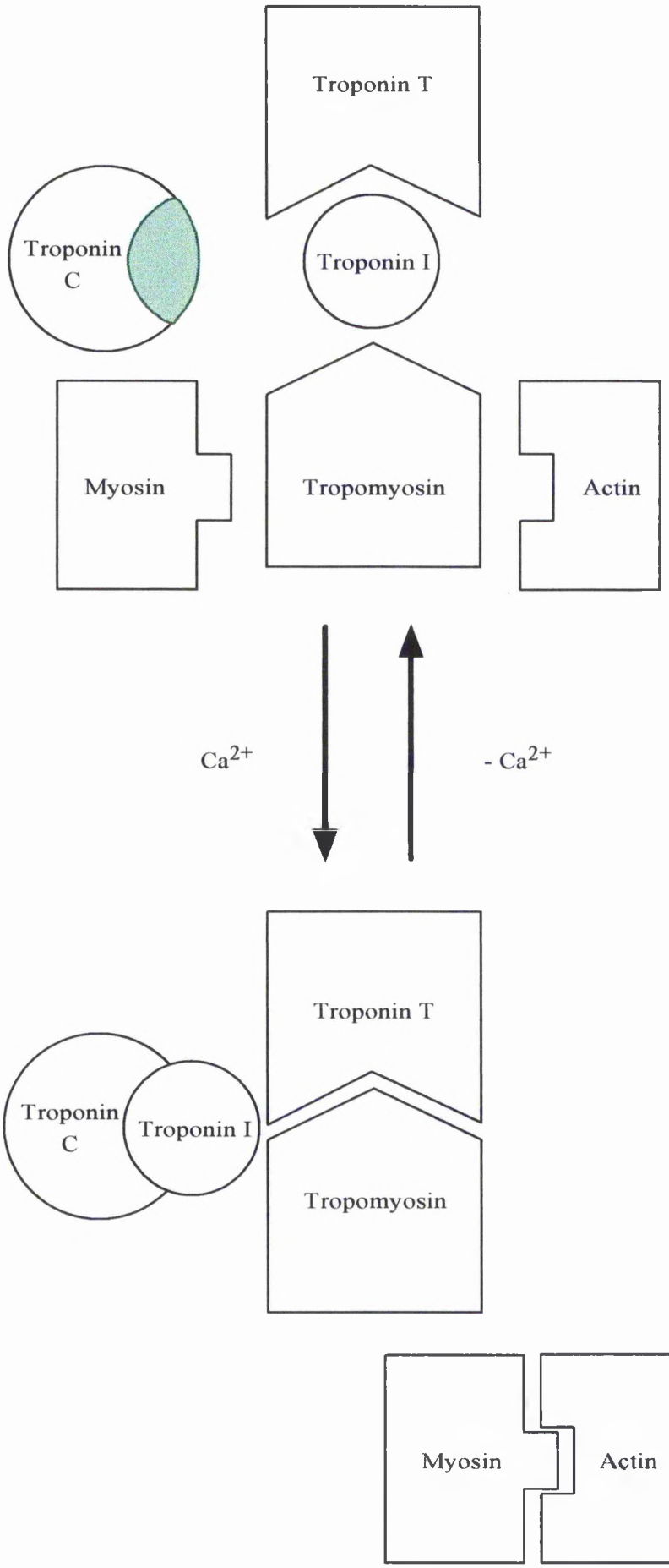
The Ca^{2+} dependence of contraction is due to the presence of the proteins tropomyosin and troponin. It is thought that troponin controls the position of tropomyosin on the actin filament. In the absence of Ca^{2+}, tropomyosin sterically blocks the actin binding site (Fig. 1.4). When Ca^{2+} concentration increases, a transformation in the shape of troponin occurs. The troponin complex acts as a Ca^{2+} sensitive switch. Calcium binds to the low affinity states of troponin-C and a hydrophobic cavity is opened (Fig. 1.4). This cavity is the site of interaction with troponin-I. When troponin-I binds to troponin-C, TnT is able to shift tropomyosin which frees the actin binding site allowing the interaction of actin and myosin (Fig. 1.4) (Grabarek *et al.*, 1992). Whether the inhibition of contraction by troponin occurs at the actin-binding step and/or subsequent to actin binding is still a matter of controversy (Chalovich, 1992; Farah and Reinach, 1995).

Overview of the thin filament

Each strand of the thin filament can therefore be thought of as a serial repetition of regulatory units; each unit containing seven actin monomers, one tropomyosin coiled coil, and one troponin complex (Fig. 1.3) (Ohtsuki *et al.*, 1986; Zot and Potter, 1987; Da Silva and Reinach, 1991; Grabarek *et al.*, 1992; Chalovich, 1992; Farah and Reinach, 1995). The organisation of the thin filament has important implications for the mechanism of control

Figure 1.4. Schematic representation of the role of tropomyosin and troponin in the calcium sensitivity of contraction. In the absence of Ca^{2+} (top of figure), the hydrophobic cavity in troponin C (shown shaded green in figure) remains closed. With this cavity closed, tropomyosin blocks the binding of myosin to actin since it is prevented from binding to troponin T by the position of Troponin I which cannot bind to troponin C.

When Ca^{2+} concentration increases (lower part of figure), calcium binds to the low affinity states of troponin-C and its hydrophobic cavity is opened. Troponin-I then binds to troponin-C, allowing TnT to shift tropomyosin which enables the interaction of actin and myosin.


Troponin T
Troponin C
Troponin I
Myosin
Tropomyosin
Actin
Ca2+
- Ca2+
Troponin T
Troponin C
Troponin I
Tropomyosin
Myosin
Actin

because Ca^{2+} binding to one troponin complex presumably controls the structure of seven actin monomers *via* tropomyosin.

1.7 The Thick Filament

The thick filament consists mainly of myosin and a small amount of another sarcomeric protein, C-protein. These two components are described in detail below.

Myosin

Skeletal muscle myosin makes up the largest part of the contractile apparatus in skeletal muscle fibres (Ohtsuki *et al.*, 1986). Myosin is a relatively large molecule of around 500 kDa which spontaneously polymerises *in vitro.* Myosin consists of a double headed globular region joined to a very long rod which is a two-stranded α-helical coiled coil (Fig. 1.5A).

Each myosin molecule consists of two heavy chains and four light chains (Gazith *et al.*, 1970; Weeds and Lowey, 1971; Focant and Huriax, 1976; Huriax and Focant, 1977). Limited digestion of myosin by trypsin splits the molecule into light meromyosin (LMM) and heavy meromyosin (HMM) (Fig. 1.5A). LMM is a two-stranded a-helical rod with a length of 134 Å. It forms filaments, lacks ATPase activity and does not bind to actin. HMM is a rod attached to a double headed globular region (Fig. 1.5A). It catalyses the hydrolysis of ATP, binds to actin and does not form filaments. Further digestion of HMM by trypsin splits it into two globular sub fragments (S1) and one rod-shaped sub fragment (S2) (Fig. 1.5A).

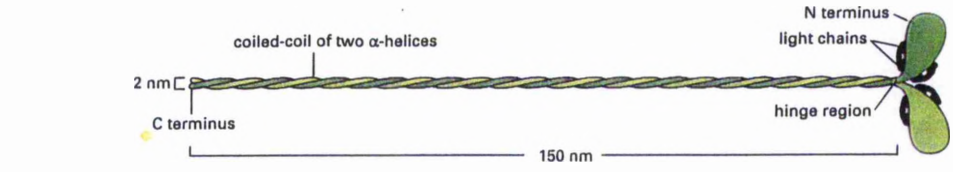
Myosin light chains

The myosin light chains are bound to the S1 fragments (Fig. 1.5B). The light chains consist of two phosphorylatable (P), 5,5'-Dithio-bis(2-

Figure 1.5A. A myosin molecule showing the double headed globular region joined to the rod region. The effects of proteolytic digestion upon myosin are presented as described in the text. (modified from Alberts, 1994)

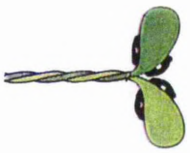
Figure 1.5B. Schematic representation of the head region of myosin in skeletal muscle. The diagram shows the position of the rod shaped subfragment (S2), the globular sub fragment (S1), the myosin light chains, C-protein and the myosin hinge (head/rod junction) as described in the text. (modified from Rayment *et. al.* 1993).

A



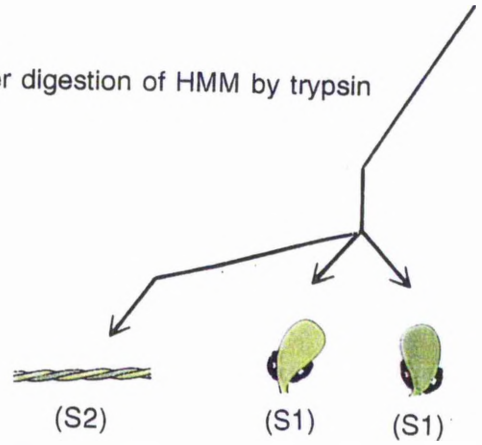
Limited digestion of myosin by trypsin

light meromyosin (LMM)



heavy meromyosin (HMM)

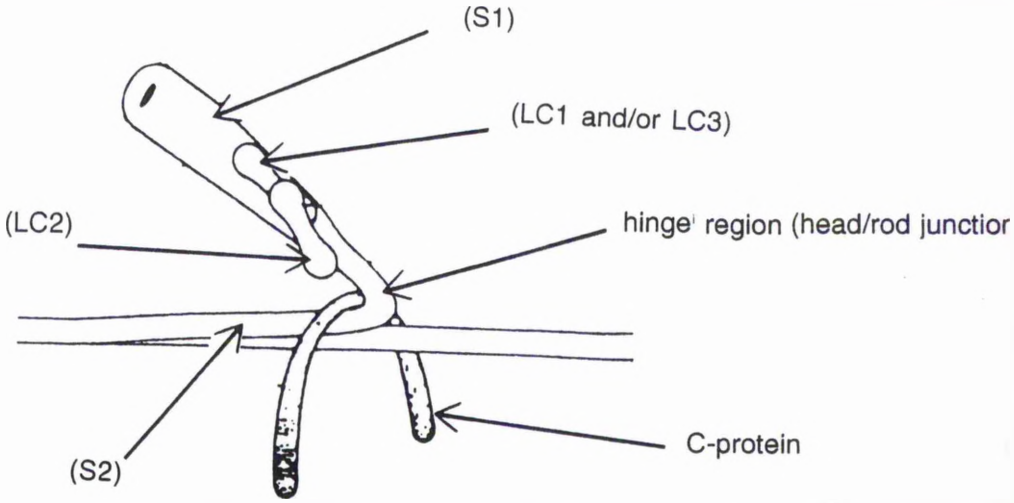
Further digestion of HMM by trypsin



rod-shaped sub fragment

two globular sub fragments

B



Nitrobenzoic acid) (DTNB) light chains, or light chain 2 (LC2) and two alkali light chains or light chain 1 (LC1) and/or light chain 3 (LC3). The phosphorylatable light chains were originally named DTNB light chains since they could be extracted from myosin using DTNB, similarly the alkali light chains were so called since they can be extracted at high pH. One alkali light chain (LC1 and/or LC3) and one P light chain (LC2) are associated with the head region of each myosin heavy chain (Fig. 1.5B). The light chains are located near the hinge region (head/rod junction) of the myosin molecule (Fig. 1.5B) (Katoh and Lowey, 1989). The myosin light chains are identified by a number which refers to their migration in SDS PAGE with light chain 1 having the highest molecular mass.

Alkali light chains (LC1, LC3)

Lowey *et al.* (1993) showed that myosin light chains play a role in transmitting force and movement from thick to thin filaments. This is in agreement with Rayment *et al.* (1993) that the light chains impart mechanical stability to the a-helical portion of the myosin head during force production. Other evidence suggests that the alkali light chains influence the rate of interaction with actin. Katoh and Lowey (1989) suggest that they may be involved in modulating interactions between myosin and actin (Schaub *et al.*, 1986).

DTNB light chain (LC2)

Mammalian muscle contains two isoforms of DTNB light chain, MLC-2fast (MLC2f) and MLC-2slow (MLC2s). Partial extraction of LC2 from rabbit skinned skeletal muscle fibres results in an increase in the Ca^{2+} sensitivity of tension (Hofmann *et al.*, 1990), and an increase in the rate of tension development at low levels of Ca^{2+} (Metzger and Moss, 1992). Removal or addition of the phosphorylatable light chains influences mechanical

properties by decreasing or increasing, respectively, the maximum velocity of shortening in skinned muscle fibres (Moss *et al.*, 1982, 1983; Hoffman *et al.*, 1990; Lowey *et al.*, 1993).

1.8 Other Sarcomeric Proteins

Other proteins in the myofibril that play a structural role are C-protein, H-protein, X-protein, M-protein, titin, nebulin, desmin, α-actinin and myomesmin.

C-, H- and X-proteins

C-protein (Mr 140 kDa), X-protein (Mr 145 kDa), and H-protein (Mr 74 kDa) occur in the sarcomere A band. C-protein regulates thick filament lengths and may tether the rod shaped S2 myosin fragment to the thick filament backbone (Fig. 1.5B). Two to three molecules of C-protein are present at each 43 nm myosin repeat (Offer *et al.*, 1973). Protein-X is an isoform of protein-C which is found exclusively in slow muscle (Yamamoto, 1983). H-protein occurs at a specific site in the thick filament, close to the M-line (Bennet *et al.*, 1986).

M-protein and myomesmin

M-protein (Mr 165 kDa) (Masaki and Takaiti, 1974) and myomesmin (Mr 185 kDa) (Gunning *et al.*, 1990) are localised at the level of the M line. These proteins bind the COOH terminal of the myosin molecule (Vinkemeier *et al.*, 1993). M-line proteins stabilise the thick filament arrangements in the sarcomere during filament assembly and provide an anchor for the titin scaffold (Vinkemier *et al.*, 1993).

Titin

Titin or connectin (Mr 3000 kDa) is the largest protein in muscle, each titin molecule extends from the Z line to the M line (Furst *et al.*, 1988). There is an inextensible segment at the level of the A band and an elastic segment at the level of the I band. Titin molecules from each half of the sarcomere are thought to connect the thick filaments to the Z-lines and position the myosin filaments at the centre of the sarcomere (Horowits and Podolski, 1987; Horowits *et al.*, 1989).

Nebulin

Nebulin is giant molecule (Mr 700-900 kDa), present in skeletal but not cardiac muscle. It extends along the thin filament as a single polypeptide from the Z disk to the free end of the thin filament (Wang and Wright, 1988). It does not stretch and may act as a framework that specifies the length during assembly of both the thin filament (Trinick, 1994) and the thick filament (Whiting *et al.*, 1989).

Z disks and desmin

The Z line shows fibre type-specific variations in structure. The thickness of the Z line decreases from slow to fast fibres (Schiaffino *et al.*, 1970; Gauthier, 1979). The variable thickness of the Z line in different muscle fibre types is related to the different amount of filament overlap from adjacent sarcomeres (Yamaguchi, 1983). The structural function of desmin is to link the Z disks together.

α-actinin

α-actinin (Mr 100 kDa) is a filamentous actin cross-linking protein associated with the barbed end of the thin filaments at the Z line. It is thought to anchor the actin filaments in the Z disc (Ebashi and Ebashi, 1965; Endo and Masaki. 1982).

1.9 Myofibrillar Proteins Occur as Isoforms

The structural and functional elements are strongly conserved in muscles such that, in vertebrates the muscles are designed according to a universal sarcomere 'plan' (Huxley, 1985). For example, myosin and actin interact along the same chemical pathway and the substrate binding and catalytic mechanisms in ATPase are always the same. In addition, the A-band lengths and the spacing of the A-filaments are virtually identical in all vertebrates (Hochachka, 1994) and individual cross bridges are capable of generating the same tension over each power stroke (Huxley, 1985).

Despite the conservation of muscle design, skeletal muscle is very diverse, and specialised for particular functions. This functional diversity is a direct reflection of the contractile properties of the fibre types from which the muscle is composed. The most basic mechanism for the functional specialisation of muscle fibres is the generation of muscle protein isoforms. These are proteins with similar structures and functions but different amino acid sequences. Virtually every muscle protein has been shown to exist as isoforms. Different myosin isoforms may alter the interactions between the thick and thin filament, between myosin and ATP, or between myosin and its anchoring M-protein (Hochachka, 1994). The control of cross bridge cycling rates, through the control of myosin ATPase catalytic activity is thought to be one way in which myosin isoform alterations can match the muscles' functional requirements (Reiser *et al.*, 1985). Variations in the myosin isoform composition are thought to affect several steps in the actin myosin cross bridge cycle (Zhao and Kawai, 1993; Wang *et al.*, 1994).

Fast muscle myosin sub-units are distinct from those of slower muscle in most vertebrates, including teleosts (Lowey and Risbey, 1971; Focant *et al.*, 1976; Hoh *et al.*, 1976, Biral *et al.*, 1982; Huriaux and Focant, 1985; Martinez

et al., 1990; Crockford and Johnston, 1993; Johnston and Horne, 1994). ATPase activity, a higher proportion of MLC3f than MLC1f, fast regulatory light chain (LC2f), general muscle actin, α and α type tropomyosin (α-αTm), and fast type slow-type myosin, slow type light chains (LC1s, LC2s LC3s), general skeletal actin, β-, γ-, and δ-type tropomyosin, and slow type troponins (TnTs, TnIs and TnCs).

Isoform genetics

The genes of myofibrillar proteins are regulated by a number of transcription factors (see Haschka, 1994, for review). Regulation may occur at transcriptional and post transcriptional levels. A change in the amount of a particular isoform may reflect a variation in the rate of transcription of the gene, the stability of the transcript or the rate of translation (see Schiaffino and Reggiani, 1996, for review). Myofibrillar protein isoform genes from the same family have a similar structure, reflecting a common evolutionary origin. Stedman *et al.* (1990) showed that the coding regions of isogenes for the same protein isoform from different species were more similar than different isoforms within a species. Stedman *et al.* (1990) state that the functional specialisation of different isoforms restricts isogene variation. The chromosome *loci* of all the myofibrillar proteins, except TnC and M protein, have been determined. With the exception of the MHC gene family, the isogenes of each family occur on different chromosomes or at distant *loci* on the same chromosome. MHC isogenes are clustered in humans (Saez *et al.*, 1987), mouse (Weydart *et al.*, 1985) and rat (Mahdavi *et al.*, 1984). The significance of MHC isogene clustering is not yet known.

Variable expression of protein isoforms among muscles of different types is a major determinant of contractile properties (Moss *et al.*, 1995). Fast muscle has been shown to differ from slow muscle in a number of measured parameters which have been attributed to the myosin isoforms present.

These include, maximum power output and maximum shortening velocity (V_{max}) (Barany *et al.*, 1965; Barany, 1967), force velocity relationships (Reiser *et al.*, 1985b; Eddinger and Moss, 1987; Greaser *et al.*, 1988; Sweeney *et al.*, 1988; Rome *et al.*, 1990; Bottinelli *et al.*, 1991; Larsson and Moss, 1993; Bottinelli *et al.*, 1994a), Ca^{2+} ATPase activities, (Gibbs and Gibson, 1972; Heilmann and Pette, 1979; Zubrzycka-Gaarn *et al.*, 1982), and the time course of tension development and the decline in response to a single electrical stimulus (Burke *et al.*, 1971, 1973; Close, 1972; Kugelberg and Lindergren, 1979).

1.10 Isoforms of the Myofibrillar Proteins

The following section provides a description of the isoforms of each myofibrillar protein.

Myosin heavy chain

The MHCs found in the myofibrils of mammalian striated muscles are all isoforms of myosin family II (Moss *et al.*, 1995). The major sarcomeric isoforms of MHC are MHC-b/slow, MHC-2A, MHC2X, MHC-2B, in adult mammalian skeletal muscles; MHC-emb, and MHC-neo in developing skeletal muscles; MHC-α, MHCeo (extra ocular) and MHC-m (mandibular) which have a restricted tissue-specific distribution.

The MHC-β/slow isoform of mammalian slow (type 1) muscle fibres is identical to that expressed in the heart (Lompre *et al., 1984*) and present in the slow and cardiac muscle of Amphibia (Casimir *et al.*, 1988). In contrast, the skeletal muscle of birds contains two slow MHC isoforms which are not the same as those in the heart (Matsuda *et al.*, 1982). A further MHC in the superfast contracting cat jaw muscle, which differs from both fast and slow MHC has been reported by Rowlerson *et al.* (1981). Unique myosin heavy chains have also been detected immunohistochemically in fibres of specific

muscles, e.g., extra ocular muscles (Wieczorek *et al.*, 1985; Sartore *et al.*, 1987).

Multiple isoforms of MHC have been identified in single fibres (Biral *et al.*, 1988; Gorza, 1990; Staron and Pette, 1987; Rushbrook and Stracher, 1979). Four different heavy chains have been separated from a single, chronically stimulated rat muscle fibre by Termin *et al.* (1989a, 1989b). Huriax *et al.* (1991) resolved four different MHC isoforms in red trunk, white trunk, ventricle and head muscles of the barbel (*Barbus barbus*) Ennion *et al.* (1995) identified a MHC gene in common carp which is specific to small-diameter white myotomal muscle fibres. Adult MHC expression is modulated by factors such as neural activity (see Pette and Vrbovà, 1985), hormonal input (see Mahdavi *et al.*, 1987) and mechanical activity (see Roy *et al.*, 1991). In single fast fibres, MHC-2B and MHC-2X produce greater power than MHC-2A fibres (Close, 1972; Ranatunga, 1982; Bottinelli *et al.*, 1991).

MHC is the major determinant of myosin ATPase activity (Lowey *et al.*, 1993; Wagner, 1981). Furthermore, Bottinelli *et al.* (1994b) established that the rate of ATP hydrolysis was correlated with the MHC isoform expression in rats; the rate of hydrolysis decreased in the order MHC2B, MHC2X, MHC2A, MHCslow. Potma *et al.* (1994) also demonstrated these trends in rabbit fast psoas fibres and slow soleus fibres.

The role of the MHC has been investigated by many groups, using *in vitro* motility assays (Warshaw *et al.*, 1990; Yamashita *et al.*, 1992; Lowey *et al.*, 1993). Research by these authors has shown that the speed of actin filament movement is correlated with the proportion of fast MHC isoforms present. Furthermore, the speed of shortening correlates with MHC composition, whereby it decreases in the order MHC2B, MHC2X to MHC2A (Sweeney *et al.*, 1986, 1988; Rome *et al.*, 1990; Larsson and Moss, 1993;

Bottinelli *et al.*, 1994a, b, c). This relationship holds true for adult, developing and hypokinetic muscles in rat and rabbit (Reiser *et al.*, 1985a, b, 1987a, 1988b; Sweeney *et al.*, 1986, 1988; Eddinger and Moss, 1987). However, in groups of pure fast fibres, which contain the same MHC isoforms, considerable variations in the velocity of shortening still exist (Bottinelli *et al.*, 1994a, b, c; Sweeney *et al.*, 1988). In these fibres the velocity of shortening is proportional to the MLC3f content (Sweeney *et al.*, 1988; Greaser *et al.*, 1988; Moss *et al.*, 1990; Bottinelli *et al.*, 1994b). Therefore, both MHC and MLC composition determine the speed of shortening.

It is thought that there are 13 genes for the myosin heavy chains of mammals (Perry, 1985). Robbins *et al.* (1986) have identified as many as 31 genes within the MHC family in the chicken. However, the structure of MHC has been shown to be highly conserved (Kavinsky *et al.*, 1983). Several MHC isoforms are produced primarily by the transcription of different genes (Rushbrook and Stracher 1979, Periasamy *et al.*, 1984, Buckingham 1985). Other groups have found that MHC isoforms are expressed by alternative splicing from a single gene (Collier *et al.*, 1990; Kelly *et al.*, 1993; Nyitray *et al.*, 1994). Bandman *et al.* (1982) state that differences in myosin heavy chain isoforms may arise from post translational modifications. Gerlach *et al.* (1990) indicated a minimum of 28 different myosin heavy chain genes were present during development in different tissues of the carp (*Cyprinus carpio* L.).

Myosin light chains

Five major alkali light chain isoforms have been recognised in mammalian skeletal muscle (see Barton and Buckingham, 1985). Fast skeletal muscle contains MLC1fast ($MLC1_f$) and MLC3fast ($MLC3_f$). Slow skeletal muscle contains MLCslow/ventricular ($MLC_{s/b}$) and MLCslow-a

($MLC_{s/a}$). MLCs/a is the major isoform in rabbit and human skeletal muscle. However, it is not expressed in mouse and is present in lower levels in the rat (Hailstones and Gunning, 1990). The other isoform is MLC1emb/atrial, which is expressed in developing skeletal muscle and atrial myocardium.

There is a correlation between the maximum speed at which a muscle can contract (V_{max}) and the alkali light chain ratio ($LC1_f/LC3_f$) in type IIB fibres from different rabbit muscles (Sweeney *et al.*, 1986, 1988; Greaser *et al.*, 1988; Moss *et al.*, 1990). Crockford *et al.* (1995) examined the maximum contraction velocity of single skinned muscle fibres from the teleost fish *Oreochromis andersoni*. The fibres of this species showed either a homozygous expression of $LC1_{f1}$ or $LC1_{f2}$, or both light chains expressed in the same fibre. The contraction velocity correlated with the proportion of LC1 present, with intermediate Vmax when both isoforms were present. No differences in any other contractile proteins were found.

At low ionic strength, the ATPase activity of myosins containing $S1MLC_{3f}$ is twice that of $S1MLC_{1f}$ (Weeds and Taylor, 1975; Hayashibara and Miyanishi, 1994). Furthermore, $S1MLC_{1f}$ has a greater affinity for actin than $S1MLC_{3f}$ at low ionic strength (Trayer *et al.*, 1987; Hayashibara and Miyanishi, 1994). However, no differences in ATPase activity at physiological ionic strengths has been shown for myosins containing different alkali light chain compositions (Weeds and Taylor, 1975). Solution biochemical work with LC1 and LC3 homodimers of intact myosin, indicate that actin-activated myosin ATPase activity is similar regardless of the alkali light chain content. Furthermore, removal of the light chains does not appear to affect ATPase activity. Lowey *et al.* (1993) concluded that the light chains are important in force generation and movement in intact systems, but they do not play a significant role in modulating ATPase activity in solution.

Three isoforms of LC2 ($LC2_f$, $LC2_s$, $LC2_e$) have been identified in mammals and fish species (Crockford and Johnston, 1993; Johnston *et al.*, 1997). Larsson and Moss (1993) showed that fast human muscles containing MLC2s shorten at a lower speed than fibres with $MLC2_f$. Faerman and Shani (1993) identified a MLC2 gene which is expressed specifically in the skeletal muscles of new-born and adult mice.

MHCs and MLCs are both encoded by a highly conserved multigene family and are expressed in muscle as multiple isoforms (Nguyen *et al.*, 1982; Wydro *et al.*, 1983; Buckingham *et al.*, 1986; Mahdavi *et al.*, 1986; Emerson, 1987). In mammals and birds $MLC1_f$ and $MLC3_f$ derive from a single gene by alternative utilisation of two transcription sites and alternative splicing of the first four exons (Nabeshima *et al.*, 1984; Periasamy *et al.*, 1984; Robert *et al.*, 1984; Strehler *et al.*, 1985; Barton and Buckingham, 1985; Andreadis *et al.*, 1987). Recently, Hirayama *et al.* (1997) demonstrated that the alkali light chains from the fast muscles of carp are encoded by different genes. Dalla Libera *et al.* (1991) suggested that the alkali light chains of the grey mullet (*Mugil capito*) are also the products of separate genes.

Actin

Actin is the most strongly conserved of the muscle proteins. Isoforms have been found in smooth muscle, however, no actin isoforms have been found in fast or slow skeletal muscle (Vandekerckhove and Weber, 1979). Cardiac muscle actin differs from skeletal muscle actin (Perry, 1985), but only 5 of the total 375 amino acids vary between rabbit slow muscle actin and bovine cardiac muscle actin (Vanderkerckhove and Weber, 1979). In skeletal muscle, actin interacts with a number of proteins, which may occur as different isoforms. Therefore the highly conserved structure of actin may be due to accommodating a number of other proteins (Perry, 1985)

Tropomyosin

In skeletal fast muscle three combinations of tropomyosin (Tm) subunits are possible α/α and β/β homodimers and the α/β heterodimer. The a/b Tm subunit ratio appears to be species-specific (Cummins and Perry, 1973) and muscle specific (Matsuda *et al.*, 1983). The a subunit predominates in fast muscles. In slow muscle it is absent or partially replaced by γ and δ. Schachat *et al.* (1987) stated that certain combinations of Tm subunits appeared to be co-expressed with specific troponin T subunit isoforms and that the α and β subunits bind to TnT with different affinities. Little variation between the isoforms of Tm has been shown. Variation between tissues is possibly related to the interaction between Tm and the troponin complex expressed in the muscle type. Rowlerson *et al.* (1983) demonstrated that the cat jaw closer muscle contains a single, distinct form of tropomyosin. Phosphorylation of the Tm isoforms is higher in foetal than adult muscle, suggesting a possible role for phosphorylation of Tm in development rather than in the regulation of contraction (Heeley *et al.*, 1985).

Four Tm genes have been identified and characterised in birds and mammals. Each of these genes can generate several transcripts expressed in striated, and smooth muscle and non muscle tissue by alternate splicing (Lees and Helfman, 1991; Pittenger *et al.*, 1994). In mammals, three striated muscle specific transcripts derived from different genes code for β-Tm, αTmfast and αTmslow isoforms (see Schiffiano and Reggiani, 1996). The β-Tm gene generates another muscle specific splicing product called β-Tm2 that is expressed at high levels in developing mouse (Wang and Rubenstein, 1992). The β-Tm subunits in skeletal muscle are derived from transcripts of a single gene with a unique promoter by alternative splicing (Libri *et al.*, 1989). At the RNA level, the α-Tm subunit exists in at least six different tissue

specific isoforms resulting from alternative splicing of the primary transcript of the α-Tm gene (Ruiz-Opazo and Nadal-Ginard, 1987; Weiczorek *et al.*, 1988).

Troponin T

The number of TnT isoforms actually identified is less than the number theoretically possible. Rabbit muscle shows at least six fast isoforms and two slow isoforms (Briggs *et al.*, 1987; Härtner *et al.*, 1989). Additional charge variants further increase the number of fast and slow TnT isoforms, most of which represent phosphorylated forms (Briggs *et al.*, 1987; Härtner *et al.*, 1989). Moore and Schachat (1985) and Moore *et al.* (1987) showed that various fast TnT isoforms follow a restricted pattern in association with the tropomyosin subunits (Moore and Schachat, 1985; Moore et *al.*, 1987). The particular fast TnT isoform in combination with the a and b-Tm subunits has been correlated with pCa/tension relationships between fibres in rabbit (Schachat *et al.*, 1987; Greaser *et al.*, 1988) and chicken muscle (Reiser *et al.*, 1987b). Thus, variation in expression of TnT isoforms results in significant alterations in the Ca^{2+} sensitivity of tension. These authors therefore concluded that isoforms of TnT and Tm influence the activation response of thin filaments to Ca^{2+}. Schmitt and Pette (1990) suggested that fast and slow isoforms are correlated with fast and slow myosin heavy chain isoforms in rabbit muscles. Studies of rabbit fast muscle, which has been chronically low frequency stimulated in order to transform it to slow muscle, have provided insights into the functional significance of TnT isoforms; the relative amounts of fast TnT isoforms reduce and the slow isoforms increase (Schachat *et al.*, 1988; Härtner *et al.*, 1989; Pette, 1990). During this transition the troponin subunits, TnC and TnI, also undergo transformation, although less pronounced than TnT (Härtner and Pette, 1988, 1990; Pette,

1990). Therefore transforming fibres may contain troponin molecules made up of both fast and slow subunits (Pette and Staron, 1990).

Isoforms of TnT are produced by alternate mRNA splicing pathways from a single gene containing two different exons, which produce two different but related amino acid sequences near the C terminus of the protein (Wilkinson *et al.*, 1984; Medford *et al.*, 1984; Breitbart *et al.*, 1985; Smillie *et al.*, 1988). Combinatorial splicing mechanisms could theoretically generate up to 64 different mRNA isoforms (Breitbart *et al.*, 1985; Breitbart and Nadal-Ginard, 1986, 1987). The differential splicing of alternative and constitutive exons is developmentally controlled by muscle-specific trans-acting factors (Breitbart and Nadal-Ginard, 1987).

Troponin C

Fast and slow isoforms of TnC are found in skeletal muscle. Cardiac TnC has the same amino acid sequence as slow muscle TnC. Two Ca^{2+} specific binding sites are possessed by fast TnC, while slow TnC and cardiac TnC possesses only one (Potter and Johnston, 1982). The filling of sites is considered necessary for contraction (Perry, 1985).

Stephenson and Williams (1982) found that at a given sarcomere length, the pCa for half maximal activation (pCa50) of tension was greater in slow soleus muscle fibres than in fast extensor digitorum longus muscles of the rat. Similarly, skinned slow twitch fibres from fish muscles have greater pCa50 values than fish fast-twitch fibres (Altringham and Johnston, 1982). Some authors have observed greater Ca^{2+} sensitivity of tension in rat fast-twitch than in slow-twitch fibres (Mousier *et al.*, 1989). Metzger and Moss (1987) observed little difference in Ca^{2+} sensitivity in rat fast twitch and slow twitch fibres. Tension-pCa relationships are generally steeper in fast-twitch muscles. Near stochiometric substitutions of slow TnC into skinned skeletal

muscle fibres does not alter the steepness of the tension-pCa relationship (Moss *et al.*, 1991). Reiser *et al.* (1992) observed no differences in Ca^{2+} sensitivity of tension in adult avian fast and slow muscles that could be attributed to different isoforms of TnC. Instead, variations in Ca^{2+} sensitivity were related to variable expression of isoforms of TnT.

Metzger (1996) studied the effects of TnC isoforms on the pH sensitivity of contraction of mammalian muscle. Endogenous TnC was partially extracted from skinned fibres and replaced with purified isoforms. Reconstitution of psoas fibres with cardiac TnC increased the pH sensitivity of contraction. Slow soleus fibres reconstituted with fast TnC showed a significant decrease in pH sensitivity. These findings provide evidence that TnC isoforms play an important role in acidic pH-mediated contractile dysfunction in striated muscle fibres.

Fast and slow TnC genes are encoded by single copy genes in the human genome and the slow TnC isoform appears to be identical to the cardiac TnC isoform (Gahlmann *et al.*, 1988).

Troponin I

TnI occurs in three forms specific to fast (TnIf), slow (TnIs) and cardiac (TnIc) muscle. In rabbit skeletal muscle TnI slow and fast isoforms are present as two molecular weight variants in slow and fast twitch muscles, respectively (Hartner and Pette, 1990). The slow isoform has a larger molecular mass. The fast TnI isoform is phosphorylatable, existing as three charge variables which are reduced to a single form by digestion with alkaline phosphatase (Härtner and Pette, 1990). TnI may indirectly account for fibre-specific Ca^{2+} binding properties (Perry, 1985). Crockford *et al.* (1991) found two isoforms of TnI (TnIf1 + TnIf2) which were both expressed in single fibres from the fast muscles of two tropical fish species,

Oreochromis niloticus and O. andersoni. Furthermore, Young and Davey (1981) found two isoforms of TnI in single fast fibres of bovine sternomandibularis muscle. The isoforms of TnI have been found to be encoded by distinct genes (Wilkinson and Grand, 1978; Koppe *et al.*, 1989; Murphy *et al.*, 1991).

Other proteins

The titin of red muscle differed in electrophoretic mobility to white muscle titin, as has been demonstrated in mammalian muscle (Wang and Wright, 1988). Titin and nebulin are produced by differential splicing from a single promoter (Medford *et al.*, 1984, Wilkinson *et al.*, 1984, Briebart *et al.*, 1985, Imai *et al.*, 1986, Bucher *et al.*, 1988).

1.11 Alterations in Isoform Expression

Isoforms and development

Various isomyosins have been observed in developing muscles. Developmental isomyosins appear sequentially during embryonic and perinatal development (Hoh and Yeoh, 1979; Whalen *et al.*, 1981; Marechal *et al.*, 1984; d'Albis *et al.*, 1989). The regulation of muscle diversification during development is regulated by a number of factors (see Gunning and Hardeman, 1991, for review). These include hormonal input or neural input, or the myoblast predetermination. The changes in contractile proteins during development may be regulated by both myogenic and neurogenic factors (Hoh, 1991) and have been attributed to the innervation of the muscles, (Van Horne and Crow, 1989), nerve regulation (Bandman *et al.*, 1982), or modulation by the thyroid hormone (Gardahaut *et al.*, 1992). Yamano *et al.* (1991) suggested that the developmental changes in the muscular tissue of metamorphosing flounder were regulated by thyroid hormone. The developmental pattern of isoform expression varies for different gene

families (see Sutherland *et al.*, 1993). Caplan (1983) suggested that developmental isoforms of contractile proteins were necessary for the development of the skeleton, tendons and other movement systems and that these isoforms were appropriate for the relatively low load bearing presented to embryonic muscles.

Isoforms and ageing

In rabbits, ageing does not alter the myosin content of the fast muscles. However the soleus muscle becomes more heterogeneous in old animals. In ageing fast muscle of rats, MHC2B decreases and MHC2X increases (Sugiura *et al.*, 1992; Larsson *et al.*, 1993). Woodhead (1979) found changes in actomyosin ATPase activity with ageing in teleosts.

Isoforms and regeneration after injury

Injured muscle undergoes regeneration. This process shows some similar features to the myogenic process of embryonic muscle development: satellite cells proliferate and fuse to form new muscle fibres that transiently express developmental myosin isoforms before switching to adult isoforms (Satore *et al.*, 1982; Carraro *et al.*, 1983). For example, chicken muscles re-express ventricular MHC (Gorza *et al.*, 1983; Stewart *et al.*, 1991), and in mammalian muscle regeneration, MHC_{emb} and MHC_{neo} are expressed (Weydart *et al.*, 1985; d'Albis, 1988; Esser *et al.*, 1993). Sartore *et al.* (1982) identified foetal myosin heavy chains in muscle regenerating after injury.

Isoforms and exercise

MHC isoform expression is sensitive to the degree of chronic mechanical activity imposed on the muscle. An increase in the chronic weight-bearing load on a muscle typically up-regulates the expression of slow myosin isoforms relative to other isoforms (Tsika *et al.*, 1987b).

Isoforms and temperature acclimation

When fish are exposed to a prolonged alteration in environmental temperature, acclimation changes may occur. However the exact nature of the response depends upon the species examined.

Alterations in the isoforms present in fish fast muscle fibres in response to temperature acclimation have been demonstrated in cyprinids and some marine teleosts (Hwang *et al.*, 1990; Gerlach *et al.*, 1990; Crockford and Johnston, 1990; Langfeld *et al.*, 1991; Watabe *et al.*, 1992; Crockford and Johnston, 1993; Johnston and Horne, 1994; Johnson and Bennet, 1995; Ball and Johnston, 1996; Imai *et al.*, 1997).

Isoforms and hormones

Izumo *et al.* (1986) investigated MHC isoform switching in response to thyroid hormone in adult rats. They found that all the MHC genes investigated were responsive to thyroid hormone, and that the same gene is regulated by thyroid hormone in a tissue specific manner. In addition, skeletal embryonic and neonatal myosin MHC genes could be re-induced by hypothyroidism in specific adult muscles.

The thyroid hormone has been shown to influence normal developmental transitions, whereby the transition from neonatal MHC to adult fast is impaired in the absence of MHC and increased in its presence (Butler-Browne and Whalen, 1984; Gambke *et al.*, 1983; Whalen *et al.*, 1985). In addition, the thyroid hormone has a significant impact on differential expression of MHC isoforms. Overall, thyroid status predominates over the other potential regulators of MHC isoform expression, such as electrical stimulation and metabolic activity (Moss *et al.*, 1995).

Stimulation alters isoform expression

Muscle has been shown to express slow isoforms of some contractile proteins by exposure to chronic low frequency 10 Hz stimulation, whilst more phasic higher frequency stimulation results in a slow to fast transformation of some muscles. Hoh and Hughes (1988) stated that it is the stimulation pattern that the muscle receives from the nerve, and not the innervation *per se*, that influences the type of myosin isoform expressed in the muscle. During development neural influences have an effect on isoform transitions in some cases but not in others. The effect varies according to embryonic stage, suggesting the existence of critical periods in development, when sensitivity to neural influence is greatest (Harris *et al.*, 1989; Condon *et al.*, 1990).

1.12 Fish Muscle

In birds and mammals the muscles generally contain a heterogeneous population of fibre types (Pette and Starron, 1990). In contrast, the fibre types of fish muscles are arranged in discrete regions. This makes fish ideal for the study of muscle fibre composition because whole muscle function reflects the contractile properties of a single fibre type. The myotomes of fish contain different muscle fibre types which are specialised for function at varying speeds (Bone, 1978; Johnston and Altringham, 1991). The various fibre types of fish muscle have different structural and functional properties and use different energy supply systems (see McMahons, 1984; Woledge *et al.*, 1985).

In fish, swimming is achieved by waves of curvature passing posteriorly along the fish and by oscillatory fin movements. Some species rely on one mode more than the other. Aquatic locomotion requires an increase in power proportional to the swimming velocity cubed (Webb,

1976), hence the majority of fish tissue is muscle. These animals are able to carry such a disproportional large amount of muscle because of being neutrally buoyant.

The red and white fibres of fish are arranged in myotomes (Nursall, 1959). The arrangement of the fibres along the length of the fish varies, in order to maintain the same amount of sarcomere shortening, and filament overlap during swimming (Alexander, 1969). In most fish species, red fibres form a superficial strip in the region of the lateral line nerve, and the remaining bulk of the myotome is composed of white muscle. These two distinct fibre types can be distinguished at the light microscopy level on the basis of histochemical staining for aerobic enzymes and myofibrillar ATPase (Johnston and Moon, 1980) However some fibre types which appear histochemically the same have different ultrastructures (Edman *et al.*, 1988), for example, pink and white fibres (Rowlerson *et al.*, 1985).

Five major fibre types have been determined in the dogfish (*Scyliorhinus canicula*) (Bone and Chub, 1978), four fibre types have been described in the perch (*Perca flavescens*) (Akster 1981), three in the carp (*Cyprinus carpio*) (Johnston *et al.*, 1977) and only two in the brook trout (*Salvelinus fontinalis*) (Johnston and Moon, 1980), the Atlantic mackerel (*Scomber scombrus.*) (Bone, 1978b), the Atlantic herring (*Clupea harengus*) (Johnston, 1993).

White fibres

The white muscle fibres run helically along the long axis of the body, following complex spiral trajectories which involve angles of up to 40 ° (Alexander, 1969; Bone and Marshal, 1982; Rome *et al.*, 1988). Alexander (1969) calculated that this complex arrangement means the inner white fibres only shorten 25 % as much as the red muscles to achieve the

equivalent body curvature. White fibres have relatively larger diameters than white muscle (about 100 μm), lack lipid reserves and myoglobin, have low mitochondrial densities, low capillary densities and high glycolytic capacities (Bone, 1978; Johnston, 1982, 1983). Biochemically the myofibrillar ATPase activity of the white fibres is around three times that of the red (Johnston *et al.*, 1972).

White fibres are recruited at burst swimming speeds and quickly fatigue (Rome *et al.*, 1988). However, in carp, multiply innervated white fibres are recruited for sustained swimming (Johnston *et al.*, 1977). This has also been shown in the brook trout by Johnston and Moon (1980).

Red fibres

Red fibres are normally recruited for sustained swimming. Greer-Walker and Pull (1975) reported that red fibres constituted between 0.5 and 29 % of the myotomal muscle mass in fish. The proportion of red fibres in the myotomes of different species is correlated positively with sustained swimming performance and negatively with the involvement of pectoral fins during swimming at low and intermediate speeds (Greer-Walker and Pull, 1975; Walesby and Johnston, 1980). In most species red fibres are located immediately beneath the skin at the outer extremities of the horizontal septum and are aligned parallel to the long axis of the body. The myofibrils of fish red muscle fibres are relatively small (about 1 μm diameter) and are regular in cross section, occupying around 50-65 % of fibre volume (Bone, 1978; Johnston, 1981). Mitochondrial volume densities are around 15-46 % (Johnston, 1983). Red muscles are polyneuronally innervated (Bone, 1964) and are normally activated by action potentials (Altringham and Johnston, 1988).

In herring larvae the red fibres have been shown to be directly involved in gaseous exchange cross the skin (Batty, 1984). The capacity of fish red muscles for anaerobic glycolysis is species dependent (Johnston, 1983). The capillary density of muscle is related to its aerobic capacity, red having a greater mean capillary fibre ratio than white in three teleost species studied by Mosse (1979). Extrapolated values for V_{max} are lower in red than in white twitch fibres (Altringham and Johnston, 1982; Johnston and Brill, 1984).

Tonic fibres

Tonic fibres are dedicated to maintaining posture and hence are common in terrestrial vertebrates. Fish are supported by the surrounding water and therefore have little need for these muscles. However, in the dogfish a few superficial large diameter fibres form an interrupted single layer external to the zone of red fibres. These fibres have a large diameter, few mitochondria and low myofibrillar ATPase activity (Bone *et al.*, 1986). They form less than half a per cent of the total number of myotome fibres. Ultrastructurally, these fibres are distinguishable in longitudinal section by the absence of M-lines and by a lack of myofilament bundle alignment when compared to red or white muscle. In addition they have intermediate sarcoplasmic reticulum volume densities between that of red and white muscles. These superficial fibres may have a role in maintaining body tone and attitude while the dogfish is not swimming (Bone *et al.*, 1986). Tonic fibres have also been described in the stickleback (*Gasterosteus aculeatus*) (Kilarski and Kozlowska, 1983), the turbot (*Scophthalmus maximus*) (Calvo and Johnston, 1992) and the plaice (*Pleuronectes platessa*) (Brooks and Johnston, 1993).

Pink fibres

Pink fibres are fast oxidative glycolytic fibres. They posses an intermediate amount of myoglobin which gives them their pink colouration. These fibres usually form part of a mosaic arrangement of fibres between the focally innervated white and multiply innervated red regions of the musculature (Bone, 1978). Pink fibres have few endplates and high aerobic capacities, and are found in all vertebrates (Johnston, 1985). The enzymatic activities of pink muscles are intermediate between those of red and white muscle in the carp (Johnston *et al.*, 1977).

Specificity of fish muscle

The musculature of teleosts is different from other types of vertebrate muscle. The A-band myosin filaments are organised in a simple lattice. The thick filaments have triangular profiles when viewed in transverse section at the bare region just next to the M band. The triangular cross sections all have the same orientation in each myofibril. The true significance of this arrangement remains to be determined (Luther*et al.*, 1996).

Structures such as the M-band and the Z-band are fibre type specific (Sjustrom and Squire, 1977). Many of the protein components and particular organelles in fibres are also fibre type specific. Myosin heavy chain isoforms appear to be fibre type specific (Scapolo and Rowlerson, 1987; Kararinski and Kilarski, 1989; Johnston *et al.*, 1990; Crockford and Johnston, 1993, Brooks and Johnston, 1995). Similarly, myosin light chain isoforms have been shown to differ between fibre types. Red muscle generally contains only two light chain components whereas white muscle contains three myosin light chains (Focant *et al.*, 1976, Rowlerson *et al.*, 1985; Huriax and Focant, 1985; Kararinski and Kilarski, 1989; Huriax *et al.*, 1990, Martinez *et al.*, 1990, Johnston and Horne, 1994). However, three myosin light chains

were found in the red muscle of plaice by Brooks and Johnston (1995). The light chain composition of plaice muscle and the myofibrillar ATPase staining suggested that the red muscle was in fact fast pink (Johnston, 1983; Brooks and Johnston, 1995). Several species of teleosts exhibit pink fibres containing three myosin light chains similar to those of the white fibres (Rowlerson *et al.*, 1985; Scapolo and Rowlerson, 1987). There are also extra proteins in myosin filaments such as C-protein and X-protein whose distribution is fibre type dependent (Bennett *et al.*, 1986; Ohtsuki *et al.*, 1986). Furthermore, isoforms of tropomyosin and Troponin T have been shown as fibre type specific in teleosts (Focant *et al.*, 1976; Crockford, 1989; Brooks and Johnston, 1995).

1.13 Aims

The primary aim of this study was to examine and characterise the myofibrillar proteins present in the skeletal muscle of specific teleost species. The influence of several factors upon the expression of myofibrillar protein isoforms was investigated. These factors included ontogeny and rearing temperature in the Atlantic herring (*Clupea harengus* L.), and body size in the short-horn sculpin (*Myoxocephalus scorpius* L.). Furthermore, the influence of evolution at sub-zero temperatures on the myosin sub-units in Antarctic fish was investigated by comparing with phylogenetically related species of the sub-Antarctic biogeographical zone.

Chapter 2

Materials and Methods

2.1 Collection of Specimens

Detailed descriptions of the collection of specimens is given in the relevant chapters. Chapter 3 describes the collection of Atlantic herring, Chapter 4, short-horn sculpin and Chapter 5, Antarctic and sub-Antarctic fish.

2.2 Preparation of Myofibrils

Myofibrils were prepared from muscle samples as described by Focant *et al.* (1976). All tissue and solutions were kept below 2 °C to minimise proteolytic breakdown during the preparation. Muscle samples were homogenised for 30 seconds at speed 5 using a 10mm diameter Polyton homogeniser in 20 volumes of ice cold buffer containing (in mmol l^{-1}): Tris-HCl, 10 pH 7.6; NaCl, 50; ethylene-diaminetetraacetic acid (EDTA), 1. The buffer also contained the following proteolytic enzyme inhibitors, 50 μg/ml n-tosyl-L-phenylanaline chloromethyl ketone (TPCK), 50 μg/ml phenyl-methylsulphonyl fluorine (PMSF), 0.5 μg/ml leupeptin, 1 μg/ml pepstatin A and 0.2 units/ml aprotinin.

Triton X-100 (0.5 % v/v) was added to the homogenate to aid the removal of membrane bound proteins. The homogenate was spun in a chilled centrifuge for 5 minutes at 1500 g, the supernatant discarded and the pellet resuspended. This was repeated an additional four times with the Triton X-100 omitted. The resulting pellet containing the washed myofibrils was resuspended in an equal volume of 100 mM NaCl, 10 mM Tris-HCl pH 7.2. This myofibrillar suspension was then immediately prepared for electrophoresis or stored at -20 °C in 50 % (v/v) glycerol until required.

2.3 Determination of Protein Content

The concentration of myofibrils was calculated to enable samples of known concentration to be prepared for electrophoresis. Protein concentration of the myofibril solutions was determined using the Sigma Diagnostics Microprotein-PR protein assay kit which is a modification of the procedure of the method of Fujita, *et al.* 1983 (Sigma. Poole, Dorset).

2.4 Electrophoresis Equipment

All slab gel electrophoresis was carried out using the Biorad Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, UK.). The first dimension of two dimensional iso-electric focusing (2-D IEF) gels was performed using the Biorad Mini-Protean 2-D electrophoresis cell (Bio-Rad Laboratories, UK.). Western blotting was carried out using the Biorad Mini Trans Blot cell (Bio-Rad Laboratories, UK.).

The equipment was cleaned prior to use by soaking overnight in a 0.5 % solution of Decon-90 (Sigma). The next day the equipment was thoroughly rinsed in ulta-pure milli-Q water and air dried. Before use, the gel plates were wiped with 70 % (v/v) ethanol. New casting tubes were used for each first dimension IEF gel.

2.5 Electrophoretic Techniques

Once mixed, the gel solutions were thoroughly degassed using a vacuum pump, immediately prior to the addition of the polymerisation agents. Gels that were cast without a comb were overlaid with 50 ml of isobutanol during polymerisation to ensure an even surface by preventing a meniscus forming at the top of the gel. The gels were left for at least 3 hours to polymerise before the addition of the stacking gel or loading of the

samples. Slab gels were 70 mm long and 80 mm wide with a thickness of 0.75 mm. Gels of 1 mm thickness were cast for second dimension iso-electric focusing (2D-IEF) and 2nd dimension peptide mapping. First dimension iso-electric focusing (IEF) gels were cast in 1 mm diameter casting tubes.

One dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

One dimensional SDS-PAGE electrophoresis was carried out according to the method of Laemmli (1970). SDS-PAGE separates proteins according to molecular weight. The anionic detergent SDS disrupts hydrogen bonding unfolding the protein and masks protein charges. Dithiothreitol (DTT) is also included in the sample preparation as this further unfolds the protein as it cleaves disulpide bonds between cystein residues. During electrophoresis there is a linear relationship between the logarithm of the molecular wieght and the relative migration of the unfolded amino acid chains.

Sample preparation: myofibrils were dissolved in 60 mM Tris-HCl pH 6.8, 2 % sodium dodecyl sulphate (SDS), 10 % glycerol, 1 mM dithiothreitol (DTT) and 0.001 % bromophenol blue (BPB) to give a final protein concentration of 2 mg/ml. The samples were heated to 100 °C for 3 minutes. Any solid particles were removed by centrifugation at 5000 g for 10 minutes. 10 µl of sample was loaded onto each lane.

Stacking gel: 0.125 M Tris-HCl pH 6.8, 4 % total acrylamide of which 2.67 % was NN'-methylenebisacrylamide (BIS) crosslinker and 0.1 % SDS. Polymerising agents were 1 µl of N,N,N'N'-Tetramethylethylendiamine (TEMED) per ml of gel and 0.5 mg ammonium persulphate per 1 ml of gel.

Resolving gel: 0.375 M Tris-HCl pH 8.8, and a varying amount of total acrylamide depending on requirements. This ranged from 10 % to 15 % of

which 2.67 % was BIS crosslinker, and 0.1 % SDS. Again polymerising agents were 1 μl of TEMED per ml of gel and 0.5 mg ammonium persulphate per ml of gel.

Electrode buffer: 25 mM Tris, 0.192 M glycine, 0.1 % SDS pH 8.3 at 20 °C.

Running Conditions: the gels were run at 50 Volts with a current limit of 50 Amps until the samples had entered the stacking gel. The voltage was then increased to 200 volts with a current limit of 50 Amps until the bromophenol blue dye front had reached the end of the gel, which usually took approximately 50 minutes.

One dimensional SDS-PAGE gels with 8 M Urea

Urea SDS PAGE was carried out as described by Sender (1971). Urea was added to the samples for SDS-PAGE electrophoresis at room temperature until a saturated solution was obtained. The gels contained the same ingredients for SDS-PAGE as described above, with the addition of 8 M urea in both the stacking and resolving gels. The addition of 8 M urea enabled the identification of tropomyosin, as this protein migrates with a relatively larger molecular weight in the prescence of urea (Crockford, 1989).

Iso-electric focusing (IEF) gels

Two dimensional iso-electric focusing (2D-IEF) gels were carried out using Crockford's (1989) modified methods of O'Farrell (1975). The first dimension IEF separates proteins in a pH gradient established in the gel. The pH gradient is produced in the gel by the electric field acting upon carrier ampholines. Carrier ampholines are available commercially and have a range of pH and net charge. When the electric field is applied the negatively charged ampholines migrate towards the anode and the

positively charged ones towards the cathode, resulting in a stable and gradual pH gradient through the gel. Since proteins are amphoteric molecules whos net charge is the sum of all the negative and positive charges of the amino acid side chains, each protein has a characteristic pH. When the proteins are applied to the IEF gel they migrate to a position in the gradient until they reach their isoelectric point (pI). At this point the protein has no net charge in the gel and its migration stops. This technique enables characterisation of the myosin light chains which migrate in the neutral to acidic pH range (Rowlerson *et al.*, 1985; Martinez *et al.*, 1990). IEF in a neutral to basic pH range separates isoforms of troponin T and troponin I from the other myofibrillar proteins (Wilkinson *et al.*, 1984; Imai *et al.*, 1986). The focused proteins were then further distinguishable by their respective molecular masses by following the IEF with SDS-PAGE.

Sample preparation: myofibrils were mixed with 8 M urea, 1 % 3-10 Ampholyte, 5 % glycerol, 1 mM DTT, 2 % Noniodet (NP-40) and 0.001 % BPB to give a final concentration of 4 mg/ml. Samples were warmed to 30 °C for 1 h and spun at 5000 g for 5 minutes prior to use. 10-100 μl of sample was loaded onto each gel tube

Resolving gels: 8 M urea, 2 % NP-40, 9 % total acrylamide of which 2.67 % was BIS crosslinker and 5 % ampholyte mixture. The gels were polymerised by the addition of 1 μl of TEMED per ml of gel and 0.5 mg of ammonium persulphate per ml of gel. The exact make-up of the ampholyte mixture used was varied depending on the proteins to be studied. For acidic proteins the Ampholyte mixture contained equal volumes of Ampholine 3-10, Ampholine 4-6.5, and Ampholine 2-5.5. For basic proteins the Ampholyte mixture contained two volumes of Ampholine 9-11 to one volume of Ampholine 3-10.

Electrode buffers: 20 mM NaOH at the cathode, thoroughly degassed and 10 mM phosphoric acid at the anode.

Running conditions for resolving acidic proteins: gels were run from the cathode to the anode. The gels were pre-run for one hour at 200 volts, followed by a further 1 h at 400 V and a current limit of 10 mA. The samples were loaded directly into the phosphoric acid electrode buffer at the anode. The gels were run at 200 V for 2 h, followed by 400 V for a further 4 h and 800 volts for another 1.25 h to give a total of 3 KVh.

Running conditions for basic proteins: gels were run from the cathode to the anode, but the power supply was reversed and the position of the buffers in the gel tank were switched over. The gels were not pre-run, because the samples would come into contact with the NaOH buffer which would precipitate the sample. To prevent the precipitation of the samples during loading, the gels were overlayed with 10 ml of half concentration sample buffer. The samples were then injected between the gel and this overlayed sample buffer. The gels were run for 1 h at 200 V, 400 V for 2 h and finally 800 V for 1.25 h. This gives a total run of 2 KVh.

The second dimension was performed using 1 mm thick SDS PAGE gels. The IEF gels were forced out of the tubes and onto the top surface of the stacking gel. The tube gel was then overlayed with 4 times concentration SDS PAGE sample buffer. The run was carried out as described above for 1D SDS PAGE gels.

Peptide mapping

Peptide maps of myosin heavy chains were produced by modifications of Cleveland *et al.* (1977). Peptide mapping provides a relatively simple and inexpensive way of further comparing proteins which have identical migration by other methods such as SDS-PAGE. The proteins

of interest are digested under identical conditions by proteolytic enzymes which cleave at specific amino acid residues. If two proteins have a different amino acid sequence then the fragments produced by the digestion will be of different lengths and will therefore have different molecular masses. The resulting peptide fragments are then resolved by SDS-PAGE and the banding pattern, or peptide map, produced is diagnostic of the protein from which peptide fragments were generated. Therefore, using this method it is possible to predict that two proteins may have different amino acid sequences.

Samples were run on a 0.75 mm thick, 8 % total acrylamide SDS PAGE gel. The gel was not fixed, but placed in a solution of 0.01 % Coomassie blue R-250 for 5 minutes. The myosin heavy chains were cut out with a razor blade and placed into the wells of a 1 mm thick SDS PAGE gels. The stacking gel was made about 2 cm deep, to allow plenty of contact time between MHC and the enzyme during the run. Bubbles in the electrode buffer between the gel piece and the stacking gel were removed with a spatula. The enzyme required for the digest was dissolved in 60 mM Tris-HCl pH 6.8, 5 % glycerol, 1 mM DTT and 0.001 % BPB. The enzyme solution was then injected onto the top of the gel pieces. The gels were initially run at 50 volts with a current limit of 50 mA for 30 minutes, by which time the bromophenol blue front had entered the gel. The power was then switched off for 1 h to allow the digestion of the MHCs. The power was restored until the dye front had entered the resolving gel. The voltage was then increased to 200 volts until the dye front ran off the bottom of the gel.

The enzymes used were trypsin from bovine pancreas (1μg per lane), endoprotease Glu-C from *Staphylococcus aureus* V8 (2 units per lane), elastase from porcine pancreas (4 μg per lane), α-chymotrypsin from bovine pancreas (1 μg per lane), papain from papaya latex (1 μg per lane),

thermolysin from *Bacillus thermoproteolyticus rokko* (7 μg per lane), clostripain from *Clostripain histolyticum* (1.5 units per lane) and ficin from fig tree latex (12 μg per lane).

Alkali-Urea gel electrophoresis

Alkali urea gel electrophoresis was performed as described by Focant and Huriax (1976) with the modifications given by Crockford (1989). These gels separate proteins by both charge and molecular mass. There main purpose in this study was to enable the identification of Troponin I in the prescence and abscence of free calcium.

Sample preparation: myofibrils were thoroughly mixed with 20 mM glycine, 1 M Tris-HCl pH 8.9, 12 M urea, 1 mM DTT, 0.001 % BPB and either 10 mM Ethylene glycol-bis(b-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA) or 5 mM $CaCl_2$, to give a final protein concentration of 2 mg/ml. Samples were warmed at 30 °C for 1 h prior to use.

Resolving gel: 8 M urea, 100 mM glycine pH 8.9 using 1 M Tris base, 10 % total acrylamide of which 2.67 % was bis crosslinker. The gels were polymerised by the addition of 1 μl of TEMED per ml of gel and 0.5 mg of ammonium persulphate per ml of gel.

Electrode buffer: 100 mM glycine pH 8.9 in 1 M Tris-HCl pH 8.9.

Running conditions: the gels were pre run at 400 V for 1 h. The loaded gels were run initially at 50 V until the samples had entered the gel and then at 400 V until the dye front reached the bottom of the gel.

Two dimensional alkali-urea gels

The first dimension alkali urea gels were rapidly stained with a solution of 0.01 % Coomassie blue R250. The bands of interest were cut out

and placed in separate eppendorf tubes containing 0.5 ml of double concentration SDS PAGE sample buffer to equilibrate. The pieces were then placed into the sample wells of a 1 mm thick SDS PAGE gel, and overlaid with 5 μl of SDS PAGE sample buffer. Gels were run at 50 volts until the dye front had entered the gel, and then 200 V until the dye front ran off the bottom of the gel.

2.6 Gel Fixing

The gels were fixed in a solution of 12 % trichloroacetic acid and 3 % sulphosalcyclic acid before thoroughly rinsing in 3 changes of milli Q water prior to staining.

2.7 Gel Staining

Coomassie blue G250 colloidal stain

From the method of Neuhoff *et al.* (1988). Gels were placed in 2 % phosphoric acid, 15 % (w/v) ammonium sulphate, 0.01 % (w/v) Coomassie blue G250 and 20 % methanol. Gels were de-stained with a 5 % methanol solution followed by rinsing in distilled water.

Silver staining

Peptide maps of herring MHC (Chapter 3) were stained using the methodology of Bloom *et al.* (1988). Gels were placed in 0.2 g/l sodium thiosulphate for 60 seconds, rinsed in large volumes of ultra-pure water three times for 20 s, followed by 0.1 g/l silver nitrate for 10 minutes before again rinsing in Milli Q water (3x30 seconds). Gels were then developed until the bands appeared with minimum background in 100 mls od developer solution which contained 0.3 g sodium carbonate, 0.4 mg sodium thiosulphate and 50 ml formaldehyde. The development was stopped by placing the gels in 5 % citric acid solution.

Peptide maps of Sculpin, Antarctic and Sub-Antarctic fish MHC (Chapters 4 and 5) were stained using the Plus One Silver Stain Kit (Pharmacia Biotech Ltd.), which is based on the methods of Phloeling and Neuhoff (1981). This method was superior to the Bloom method because the staining was more reproducible and produced virtually no background staining.

'Stains all' staining

This stain was used to detect Troponin C, which does not stain well with Coomassie blue. The method is taken from Campell *et al.* (1983). SDS interferes with this stain and so the gels were rinsed for at least 24 hours in several changes of water before staining. The staining solution is light sensitive and so the gels were stained and stored in a light proof container. The stain contains 30 mM Tris-HCl pH 8.8, 25 % isopropanol, 7.5 % formamide and 0.0025 % 'Stains All' (1-ethyl-2-[-3-(ethylnaptho[1,2-d]-thiazolin-2-ylidene)-2-methylpropenyl]naptho[1,2-d]-thiazolium bromide).

2.8 Long Term Storage of Gels

The gels were stored wet in a solution containing 15 % (w/v) ammonium persulphate and 0.0001 % sodium azide, or dried between sheets of Whatman Gel Drying Film (Whatman, U.K Ltd.). The gel and two sheets of the gel film were equilibrated in a solution of 5 % (v/v) ethanol and 5 % (v/v) glycerol. The gel was clamped between the 2 sheets of the gel film and left to air dry for 24 hours.

2.9 Western Blotting

Western blotting was carried out from the basic methodology in Towbin *et al.* (1979) and Gershoni and Palade (1983). In each case a SDS

PAGE was run as described previously, before the blotting procedure was begun.

Protein transfer

The transfer buffer used contained 25 mM Tris, 192 mM glycine, 20 % methanol, pH 8.3. The gel was equilibrated in this transfer buffer for 10 minutes. The membrane used was nitrocellulose with a pore size of 0.45 microns. A piece of nitrocellulose was cut just larger then the gel and soaked in transfer buffer. Two pieces of pre-cut 7.5 x 10.5 x 1 mm thick filter paper was soaked in the transfer buffer for 5 minutes. The nitrocellulose was placed onto one of the filter paper pieces, and the equilibrated gel placed onto the nitrocellulose. The final piece of filter paper was then placed on top of the nitrocellulose. Care was taken to ensure that no air bubbles were trapped during this process. This sandwich was then placed in the Biorad Mini Trans-Blot cell with the Bio-Ice cooling unit. The was run for 12 hours at 30 volts.

Whole protein detection

The efficiency of the transfer and the position of the proteins on the nitrocellulose was detected by 0.2 % Ponceau S (3-hydroxy-4-[2-sulfo-4-(sulfo-phenylazo-]2,7-naphthalene disulfonic acid) in 3 % trichloroacetic acid, 3 % sulfosalicyclic acid. The position of the protein was then marked on the nitrocellulose with a permanent marker for reference. The blot was then rinsed with phosphate buffered saline (PBS).

Blocking non specific binding sites on the blot

The nitrocellulose was blocked after transfer using a solution of 5 % w/v milk powder, and 1 % bovine calf serum in PBS for 1 h at room temperature. The blot was then rinsed with PBS.

Application of the antibodies

Primary antibody: the relevant primary antibody was diluted in PBS and the blot incubated in this solution for 4 hours at room temperature or as instructed by the manufacturer. A complete description of the antibodies used is given in the relevant chapters.

Secondary antibodies: the blot was rinsed 3 times for 15 minutes each in PBS to remove unbound primary antibody. The secondary antibody used was anti-mouse immunoglobulin G (IgG), horse radish peroxidase (HRP) linked ploYclonal antibody (from sheep). This was diluted in PBS and incubated with the blot for 1 hr.

Detection methods

The blot was rinsed 3 times for 15 minutes each in PBS to remove unbound antibody. Three different detection methods were used with varying degrees of success. These were either 3,3'- diaminobenzidine (DAB) (Tsang *et al.*, 1985), or 4-chloro-1-napthol (4CN) (Hawkes *et al.*, 1982) or ECL-chemoluminescence (Amersham Life Sciences).

3,3'-diaminobenzidine (DAB): the developing solution contained 0.6 mg/ml of DAB in Tris-HCl, pH 7.6 plus 1 μl/ml of 30 % hydrogen peroxide. Development occurred between 1 and 5 minutes.

Chloronaphthol (4-chloro-1-naphthol): to 10 ml of 50 mM Tris-HCl pH 7.6, 0.1 ml of stock (300 mg chloronaphthol in 10 ml ethanol) was added plus 1 μl of 30 % H_2O_2. Development was complete after about 30 minutes.

ECL Chemoluminescent Detection: this method was superior to the above methods as it was very sensitive, gave a high contrast signal and could be produced repeatedly on film from the same blot. The developer was an ECL developing kit RPN 2109 (Amersham Life sciences Ltd.) which was

used as instructed by the manufacturer. The blot was placed onto a sheet of plastic wrap, antibody side up. The two solutions in the kit were mixed in equal proportions and poured onto the blot. The blot was wrapped in the plastic wrap and placed into a light proof box in the dark room. In the dark, Kodak X-omat film was placed onto the blot for between 30 seconds and 15 minutes, depending on the strength of the signal. The film was then developed using standard dark room techniques.

2.10 Quantitative Analysis of Gels

Densitometry

Gels stained with Coomassie Blue were scanned at 600 nm using a Shimadzu CS-9000 densitometer. Interpretation of scanned results was performed using NIH Image gel electrophoresis analysis software on the Apple Macintosh.

Estimate of difference

The following method was used to compare the peptide maps of myosin heavy chain(s). Photographs of the gels were photographically enlarged so that the closest two bands were 1 mm apart. The gel was then divided into 1 mm elements and scored according to whether a band was absent or present in that element. In order to compare two lanes, the total number of bands in both lanes (t) and the number of bands unique to each lane (u) were measured. An estimate of difference (D) was given by twice the number of unique bands divided by the total number of bands plus the number of unique bands, i.e. $D = 2u / (t + u)$. Values of D were zero if the banding patterns of the two lanes are identical and 1 if the banding patterns of the two lanes were completely different from each other.

Chapter 3

Temperature and the plasticity of myofibrillar proteins during ontogeny in the Atlantic herring (*Clupea harengus* L.)

3.1 Introduction

"In the middle ages, Amsterdam was said to have been built on the bones of herring," (Pitcher and Hart, 1982).

The Atlantic herring (*Clupea harengus*) is a pelagic schooling fish, which was once the foundation of a huge fishing industry. The population structure of the species is complex. Numerous races, tribes and stocks have been identified which differ in morphological, ecological and behavioural characteristics (Parrish and Seville, 1967; Jørstad *et al.*, 1991). Spawning occurs in shallow water and each stock spawns at a different time of year.

Embryos of Clyde herring stock develop on the sea bed during March each year. As a result of natural climatic variation, the embryos have been subjected to varying seawater temperature from 4.8 °C to 10 °C over the last 40 years (Jones and Jeffs, 1991). The newly hatched larvae of herring are 6-9 mm in total length (TL) and are more dense than sea water and hence they must swim continuously to avoid sinking. They lack gills, which form at around 20 mm TL, and thus the swimming muscles receive oxygen directly through the skin. The larvae are planktonic until they metamorphose at approximately 29 mm TL (De Silva, 1974). Metamorphosis occurs during the late summer when the sea temperature has risen to 12-16 °C.

Many studies have shown that temperature significantly alters the rate and degree of expression of the developmental program in fish embryos

(Blaxter, 1988; Johnston, 1993; Johnston *et al.*, 1996; Johnston *et al.*, 1997). Aspects of the developmental program, which have been shown to be particularly temperature sensitive, include development time (Blaxter and Hempel, 1963; Herzig and Winkler, 1986; Blaxter, 1988; Johnston *et al.*, 1996); body length at hatching (McIntosh and Masterman, 1897; Blaxter and Hempel, 1963; Collins and Nelson, 1993; Nathanailides *et al.*, 1995; Johnston *et al.*, 1995b, 1997), rate of yolk absorption (Jones, 1972; Fukuhara, 1990), growth rate (Kinne and Kinne, 1962; Alderdice and Forrster, 1967; Blaxter, 1988), vertebrae number (Dannevig, 1950; Tanning, 1942), pigmentation (Schmidt, 1919), fin ray number (Pavlov and Moksness, 1996), and innervation of somites (Johnston *et al.*, 1997). Every species has an optimum developmental temperature range outside which the number of deformities and abnormalities increases (Stockard, 1921; Hempel, 1979; Polo *et al.*, 1991). In plaice, Ryland and Nichols (1967) showed that the rearing temperature which produced the most survivors was 8 °C. This was the same temperature at which yolk absorption in embryos and larvae was the most efficient (Ryland *et al.*, 1975). Alderdice and Forrester (1967) demonstrated that eggs of the Pacific cod have an optimum developmental temperature range of 3-5 °C. Within this range a greater number of the eggs survive to hatching and the larvae are larger than those developed at sub optimal temperatures.

Johnston (1993) found that in developing herring, rearing temperature did not affect the relative timing of the appearance of the gut, notochord, eyes and haemocoel. However, formation of the spinal chord, pronephros, pectoral fin buds and muscle fibres, was retarded at lower temperatures with respect to somite stage. Fukuhura (1990) showed the sequence of pectoral fin formation, mouth opening, and pigmentation of the eye, varied with rearing temperature in tropical, marine fish larvae. In contrast, Brooks and

Johnston (1993), found no alteration in the order of appearance of anatomical features during development in the plaice.

Several groups have looked at the effects of rearing temperature upon the musculature of developing fish. For example, Johnston *et al.*, (1995) observed myofibrils at later somite stages in the rostral myotomes of herring embryos incubated at 5 °C, than in those incubated at 12 °C. Furthermore, the number of myoblasts present at hatching was significantly higher at 8 °C than at either 5 °C or 12 °C (Johnston *et al.*, 1997). The volume density and spacial distribution of fibre organelles at hatching has also been found to vary with rearing temperature in herring (Vieira and Johnston, 1992).

There have been many studies on larval muscle structure and function during ontogeny. El-Fiky and Weiser (1988) showed that the aerobic capacity of both the superficial layer of red and inner white muscle fibres decreased during development. Examinations of larval myofibrillar proteins from inner white and superficial red muscle fibres has revealed a gradual ontogenetic expression of characteristic proteins from adult white and red muscle fibres, respectively (Rowlerson *et al.*, 1985; Chanoine *et al.*, 1992; Johnston and Horne, 1994; Martinez and Christiansen, 1994; Mascarello *et al.*, 1995).

The myotomes of herring larvae contain a single, superficial layer of red muscle fibres which surround the larger diameter inner muscle fibres (Vieira and Johnston, 1992). This fibre arrangement has also been observed in the seabream (Matsuoka and Iwai, 1984) and the roach (El-Fiky and Weiser, 1988). In the herring, both fibre types are highly aerobic at hatching (Vieira and Johnston, 1992). Johnston and Horne (1994) showed that the red muscle fibres express myosin light chains (MLC) characteristic of adult

white muscle, and Crockford and Johnston (1993) showed that inner white fibres contained distinct larval myosin heavy chain (MHC) isoforms.

Previous work on herring by Crockford and Johnston (1993) has shown that adult fast muscle, adult slow muscle, and the inner muscle of larvae on the day of hatching, contained unique isoforms of myofibrillar proteins. These authors also demonstrated that the particular combination of myofibrillar proteins present at hatching in white muscle was dependent upon the rearing temperature. The aim of this study was to systematically characterise the myofibrillar proteins present in myofibrils during ontogeny of the Atlantic herring. In addition, to determine whether the transition from larval to adult phenotype was influenced by rearing temperature, and could be correlated with other events during development, such as metamorphosis.

3.2 Materials and Methods

Collection of adult fish

Spring spawning Atlantic herring (*Clupea harengus* L.), mean standard length 32.50 cm (S.D=3.68, n=10) were caught in the Firth of Clyde, Scotland during March of 1995. The gonads were dissected at sea and placed on ice. Gonads and fish were immediately transported to the Dunstaffnage Marine Laboratory, Oban, Scotland.

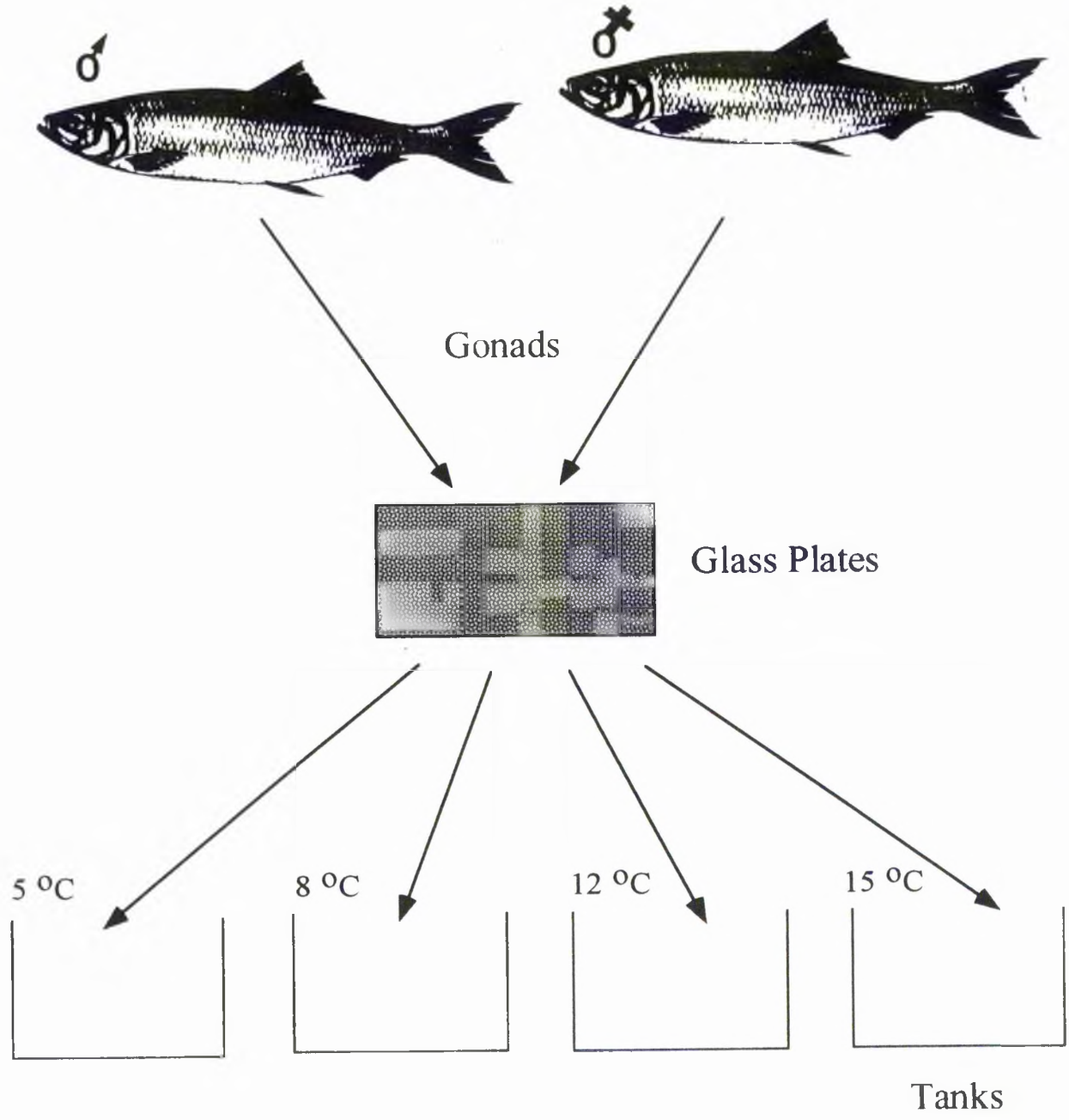
Adult fish muscle sample

The skin was removed from the fish and approximately 2 g of fast muscle was dissected on ice from the myotomes between the lateral line and the dorsal fin. Care was taken to avoid contamination from slow muscle fibres. A 2 g sample of slow muscle fibres was then dissected on ice from the lateral line region. Myofibrils were then prepared as described in Chapter 2.

Production of embryos

The collected gonads were split into two groups and the eggs fertilised 12 hours apart to allow observation of all developmental stages during the working day. The eggs from six female gonads were scattered onto glass plates and slides, submerged in sea water, using a dry spatula. The eggs became sticky in contact with seawater and attached firmly to the glass. Egg density was kept to a maximum of approximately 10 eggs/cm^2 and any clumps of eggs were removed. Male gonads were broken up by hand and pushed through a 0.25 cm^2 fine mesh sieve into sea water to create a sperm solution. To fertilise the eggs, the plates and slides were dipped into the sperm solution for 30 seconds. The eggs were then incubated in black-walled 500 litre tanks with constant sea water

Figure 3.1. A diagrammatic representation of the methods used. Eggs from ripe female herring were spread onto glass plates and fertilised with the sperm of ripe males. Plates were then placed in rearing tanks at controlled temperatures of 5, 8, 12 and 15 °C.


♂
♀
Gonads
Glass Plates
5 °C
8 °C
12 °C
15 °C
Tanks

temperatures of 5 °C, 8 °C, 12 °C and 15 °C (±0.4 °C) (Fig. 3.1). Eggs were subjected to a light regime of 10 hours light and 14 hours dark.

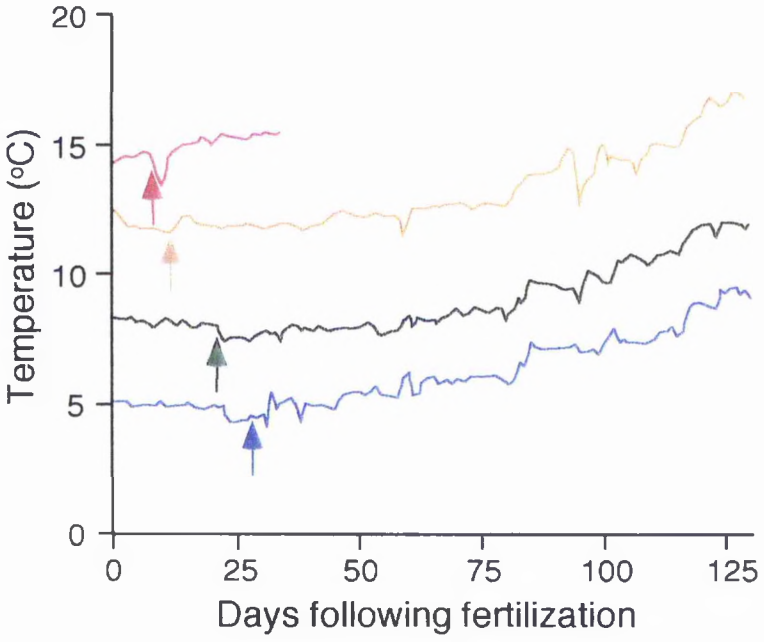
Observation of embryonic development

Living embryos were studied at regular 1-2 hour (h) intervals during development under dark and light field using a Wild M3 stereo microscope. The names of embryonic stages are based on those for zebrafish staging described by Westerfield (1994). Development of embryos was easily observed under the microscope due to the transparent chorion and tissues of the herring. Heating of the embryos during examination was prevented by frequent changes of the sea-water and after observation the slides were returned to the holding tank. Once the embryos had reached the 30-somite stage they were removed from the chorion by dissection with jewellers forceps. The embryo to be observed was anaesthetised in 0.02 % bicarbonate buffered MS222 (ethyl m-aminobenzoate), pH 8.2, in seawater and mounted in glycerol. The number of somites formed at any point in development at any rearing temperature was then counted in samples observed under 40 times magnification. Ten eggs were examined for each sample and the developmental stage of the embryos was recorded against the time since fertilisation. The effect of incubation temperature on the rate of development at each temperature was then calculated.

Larval rearing

Following hatch, the tank temperature of each group was allowed to slowly rise until it reached the ambient seawater temperature in mid-June. This mimics the natural warming of the seawater from spring to summer and improved larval survival. The temperature record for the experiment is shown in Figure 3.2. The groups of fish examined are referred to by their initial tank temperatures. Total fish length (TL) was measured from the snout to the tip of

Figure 3.2. The temperature regimes used to rear embryos and larvae of Atlantic herring. The four traces show the temperature regimes for each of the four groups of larvae with initial rearing temperatures of 5, 8, 12 and 15 °C. The arrows denote the day of hatching.


20
15
10
5
0
Temperature (°C)
0
25
50
75
100
125
Days following fertilization

the primordial or caudal fin. Fish were fed on natural zooplankton and daily to excess with live *Artemia* sp. nauplii beginning a few days after hatching.

Sampling of embryos and larvae

Embryos and larvae were sampled at the end of somitogenesis (60 somite stage), on day of hatching and at 11, 13, 15, 17, 20, 25 and 40 mm total length (TL). The age of the fish (given as days after hatching) at these total lengths are shown in Figure 3.4. To sample the 60 somite stage embryos, eggs were dechorinated with jewellers forceps and the head and yolk sac removed. Approximately 100 embryos were used per sample. Larvae were killed by over anaesthesia in a 0.002% solution of bicarbonate buffered MS222, pH 8.2, in seawater. The yolk sac, head, caudal fin and gut was removed on a cooled microscope stage (0-4 °C). The skin was removed by peeling it off in a rostral to caudal direction. This also removed the superficial muscle layer immediately beneath the skin (Crockford and Johnston, 1993). The remaining muscle was collected for analysis. At least two samples per body size class were collected, and each sample contained 20-30 larvae to give approximately 1 g of tissue. Myofibrils were then prepared as described in Chapter 2.

Electrophoresis of myofibrillar proteins

Myofibrils from larval and adult muscle were analysed, and the proteins identified using the methods described in Chapter 2. The methods used were; one dimensional SDS PAGE, two dimensional iso-electric focusing (2D-IEF) with extended acidic and basic ranges, and peptide mapping. In addition, adult muscle proteins were identified by Western blots developed using antibodies raised against actin, tropomyosin, troponin T and troponin I (Fig. 3.7B-G). Myosin heavy chain digests produced by

peptide mapping were analysed and compared by calculating the estimate of difference (D) as described in Chapter 2.

3.3 Results

3.3.1 Development

Over 90 % of the eggs were fertilised successfully in both groups. A description of the embryonic development of the herring is given below which has been separated into several named stages for clarity. The time taken for embryos reared at each temperature to reach the stage is given.

Fertilisation

A few minutes after the eggs were fertilised they became polar. The animal pole, which contained the cytoplasmic cap, was transparent compared to the vegetal pole which contained the more opaque egg yolk (Fig. 3.3A).

Cleavage

A short time after the egg became polar the cleavage period began, where the cytoplasm divided into individual cells. The first few divisions were at regular intervals but after about the fifth cleavage the divisions were less synchronous. The first cleavage (Fig. 3.3B) occurred just over 2 h post fertilisation (pf) at 12 and 15 °C, 4 h pf at 8 °C, and 6 h pf at 5 °C (Table 3.1). The animal pole cytoplasmic cap divided vertically and along its base, to produce a 2 cell blastodisc, above the yolk cell (Fig. 3.3B).

The second cleavage occurred vertically, but perpendicular to the first division which produced a 4 cell blastodisc (Fig. 3.3C). The third division occurred vertically in each of the four cells to produce an eight cell elliptical blastodisc (Fig. 3.3D). The fourth division occurred in each of the eight cells, perpendicular to the last division, and produced a 16 cell blastodisc (Fig. 3.3E). The fifth cleavage produced 32 cells in a single layer. The sixth cleavage was horizontal and produced two layers of 32 cells, one layer on

top of the other. The sixth cleavage occurred at 7 h pf at 15 °C, 7.5 h at 12 °C, 14 h at 8 °C, and 21 h pf at 5 °C (Table 3.1). This produced a blastodisc with 64 cells (Fig. 3.3F).

The division of the cells in the blastodisc became less synchronous after this point, however an approximation of the number of cells was possible.

Blastula

At approximately 500 cells, the blastula period began (Fig. 3.3G). Blastulation began at approximately 10 h pf at 15 and 12 °C, 20 h at 8 °C, and 30 h at 5 °C. The blastodisc became multi-layered, and the cells continued to divide giving approximately 500, 1000, 2000 and 4000 cells. During these stages the embryos had 'figure of eight' silhouettes.

Epiboly

Eventually the individual cells were no longer visible with a low power light microscope. The blastodisc deformed the yolk from its circular shape and the cells of the blastodisc began to spread over the egg yolk. This was the beginning of epiboly (Fig. 3.3H). Epiboly began at around 15 h pf in 15 and 12 °C-reared fish, 30 h pf at 8 °C, and 55 h pf at 5 °C. As development proceeded, more of the egg yolk was covered with cells from the blastodisc. This allowed any point of epiboly to be named according to the percentage of the yolk which was covered by the animal cells. For example, at 45 % epiboly (Fig. 3.3I), 45 % of the yolk was covered by the animal cells. At this point gastrulation occurred, as the spread of animal cells over the yolk paused and the animal cells thickened around the yolk margin to form 2 layers. Gastrulation occurred at 23 h pf at 15 °C, 24 h pf at 12°C, 45 h pf at 8 °C, and 72 h pf at 5 °C (Table 3.1). Figure 3.3J shows 90 % of the yolk covered by cells. Epiboly ended when 95 % of the yolk was covered by

animal cells at 33 h pf at 15 °C, 34 h pf at 12 °C, 59 h pf at 8 °C, 107 h pf at 5 °C (Table 3.1). Eventually, a single layer of cells, which formed the yolk sac, completely covered all of the yolk. At 95 % epiboly the anterior of the embryo expanded and the dorsal and axial mesoderm began to thicken.

Somitogenesis

After epiboly ended, somitogenesis began, which was marked by the formation of the first somite. Somites formed in an anterior to posterior direction. During somitogenesis the organ systems began to develop. Somite number was determined by counting the number of complete somites present in the embryo. At least five embryos were counted for each data point and the mean number of somites recorded. Somitogenesis ended after approximately 61 somites had developed in each embryo.

The first somite (Fig. 3.3K) was visible at 41 h pf at 15 and 12 °C, 69 h pf at 8 °C, and 120 h pf at 5 °C. The eye fold was visible at this point. The sixth somite (Fig. 3.3L) formed at 45 h pf at 15 and 12 °C, 75 h at 8 °C, and 130 h at 5 °C. The tenth somite (Fig. 3.3M) formed at 50 h pf at 12 and 15 °C, 85 h pf at 8 °C and 135 h pf at 5 °C. The anterior and posterior of the embryo were now clearly defined and the eye and otic vesicle became apparent. At the 20 somite stage (Fig. 3.3N) the lens of the eye began to form. The 32 somite (Fig. 3.3O) formed 70 h pf at 15 and 12 °C, 130 h pf at 8 °C and 190 h at 5 °C. At this stage the lens was clearly defined, and the otoliths were visible. In addition, myotubes developed in the anterior somites and the hatching glands were first visible on the head. The tail bud formed and new somites formed posteriorly, extending the tail bud away from the yolk instead of around it.

Forty somites (Fig. 3.3P) formed 75 h pf at 12 and 15 °C, 150 h pf at 8 °C, and 215 h pf at 5 °C. At this point the heart and presumptive dorsal aorta were first visible.

At the point where the 50th somite formed (Fig. 3.3Q) there was occasional movement, the heart began to beat and the anal pore was visible. The 50th somite formed at 95 h pf at 15 and 12 °C, 170 h pf at 8 °C, and 245 h pf at 5 °C.

When the final somite formed the embryo was long enough for the tip of the tail to touch the head (Fig. 3.3R). Somites now extended posterior to the tip of the tail. The eye began to be pigmented and hatching glands covered the head. The organ systems began to develop, circulation of fluid from the heart was visible and the embryo moved regularly within the chorion. Somitogenesis ended with the formation of the final somite 12 h pf at 12 and 15 °C, 200 h pf at 8 °C, and 300 h pf at 5 °C.

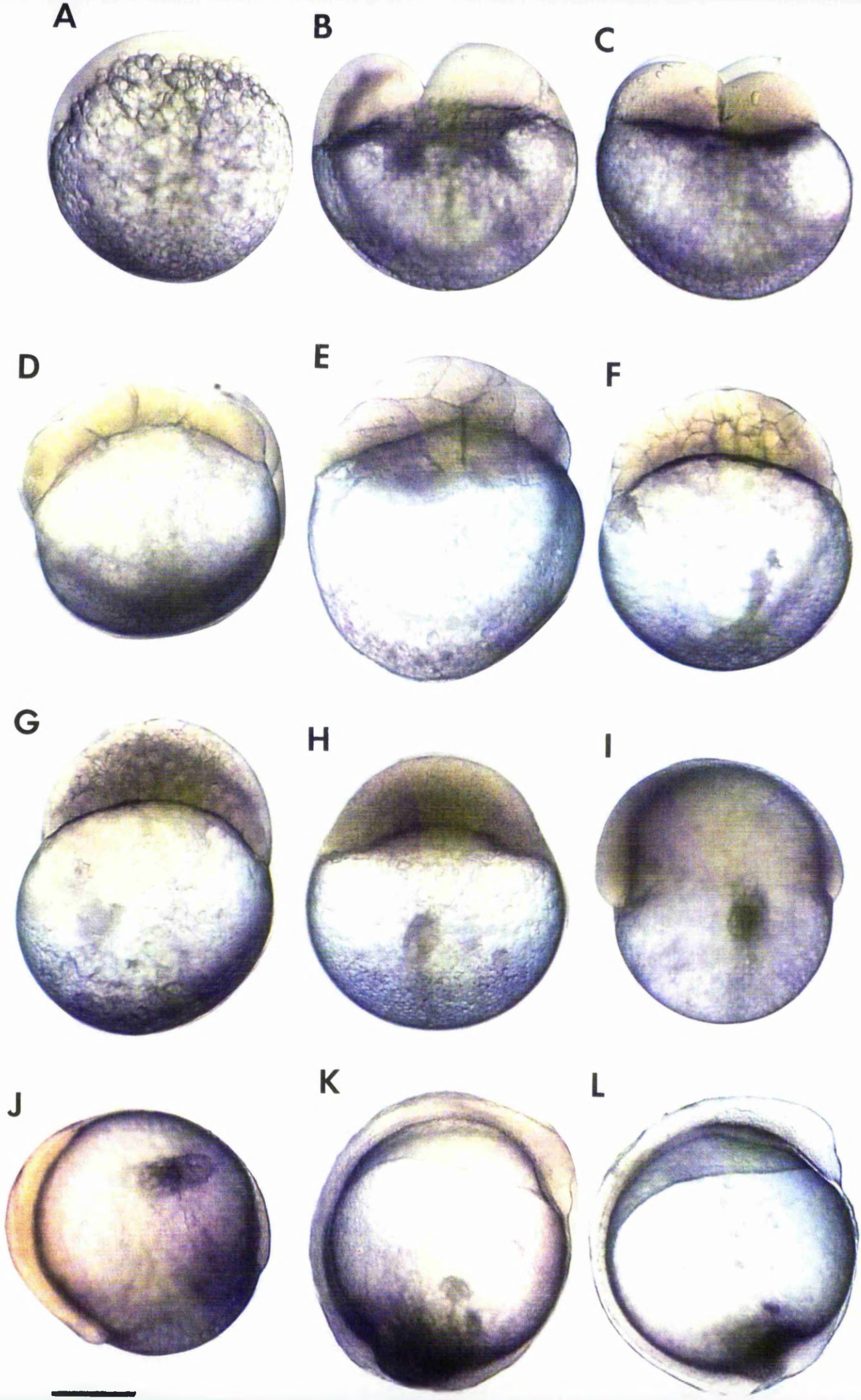
Organogenesis

Before hatch there was a period of organogenesis during which the organs developed. The eye became fully pigmented with melanin and the otoliths became anchored to the otic vesicle. Flagella became visible in the gut under high power, and the gut became pigmented and fully looped before it connected to the buccal cavity and opening at the anus.

Hatching

Larvae hatched (Fig. 3.3S) relatively earlier in fish incubated at higher temperature. Embryos hatched 8 days after fertilisation at 15 °C, 9 days pf at 12 °C, 16 days pf at 8 °C, and 28 days pf at 5 °C (Table 3.1).

Figure 3.3. Stages of development of Atlantic herring from fertilisation to early somitogenesis. Scale bars = 0.5 mm (A) One cell stage, (B) 2-cell stage (1st cleavage), (C) 4-cell stage (2nd cleavage), (D) 8-cell stage (3rd cleavage), (E) 16-cell stage (4th cleavage), (F) 64-cell stage (6th cleavage), (G) approximately 512-cell stage, mid-blastula, (H) dome, late blastula, epiboly begins, (I) 45 % epiboly, gastrulation begins, (J) 90 % epiboly, (K) first somite, somitogenesis begins, (L) 6 somite, (M) 10 somites, (N) 20 somites, (O) 32 somites, (P) 40 somites, (Q) 50 somites, (R) 62 somites (final somite formed), (S) hatched larvae.


A
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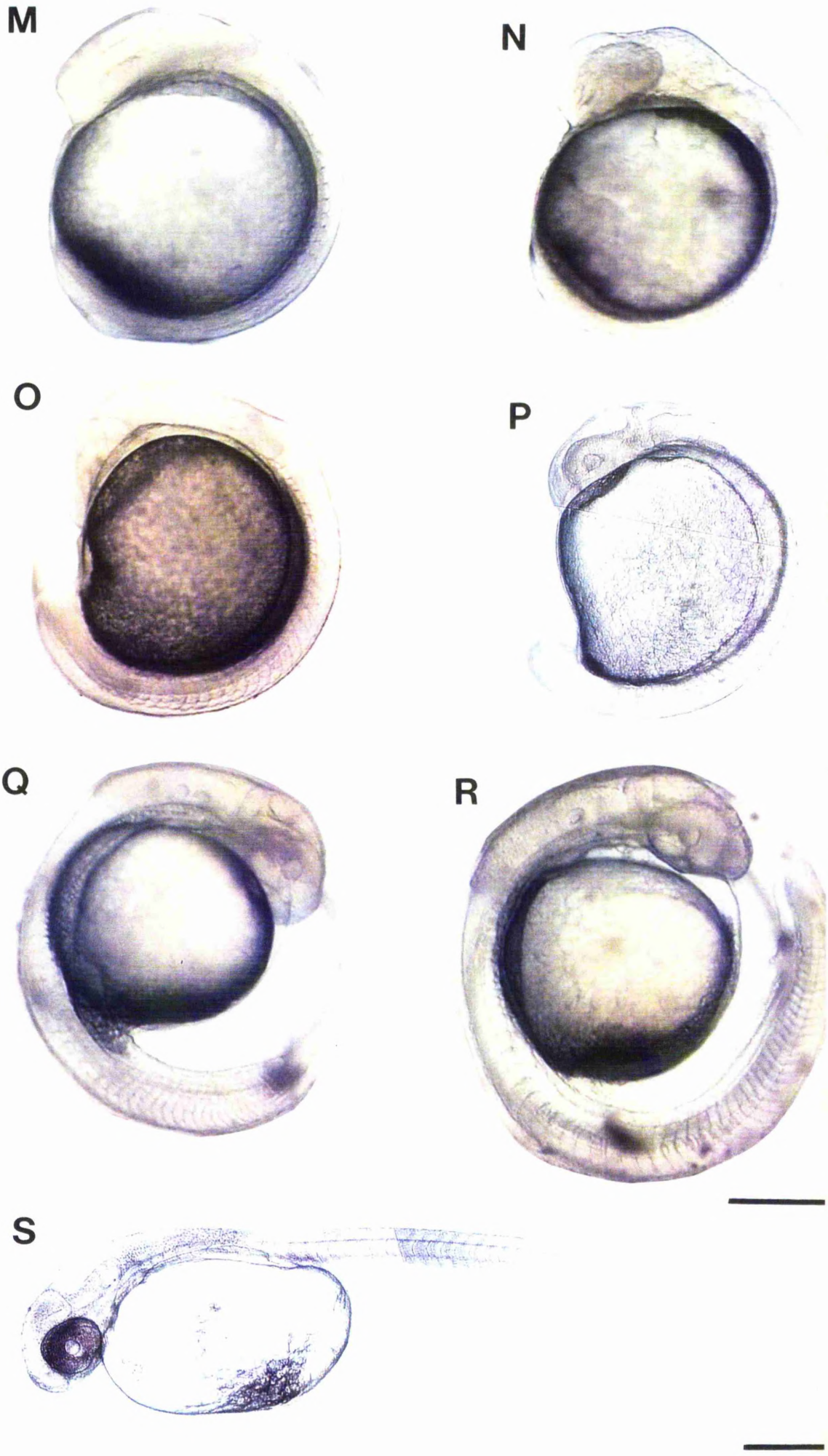
K
L


M
N
O
P
Q
R
S

Rearing temperature was found to greatly influence the rate of development (Table 3.1). The higher the incubation temperature the greater the rate of development. In addition, the rate of somitogenesis was highly temperature dependent (Fig. 3.4). Johnston *et al.* (1995) showed that in these fish, somites were formed at a rate of one every 52 minutes at 15 °C, 65 minutes at 12 °C, 69 minutes at 8 °C and 120 minutes at 5 °C.

The influence of temperature upon the rate of herring development is perhaps best illustrated in Figure 3.5. This shows herring embryos reared at 5 °C, 8 °C and 12 °C. Each fish has developed for exactly 96 hours at each temperature and is at a very different stage of development (Fig. 3.5A-C). The 5 °C-reared embryo is at 80 % epiboly (Fig. 3.5A), the 8 °C embryo has 20 somites (Fig. 3.5B), and the 12 °C embryo has 52 somites (Fig. 3.5C).

3.3.2 Electrophoresis of Adult Muscle Proteins

In order to analyse the proteins present in larval herring and any changes in isoform expression during ontogeny, the proteins present in adult white and red muscle were first identified.

Myosin heavy chain

The myosin heavy chains (MHCs) of adult white and red muscle were clearly visible as a dense band at the top of the gels (Fig. 3.6). Each protein had a similar molecular mass of approximately 200 kDa. (Fig. 3.6). Peptide digests of adult white and adult red MHC produced very different banding patterns with each enzyme (Fig. 3.7A-D). It was apparent by simple observation of the gels that the MHC banding pattern produced by digestion was different for red and white muscle, indicating that they are different isoforms.

Figure 3.4. Plot of somite number against developmental time showing the rate of somitogenesis of herring larvae reared at 5, 8, 12 and 15 °C. Values are mean ± S.D., N = 15-20 fish at each temperature.

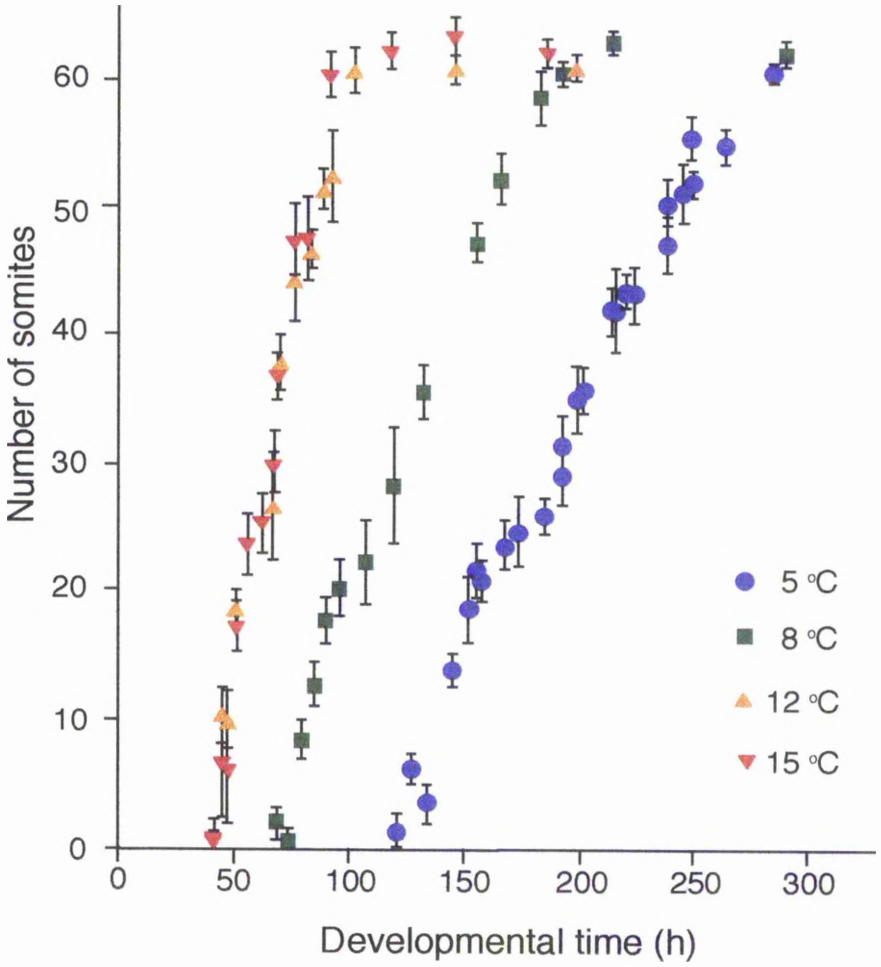

Number of somites
0
10
20
30
40
50
60
0
50
100
150
200
250
300
Developmental time (h)
5 °C
8 °C
12 °C
15 °C

Figure 3.5. Illustration of variation in development rate with rearing temperature. The photographs show herring embryos which were fertilised at the same time, but were reared at different temperatures 96 hours after fertilisation. (A) 5 °C (80 % epiboly), (B) 8 °C (23 Somites), (C) 12 °C (55 somites). Scale bar = 0.5 mm.

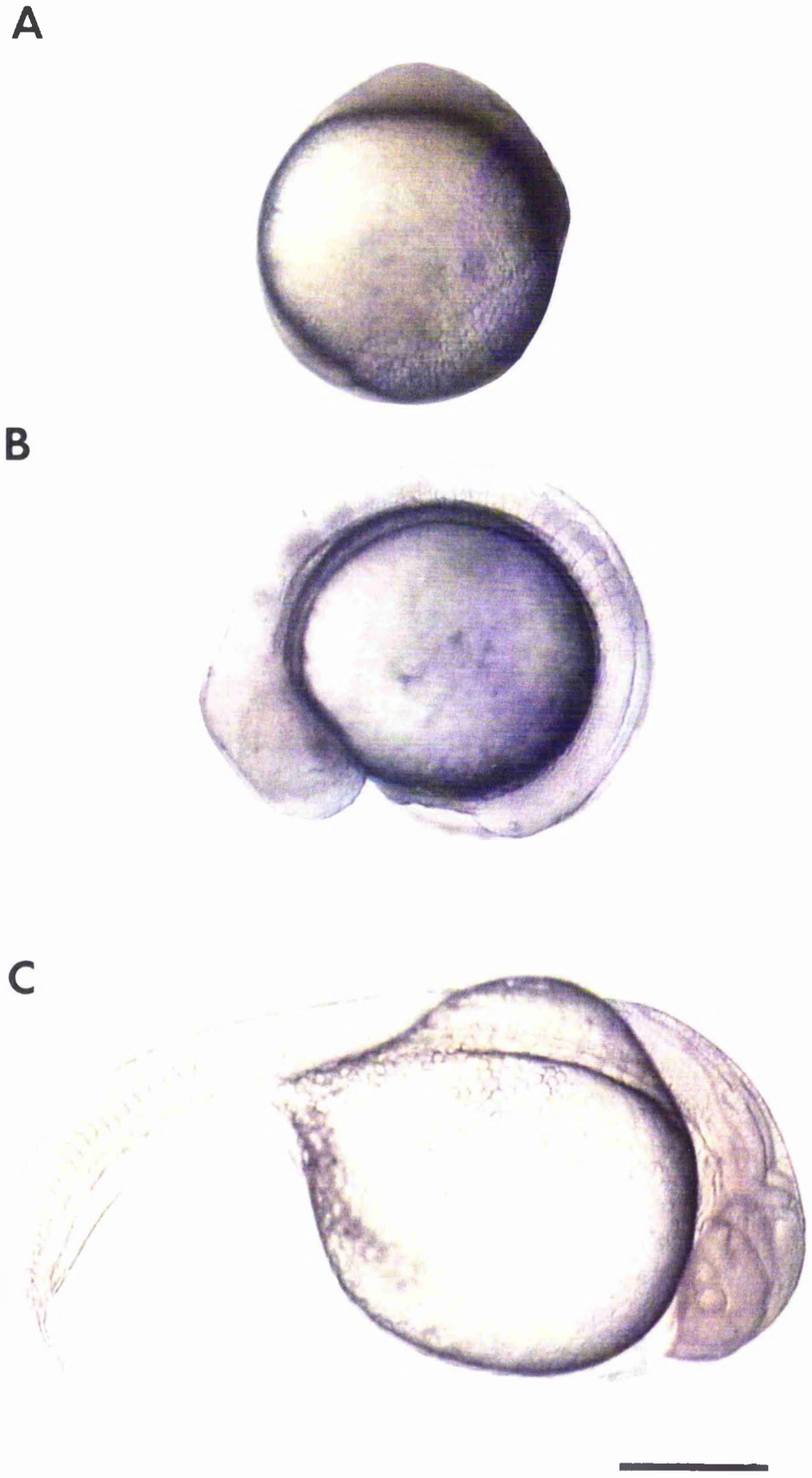

A
B
C

Table 3.1. Table which shows the number of hours taken for embryos at each rearing temperature to reach the developmental stages shown in Fig. 3.3A-S.

		Rearing Temperature (Degrees Celsius)			
		5	8	12	15
Developmental Stage	**Illustrated in Figure 3.3**	**Hours post fertilisation**			
Zygote Period					
1 cell	A	0	0	0	0
Cleavage Period					
2 cell	B	6	4	2.25	2.25
4 cell	C	9	6	3	3
8 cell	D	12	8	4.25	4
16 cell	E	15	10	5.5	5
32 cell		18	12	8	6.25
64 cell	F	21	14	8.5	7
128 cell		24	17	9	8
Blastula Period					
512 cell	G	30	20	10.25	10
4000 cell		46	26	13.5	13
dome	H	55	30	15.25	15
10 % epiboly		60	33	16.5	16
50% epiboly	I	72	45	24	23
90% epiboly	J	100	56	32	31
100% epiboly		107	59	34	33
Segmentation Period					
1st somite	K	120	69	41	40
6 somites	L	125	75	46	44
10 somites	M	136	83	50	47
20 somites	N	155	100	61	55
32 somites	O	196	123	70	61
40 somites	P	214	141	78	69
50 somites	Q	240	163	90	74
62 somites	R	276	180	104	90
hatching	S	28 days	16 days	9 days	8 days

Actin

The actin of adult white and adult red muscle had the same relative molecular mass of approximately 44 kDa. This was shown by one dimensional SDS PAGE (Fig. 3.6A) and by Western blotting (Fig. 3.6B, lanes 2-3).

Tropomyosin

The tropomyosin (Tm) from red and white muscle differed in both molecular mass and iso-electric point. This was shown by one dimensional SDS PAGE (Fig. 3.6A) and by Western blotting using a Tm antibody (Fig 6B, lanes 4-5) Two dimensional gels (iso-electric focusing in the first dimension followed by SDS PAGE) (Fig. 3.8A-C) revealed that red and white muscle Tm were acidic proteins. White muscle contained a single Tm isoform of molecular mass 40 kDa. Red muscle contained two Tm isoforms of molecular masses 38 and 37.8 kDa. Red muscle Tm isoforms were not expressed in equal proportions and varied in iso-electric point (Fig. 3.8C)

Troponin T

One dimensional SDS PAGE gels (Fig. 3.6A) and Western blots reacted with troponin T (TnT) monoclonal antibody which were stained with either chloronapthanol (Fig. 3.6C) or ECL chemiluminescence (Fig. 3.6G), revealed that the TnT isoforms present in red and white muscle were different. White muscle contained a single isoform of TnT with a molecular mass of 32 kDa, whereas red muscle contained two isoforms of TnT with molecular masses of 30 and 32 kDa (Fig. 3.6C). The two dimensional gel (Fig. 3.9) showed only a single isoform of white muscle TnT with a basic iso-electric point.

Troponin I

Red and white muscle Troponin I (TnI) differed in relative molecular mass (Fig. 3.6A, D). White muscle TnI had a molecular mass of 22 kDa whereas red muscle had a molecular mass of 20 kDa (Fig. 3.6A). Two dimensional gels (Fig. 3.9) showed that there were two TnI isoforms in white muscle. These isoforms had the same molecular mass but differed in iso-electric point.

Myosin alkali light chains

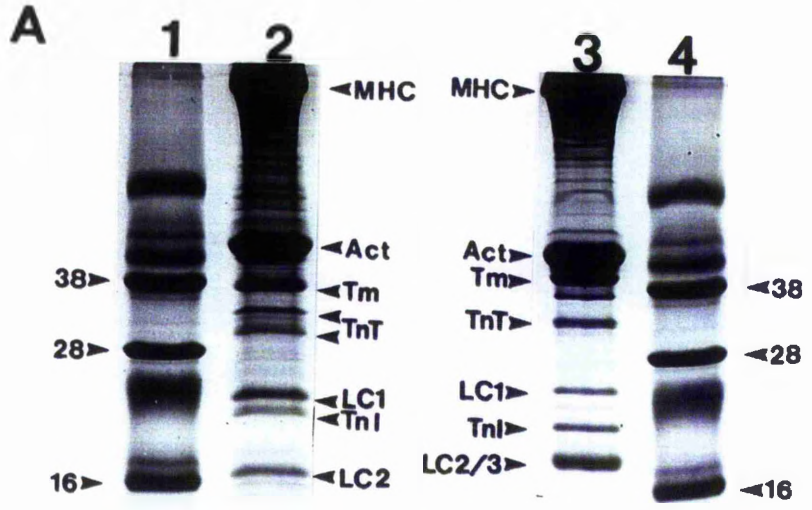
White muscle myofibrils contained a single isoform of myosin light chain 1 (LC1) which had a molecular mass of 25 kDa (Fig. 3.6A, 3.8A). Red muscle had a single LC1 which had a molecular mass of 23.5 kDa (Fig. 3.6A, 3.8C).

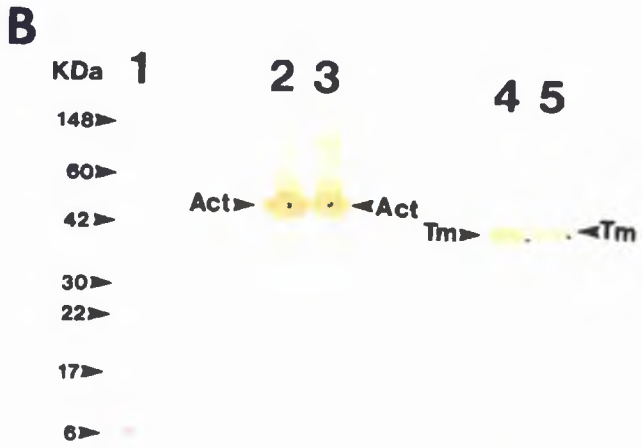
Myosin light chain 3 (LC3) was present as a single isoform in white muscle (Fig. 3.6A, 3.8A). It had a molecular mass of 19 kDa and had the most acidic iso-electric point of all the proteins examined (Fig. 3.8A). Red muscle did not posses a third light chain (Fig. 3.6A, 3.8C).

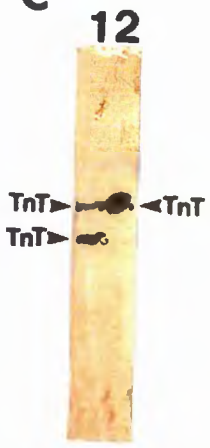
Regulatory myosin light chain

White muscle contained two isoforms of myosin light chain two (LC2) (Fig. 3.8A). Each isoform had a relative molecular mass of approximately 19 kDa (Fig. 3.6A, 3.8A). The isoforms were expressed in relatively different amounts. The major isoform had a more basic iso-electric point than the minor one (Fig. 3.8A). Red muscle contained a single LC2 isoform. (Fig. 3.8C). This isoform had a molecular mass of 17 kDa and a more basic iso-electric point than both of the white muscle LC2 isoforms.

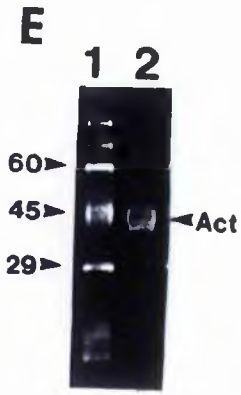
Figure 3.6. (A) 13 % SDS polyacrylamide gel of red and white muscle myofibrils from adult herring run together with molecular mass markers. Gel was stained with Coomassie blue G-250; lane 1, molecular mass markers; lane 2, red muscle myofibrils; lane 3, white muscle myofibrils; lane 4, molecular mass markers. (B) Western blots of 13 % SDS polyacrylamide gel of adult fast and slow muscle reacted with actin antibody and tropomyosin antibody stained with DAB; lane 1, colour mark prestained molecular mass marker; lane 2, red muscle myofibrils reacted with actin antibody; lane 3, white muscle myofibrils reacted with actin antibody; lane 4, red muscle myofibrils reacted with tropomyosin antibody; lane 5, white muscle myofibrils reacted with tropomyosin antibody. (C) Western blots of 13 % SDS polyacrylamide gel of adult fast and slow muscle reacted with troponin T antibody and stained with chloronaphthanol; lane 1, red muscle myofibrils reacted with troponin T antibody; lane 2, white muscle myofibrils reacted with Troponin T antibody. (D) Western blots of 15 % SDS polyacrylamide gel of adult fast and slow muscle reacted with troponin I antibody and stained with DAB; lane 1, red muscle myofibrils reacted with troponin I antibody; lane 2, white muscle myofibrils reacted with Troponin I antibody. (E) Western blots of 13 % SDS polyacrylamide gel of biotinylated molecular mass marker and adult fast muscle reacted with actin antibody and stained with ECL luminescence onto X-ray film; lane 1, biotinylated molecular mass marker; lane 2, white muscle myofibrils reacted with actin antibody. (F) Western blots of 13 % SDS polyacrylamide gel of biotinylated molecular mass marker and adult fast muscle reacted with tropomyosin antibody and stained with ECL luminescence onto X-ray film; lane 1, biotinylated molecular mass marker; lane 2, white muscle myofibrils reacted with tropomyosin antibody. (G) Western blots of 13 % SDS polyacrylamide gel of biotinylated molecular mass marker and adult fast muscle reacted with troponin T antibody and stained with ECL luminescence onto X-ray film; lane 1, biotinylated molecular mass marker; lane 2, white muscle myofibrils reacted with troponin T antibody. (H) One dimensional 13 % SDS PAGE gel of adult fast muscle and chromatographically purified troponin I from adult fast muscle. The gel was stained with Coomassie blue G250; lane 1, adult fast muscle; lane 2, chromatography purified troponin I. MHC, myosin heavy chain; Act, actin; Tm, tropomyosin; TnT, troponin T; LC1, myosin light chain 1; TnI, troponin I; LC2/3, myosin light chain 2 and myosin light chain 3. Molecular masses are given in kDa.

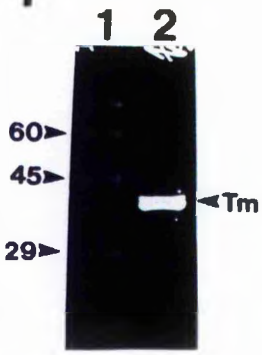

A
1
2
3
4
MHC
Act
Tm
TnT
LC1
TnI
LC2
38
28
16
LC2/3

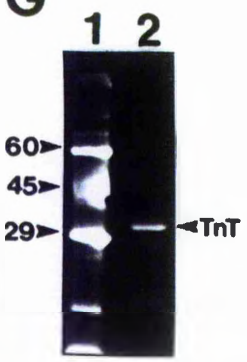

B
KDa
1
2 3
4 5
148
60
42
30
22
17
6
Act
Tm


C
12
TnT


D
1 2
TnI


E
1 2
60
45
29
Act


F
1 2
60
45
29
Tm


G
1 2
60
45
29
TnT


H
1 2
TnI

Figure 3.7 Peptide maps on one dimensional 15 % SDS-PAGE gels of purified myosin heavy chains (MHCs) from Atlantic herring; lane 1, adult white muscle; lane 2, adult red muscle. The arrowheads indicate bands which are unique to each lane. The gels are silver stained. (A) MHCs digested with bacterial V8 protease, (B) with α-chymotrypsin protease, (C) with papain protease, (D) with trypsin protease.

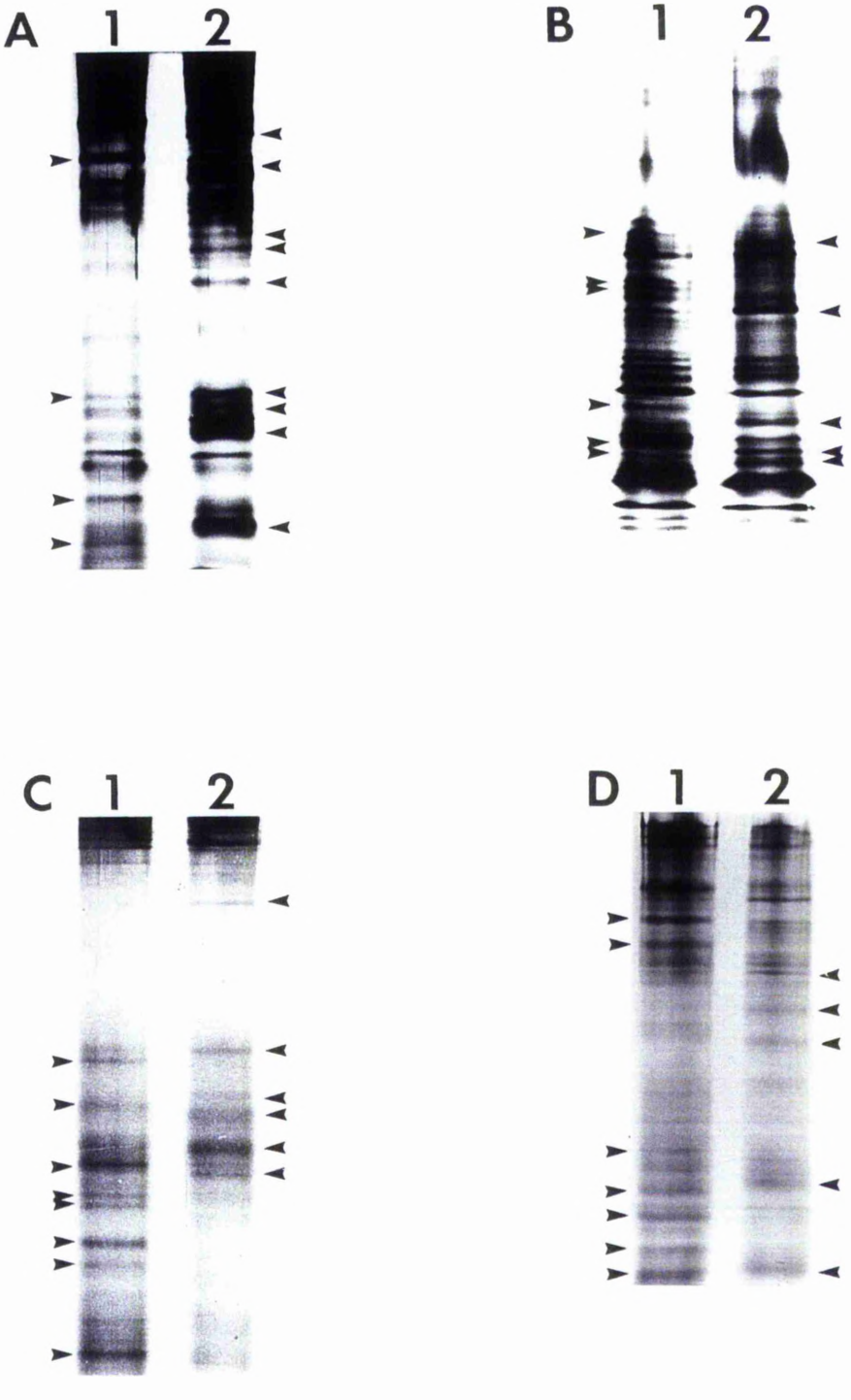

A
1
2
B
1
2
C
1
2
D
1
2

Figure 3.8. Two dimensional PAGE of acidic myofibrillar proteins. Iso-electric focusing in the first dimension and 14 % acrylamide SDS PAGE in the second dimension. (A) White muscle myofibrils from adult herring. Molecular mass markers are shown on the left edge of the gel. (B) A mixture of adult white and adult red muscle myofibrils from adult herring. Molecular mass markers are shown on the left edge of the gel. Downward-facing arrowheads indicate adult white muscle proteins. Upward-facing arrowheads indicate adult red muscle proteins. (C) Red muscle myofibrils from adult herring. Red muscle myofibrils are run on the left edge of the gel. Tm, tropomyosin; LC1, myosin light chain 1; LC2, myosin light chain 2; LC3, myosin light chain 3. Molecular masses show are in KiloDaltons. The gels were stained with Coomassie Blue G-250.

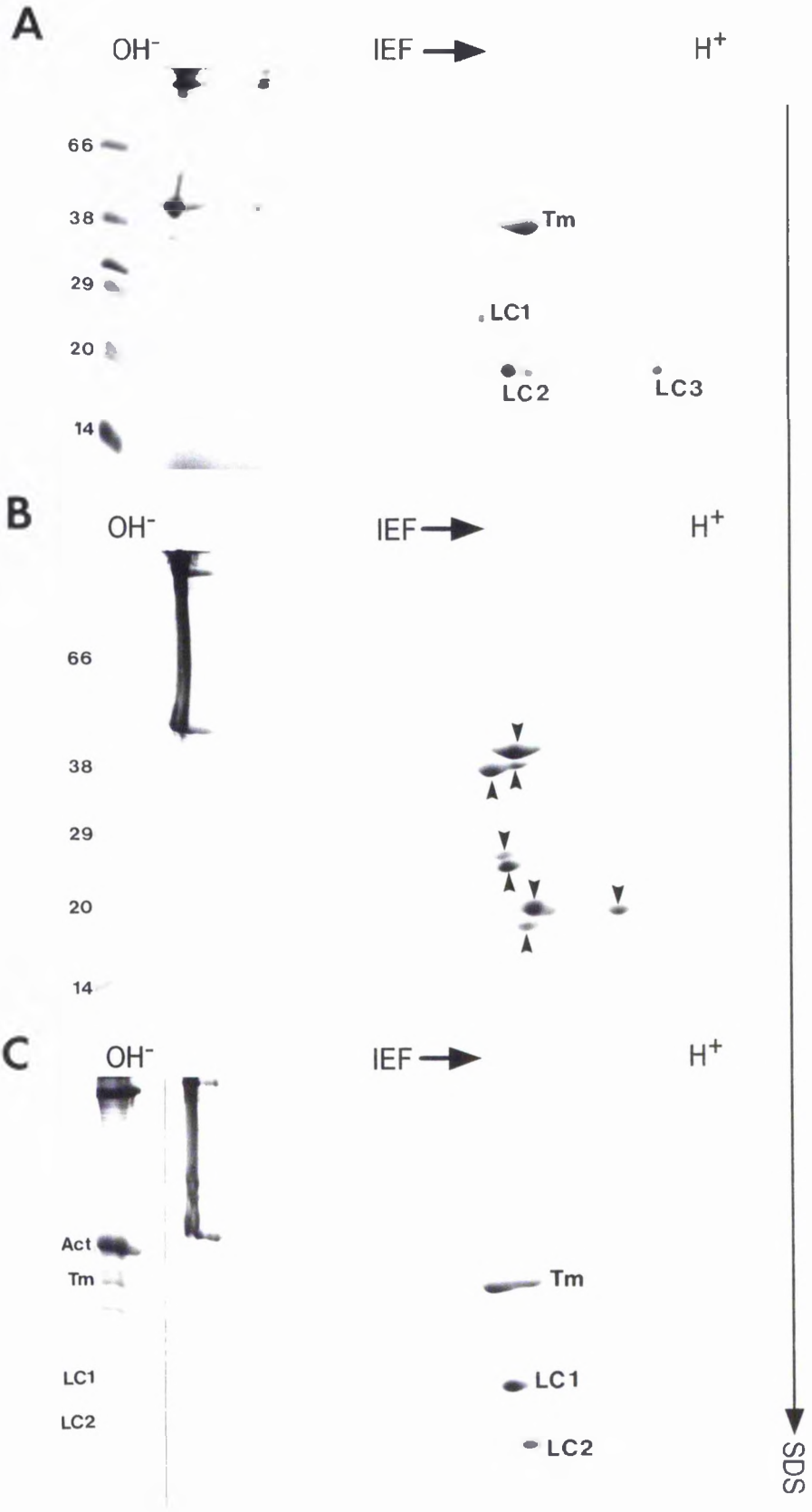

A
OH⁻
IEF
H⁺
66
38
29
20
14
Tm
LC1
LC2
LC3
B
OH⁻
IEF
H⁺
66
38
29
20
14
C
OH⁻
IEF
H⁺
Act
Tm
LC1
LC2
Tm
LC1
LC2
SDS

Figure 3.9. Two dimensional PAGE of white muscle myofibril basic proteins from adult herring. Iso-electric focusing in the first dimension and 14 % acrylamide SDS PAGE in the second dimension. The gels were stained with Coomassie Blue G-250. Molecular mass markers are shown on the left edge of the gel. Molecular masses show are in KiloDaltons. TnT, troponin T; TnI, troponin I.

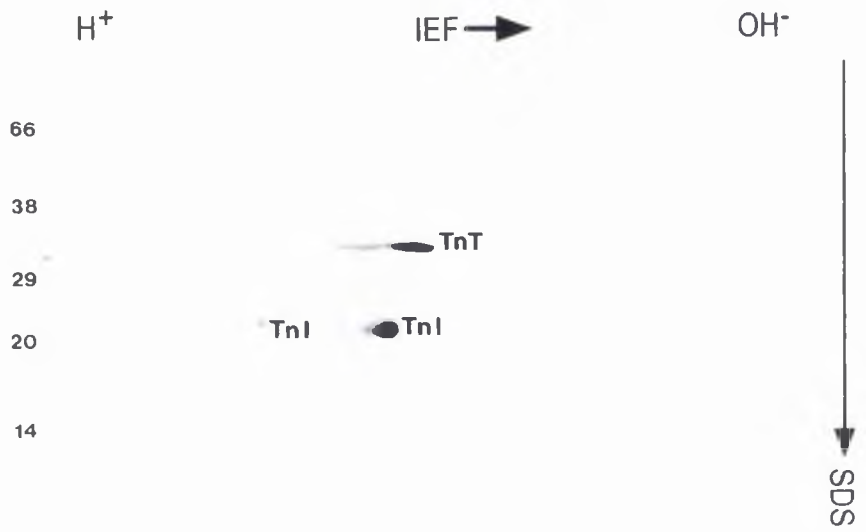

H+
IEF
OH-
66
38
29
20
14
TnT
TnI
TnI
SDS

Table 3.2. Table which shows the relative molecular masses of the myofibrillar proteins examined in adult red and white myotomal muscle.

Protein	Red Muscle	White Muscle
Myosin heavy chain	200	200
Actin	44	44
Tropomyosin	38, 37.8	40
Troponin T	32, 30	32
Light chain 1	23.5	25
Troponin I	22	20
Light chain 2	17	19
Light chain 3		19

Troponin C

Troponin C (TnC) was not investigated in this study. Crockford and Johnston (1993) reported no difference between the troponin C of adult fast muscle and the inner muscle of larval herring.

The myofibrillar proteins of adult herring red and white muscle had now been identified and characterised. The molecular masses of each protein from red and white muscle are given in Table 3.2. This information was then used to systematically identify the myofibrillar protein composition of the inner presumptive white muscle of embryos and larvae reared at different temperatures.

3.3.3 Electrophoresis of Embryonic and Larval Myofibrils

The larvae sampled are shown in Figure 3.10. The actual lengths of the fish after hatching at each temperature are shown in Figure 3.11. The rates of growth at each temperature showed very little difference (Fig. 3.11). The 15 °C-reared fish died approximately 4 weeks after hatching, the exact reason for this was not known. However, 4 weeks provided enough sampling time to follow the expression of light chains and troponins through from the larval to the adult pattern.

A large number of developmental-stage-specific isoforms were found. Where necessary, isoforms of the embryo, larval and adult stages have been given the subscripts e (for embryonic), l (for larval) and f (for fast muscle from adult), respectively. Where multiple isoforms are present each isoform is numbered corresponding to their relative iso-electric point (pI) with 1 representing the most acidic isoform.

Figure 3.10. Diagrammatic representations of the fish sampled. (A) 60 somite embryo, (B) larvae on day of hatching (8.5 mm), (C) larvae at first feeding (13 mm), (D) 17 mm larvae, (E) 20 mm larvae, (F) 25 mm larvae, (G) 40 mm juvenile just after metamorphosis.

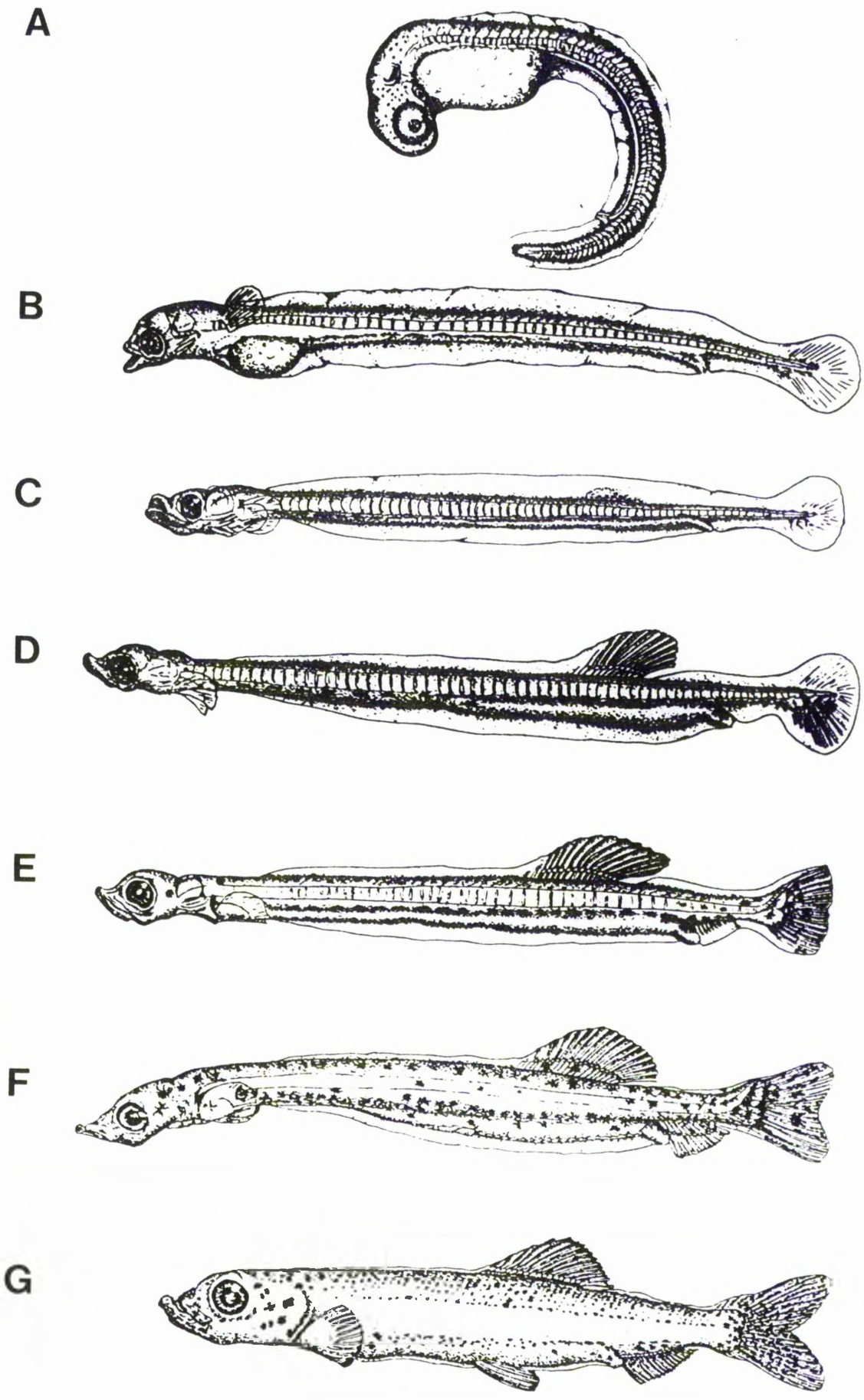
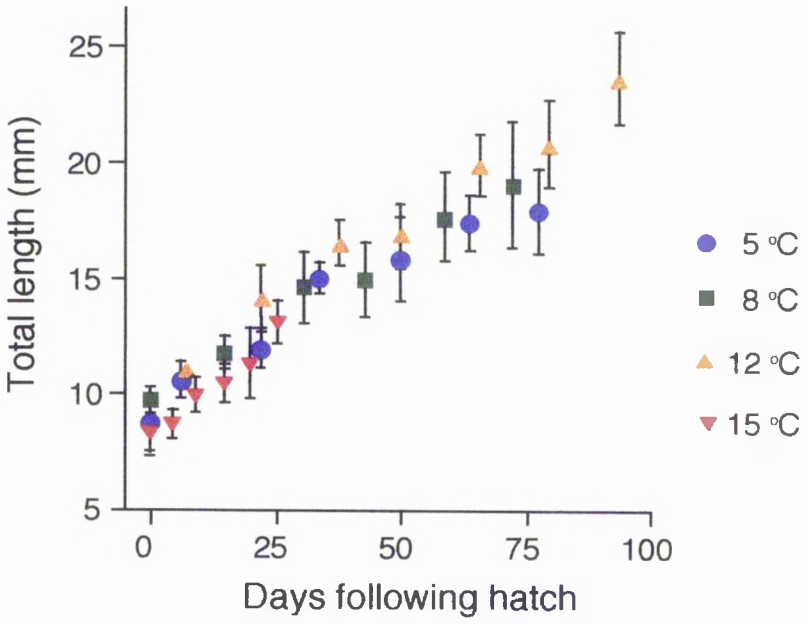

A
B
C
D
E
F
G

Figure 3.11. Graph of herring larvae total lengths when reared at 5 °C, 8 °C, 12 °C and 15 °C. Time zero represents hatching. Values represent mean live total length ± S.D., N = 20 larvae per sample.

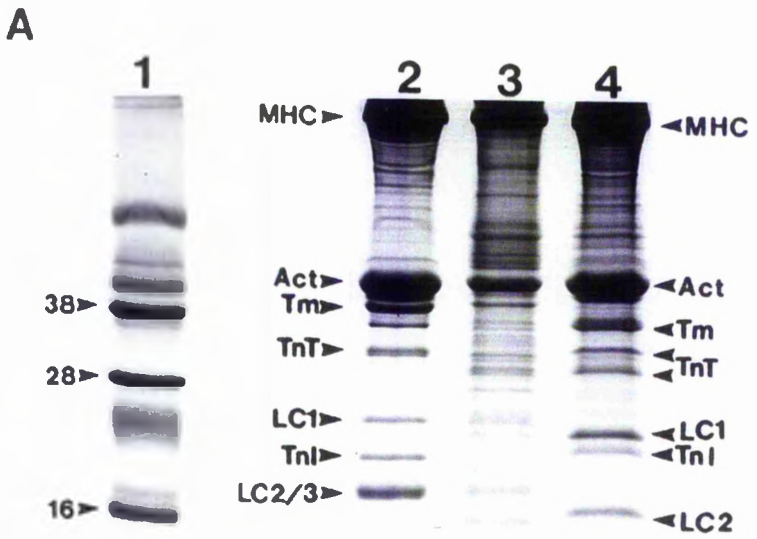

Total length (mm)
25
20
15
10
5
0
25
50
75
100
Days following hatch
5 °C
8 °C
12 °C
15 °C

SDS-PAGE gels of well washed myofibrils from 60-somite embryos revealed a large number of unknown minor bands compared to adult white and adult red muscle (Fig. 3.12A, lanes 2-4). The number of these additional bands gradually reduced during ontogeny and bands corresponding to the adult proteins predominated (Fig. 3.12B). This general trend was observed at all rearing temperatures. The myofibrillar proteins expressed during the development of 8 °C-reared fish are shown in Figure 3.12B. One dimensional SDS PAGE did not indicate with sufficient clarity the influence of rearing temperature upon the timing of these isoform changes. Therefore, the effect of rearing temperature on myofibrillar protein composition was further investigated by peptide mapping and two-dimensional electrophoresis.

Myosin heavy chain(s)

MHCs were electrophoretically purified from adult red, larval, and adult white muscle. Peptide maps of MHCs produced by digestion with papain (Fig. 3.13A), endoprotease Glu-C (Fig. 3.13B), α-chymotrypsin (Fig. 3.13C) and clostripain (Fig. 3.13D) were different between adult red and white muscles (compare lanes 6 and 7) with an estimate of difference (D) of 0.75. The embryonic white muscle fibres in 1 day old larvae contained a distinct isotype of MHC from that found in adult white muscle (compare lanes 1 and 6, Fig. 3.13A-D) (D = 0.28). The transition from the embryonic to the adult pattern of MHC expression in white muscle occurred between 20 and 25 mm TL. Peptide maps of MHCs prepared from the white muscle of larvae 15 mm TL (lane 2) and 20 mm (lane 3) were identical to that of the embryonic MHC isoform(s) (lane 1) (D = 0). Peptide maps of MHCs from 25 mm TL (lane 4) and 40 mm TL juveniles (lane 5) were identical to the adult pattern (lane 6) (D = 0) (Fig. 3.13, lanes 4-6).

Figure 3.12. (A) 13 % acrylamide one-dimensional SDS PAGE gel of herring muscle myofibrils; lane 1, molecular weight marker; lane 2, adult white muscle; lane 3, presumptive white muscle from 60 somite embryo incubated at 8 °C; lane 4, adult red muscle. (B) 13 % acrylamide one-dimensional SDS PAGE gel of myofibrils from embryonic and larval herring reared at 8 °C; lane 1, molecular weight marker; lane 2, presumptive white muscle from 60 somite embryos; lane 3, presumptive white muscle from larvae on day of hatching; lane 4, presumptive white muscle from 13 mm larvae; lane 5, presumptive white muscle from 17 mm larvae; lane 6, presumptive white muscle from 20 mm larvae; lane 7, presumptive white muscle from 25 mm larvae; lane 8, presumptive white muscle from 40 mm larvae; lane 9, adult white muscle myofibrils. MHC, myosin heavy chain; Act, actin; Tm, tropomyosin; TnT, troponin T; LC1, myosin light chain 1; TnI, troponin I; LC2, myosin light chain 2; LC2/3, myosin light chain 2 and myosin light chain 3. Molecular masses are given in kDa. The gels were stained with Coomassie Brilliant Blue G250.


A
1
2
3
4
MHC
MHC
Act
Act
Tm
Tm
TnT
TnT
LC1
LC1
TnI
TnI
LC2/3
LC2
38
28
16

B

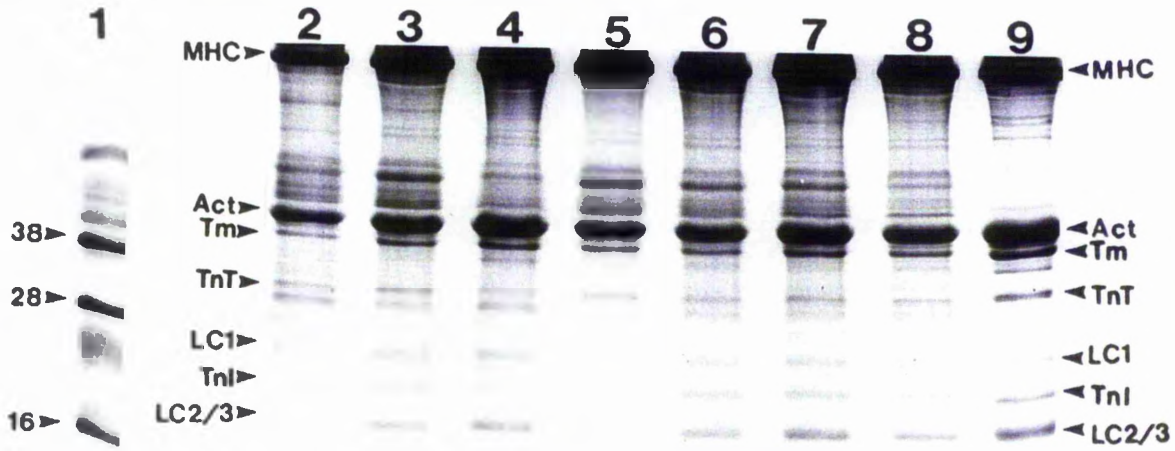
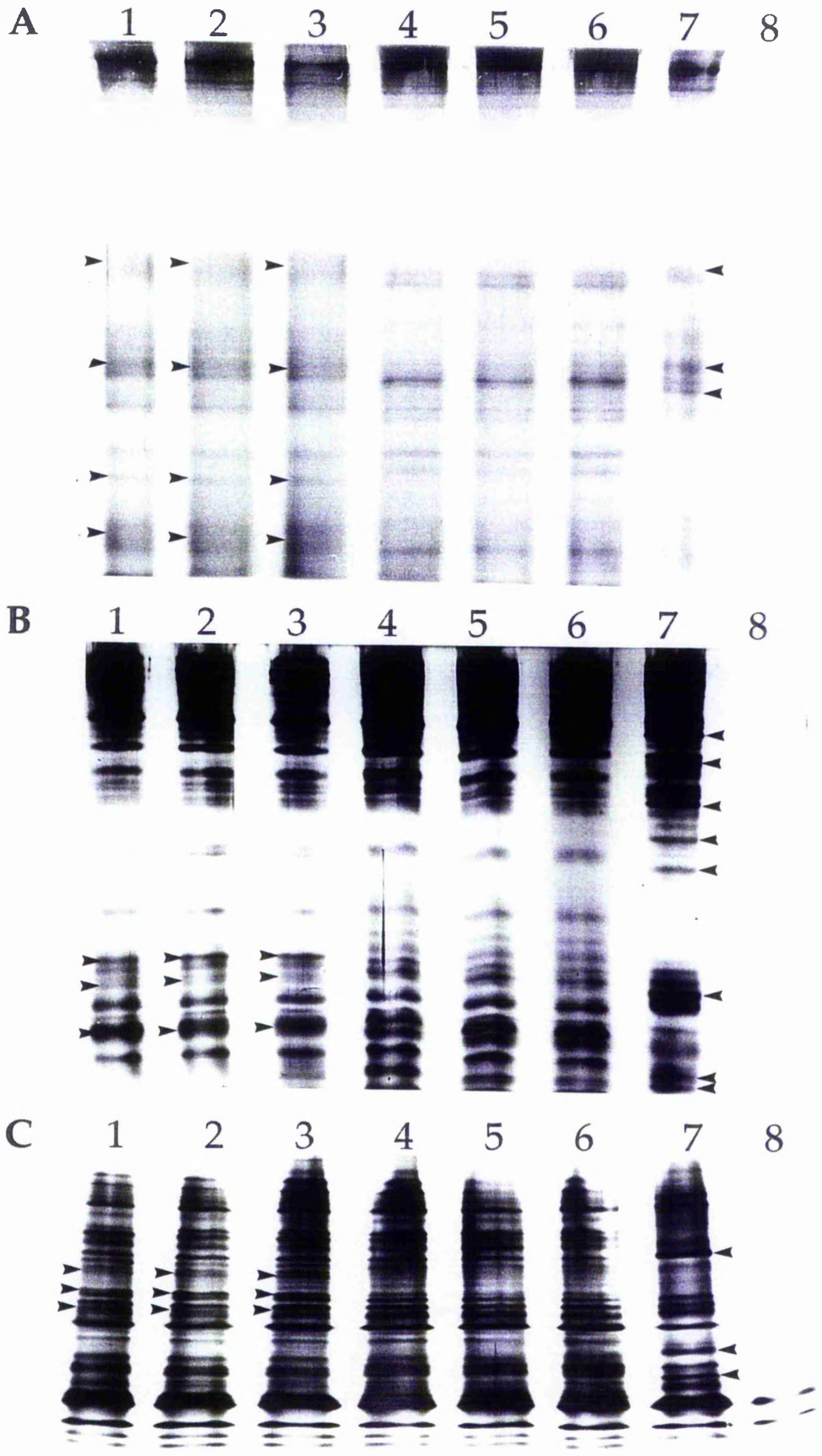

1
2
3
4
5
6
7
8
9
MHC
Act
Tm
TnT
LC1
TnI
LC2/3
38
28
16
MHC
Act
Tm
TnT
LC1
TnI
LC2/3

Figure 3.13. Peptide maps, resolved on 15 % acrylamide SDS PAGE gels, of myosin heavy chains (MHCs) purified from the white muscle of Atlantic herring reared at 8 °C. (A) MHCs digested with the protease papain (12.5 mg ml^{-1}) from *Papaya latex*, (B) digested with the protease endoprotease Glu-C (25 mg ml^{-1}) from *Staphylococcus aureus* strain V8, (C) digested with the protease α-chymotrypsin (2.5 mg ml^{-1}) from bovine pancreas, (D) digested with the protease clostropain (5 mg ml^{-1}) from *Clostridium histolyticum*; lane 1, larvae on the day of hatching; lane 2, 15 mm TL larvae; lane 3, 20 mm TL larvae; lane 4, 25 mm TL larvae; lane 5, 40 mm TL juvenile; lane 6, white muscle from adult fish 30-35 cm TL; lane 7, red muscle from adult fish 30-35 cm TL; lane 8, 10 μl of enzyme solution alone. Rightward facing arrowheads indicate differences in the gel patterns between embryonic and adult white muscle. Leftward facing arrowheads indicate differences in the banding pattern between MHCs from adult red and white muscles. The gels were silver-stained.


A
1
2
3
4
5
6
7
8
B
1
2
3
4
5
6
7
8
C
1
2
3
4
5
6
7
8

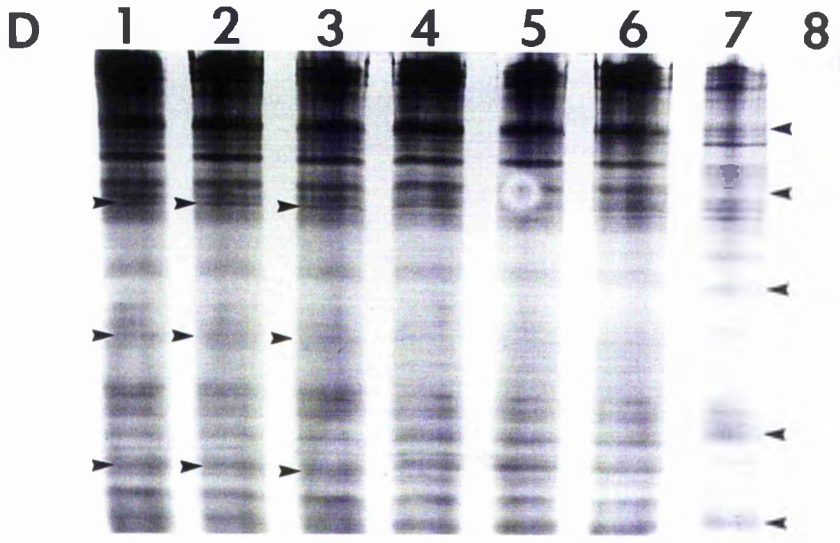
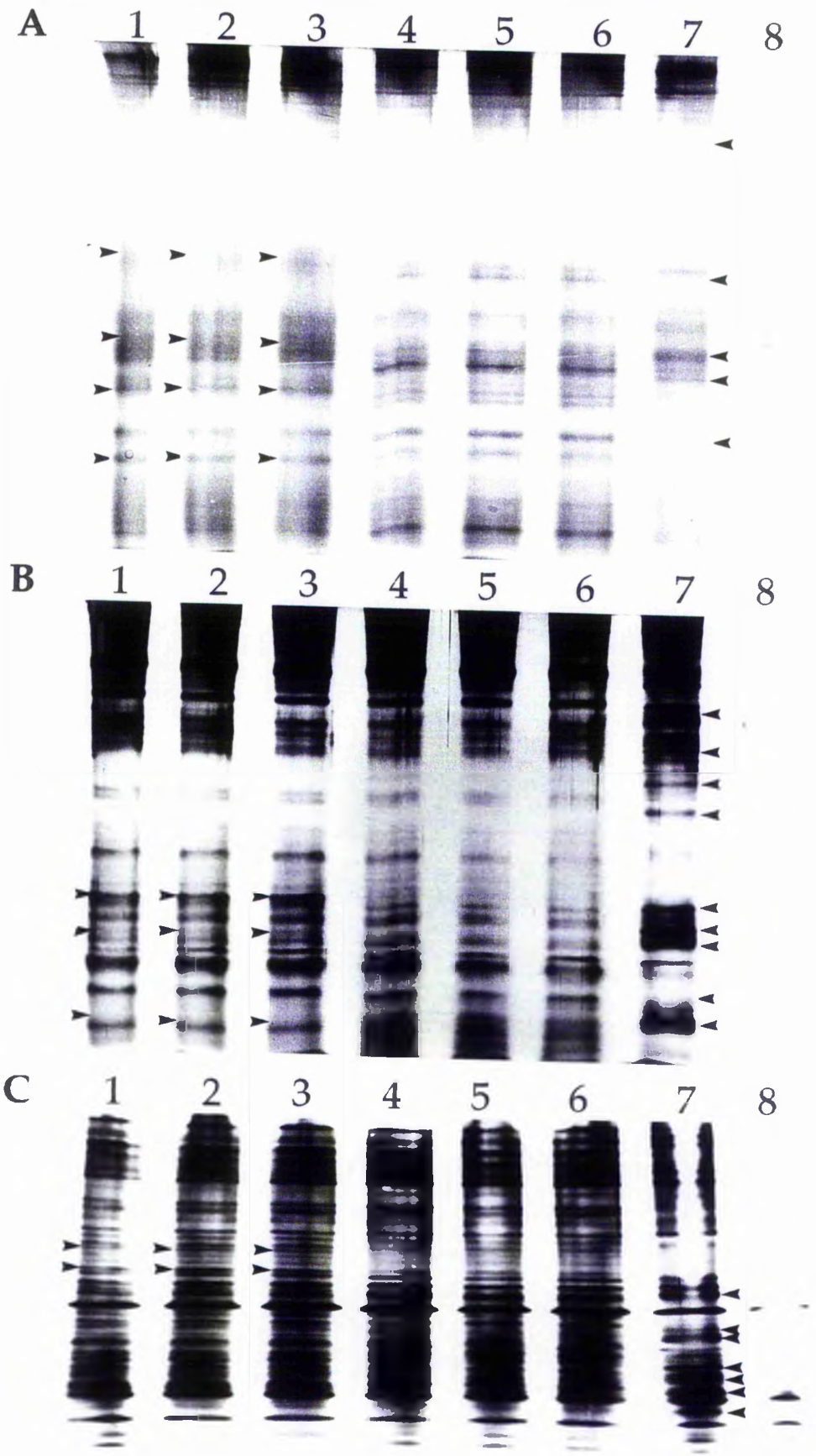

D
1
2
3
4
5
6
7
8

Figure 3.14. Peptide maps, resolved on 15 % acrylamide SDS PAGE gels, of myosin heavy chains (MHCs) purified from the white muscle of Atlantic herring reared at 5, 8, or 12 °C. (A) MHCs digested with the protease papain (12.5 mg ml^{-1}) from *Papaya latex*, (B) digested with the protease endoprotease Glu-C (25 mg ml^{-1}) from *Staphylococcus aureus* strain V8, (C) digested with the protease α-chymotrypsin (2.5 mg ml^{-1}) from bovine pancreas; lane 1, 20 mm TL larvae reared at 5 °C; lane 2, 20 mm TL larvae reared at 8 °C; lane 3, 20 mm TL larvae reared at 12 °C; lane 4, 25 mm TL larvae reared at 5 °C; lane 5, 25 mm TL larvae reared at 8 °C; lane 6, 25 mm TL larvae reared at 12 °C; lane 7, red muscle from adult fish 30-35 cm TL; lane 8, 10 ml of enzyme solution alone. Rightward facing arrowheads indicate differences in the gel patterns between white muscle in 20 mm and 25 mm TL larvae. Leftward facing arrowheads indicate differences in the banding pattern between MHCs from adult red and white muscles MHCs. The gels were stained with silver.


A
1 2 3 4 5 6 7 8
B
1 2 3 4 5 6 7 8
C
1 2 3 4 5 6 7 8

Similar results were obtained in larvae reared at 5 and 12 °C. The transition from the embryonic to the adult pattern of MHC expression in white muscle also occurred between 20 and 25 mm TL (Fig. 3.14A-C). Unfortunately the frequency of sampling was not sufficient to determine the body length between 20 and 25 mm at which the transition occurred.

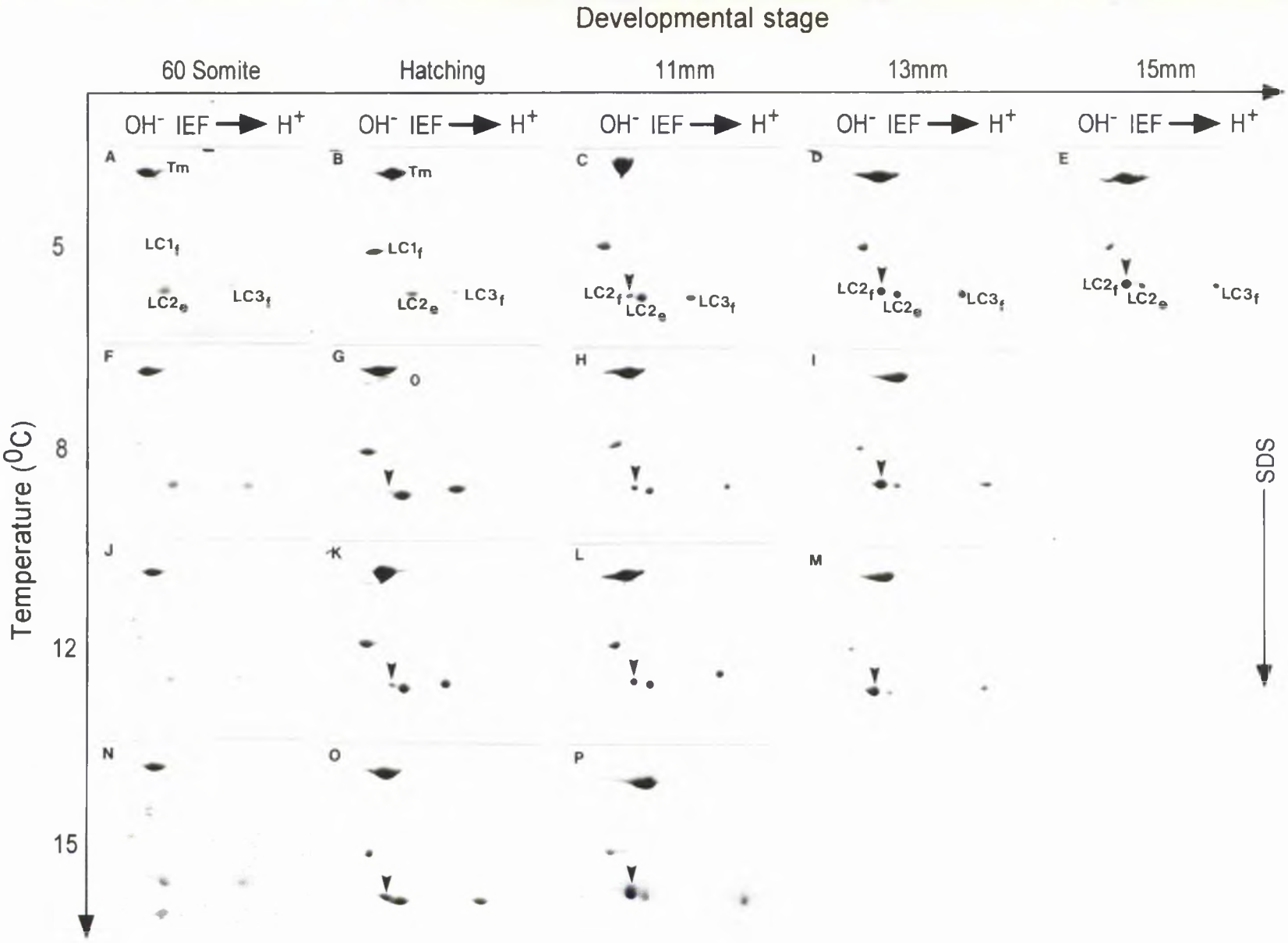
Alkali light chains

There was no evidence for developmental isoforms of the alkali light chains (LC1 and LC3). LC1 and LC3 from the inner muscle of 60-somite embryos, larvae and adults were indistinguishable on the basis of molecular mass and iso-electric point (compare Fig. 3.15A-P with Fig. 3.8A).

Regulatory light chain

60-somite embryos had a single LC2 isoform with an M_r of 19 kDa ($LC2_e$) (Fig. 3.15A, F, J, N). At hatching, an additional LC2 band appeared in larvae reared at 12 °C and 15 °C, corresponding to the adult isoform ($LC2_f$) (Fig. 3.15K, O). This isoform was present in trace amounts in larvae reared at 8 °C (Fig. 3.15G), but was not present in larvae reared at 5 °C (Fig. 3.15B). In 11 mm TL larvae, the expression of these LC2 isoforms was a function of rearing temperature (Fig. 3.15C, H, L, P). $LC2_e$ was the predominant isoform at 5 °C (Fig. 3.15C), $LC2_e$ and $LC2_f$ were expressed in equal amounts at 8 °C and 12 °C (Fig. 3.15H, L), and $LC2_f$ was the major isoform at 15 °C (Fig. 3.15P). The expression pattern in 15 °C-reared fish at 11 mm TL was similar to that found in adult white muscle (Fig. 3.15B). By 13 mm TL, larvae reared at 8 °C and 12 °C also showed the adult pattern of LC2 expression (Fig. 3.15I, M) whereas $LC2_e$ and $LC2_f$ were still equally expressed in the 5 °C-reared larvae (Fig. 3.15D). The adult pattern of LC2 expression was not found in the 5 °C-reared larvae until they reached 15 mm TL, 34 days after hatching (Fig. 3.15E).

Figure 3.15. Two dimensional PAGE gel of acidic myofibrillar proteins from presumptive white muscle of Atlantic herring. (A-E) Fish reared at 5 °C, (F-I) 8 °C, (J-M) 12 °C, (A-E) 15 °C, (A, F, J, N) 60 somite embryos, (B, G, K, O) larvae on day of hatching, (C, H, L, P) 11 mm TL larvae, (D, I, M) 13 mm larvae, (E) 15 mm TL larvae. The arrowhead indicates the position of myosin $LC2_f$. Tm, tropomyosin; $LC1_f$, myosin light chain 1; $LC2_e$, embryonic myosin light chain 2; $LC2_f$, adult white (fast) myosin light chain 2; $LC3_f$, myosin light chain 3; IEF, iso-electric focusing. The gels were stained with Coomassie Brilliant Blue G250.


Developmental stage
60 Somite
Hatching
11mm
13mm
15mm
OH⁻ IEF → H⁺
Temperature (°C)
5
8
12
15
SDS
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Tm
LC1f
LC2e
LC3f
LC2f
0

Tropomyosin

Tm was present as a single spot on two dimensional gels at all stages examined (60 somite stage embryos to adults) (Fig. 3.15A-P). It had a relative molecular mass of 40 kDa.

Troponin T

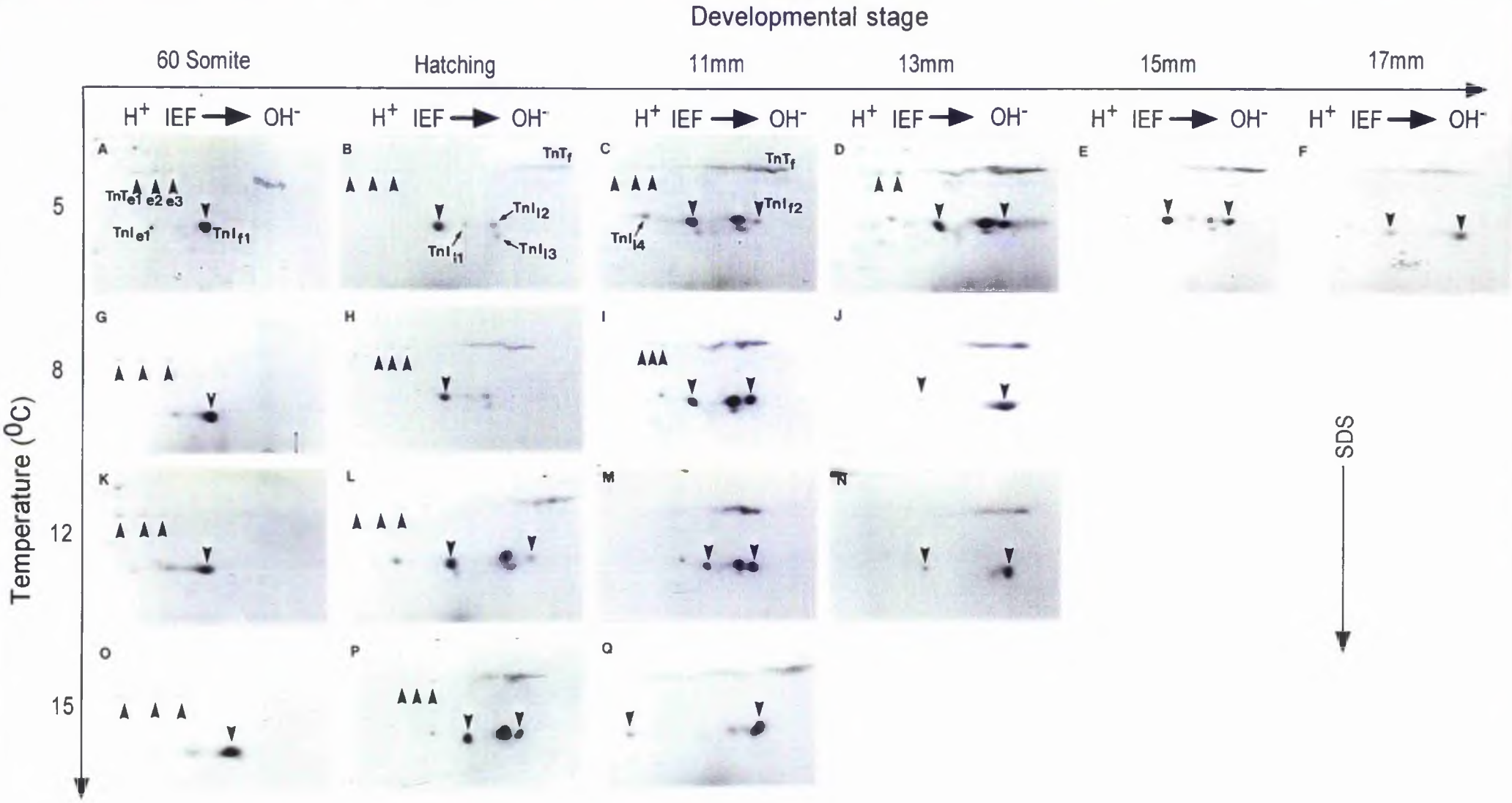
TnT was present as three spots in 60 somite embryos, all with a relative molecular mass of 32 kDa (Fig. 3.16 A, G, K, O). These isoforms were distinct from those found in larvae greater than 15 mm TL and were thought to represent embryonic isoforms. They have been designated TnT_{e1}, TnT_{e2}, TnT_{e3} in order of decreasing acidic iso-electric point (Fig. 3.16A-C, G-I, K-L, O-P). Following hatching there was a gradual decrease in the expression of embryonic isoforms and an increase in the expression of the adult white muscle isoform (TnTf) with increasing larval length. The ratio of embryonic to adult isoforms at any given length decreased in the order 5 °C > 8 °C > 12 °C > 15 °C (Fig. 3.16B-D, H, I, L, M, P). The adult pattern of TnT expression was established at 17 mm TL at 5 °C, 13 mm TL at 8 and 12 °C, and at 11 mm TL at 15 °C (Fig. 3.16F, J, N, Q).

Troponin I

There was one minor and one major spot corresponding to TnI isoforms in the inner muscle of 60-somite stage embryos. Both these isoforms had a molecular mass of 22 kDa. The major TnI isoform corresponded to one of the adult isoforms (TnI_{f1}) (Fig. 3.16A, G, K, O). The other spot is assumed to represent an embryonic isoform (TnI_{e1}) (Fig. 3.16A, G, K, O).

At hatching, additional TnI isoforms appeared which were not present in embryos. These were designated larval isoforms TnI_{l1}, TnI_{l2}, TnI_{l3}, and

Figure 3.16. Two dimensional PAGE gel of basic myofibrillar proteins from presumptive white muscle of Atlantic herring. (A-F) reared at 5 °C, (G-J) 8 °C, (K-N) 12 °C, (O-Q) 15 °C, (A, G, K, O) 60 somite embryos, (B, H, L, P) larvae on day of hatching, (C, I, M, Q) 11 mm TL larvae, (D, J, N) 13 mm TL larvae, (E) 15 mm TL larvae, (F) 17 mm TL larvae. The upward facing arrowheads indicate the positions of embryonic troponin T isoforms. The downward facing arrowheads indicate the position of larval troponin I isoforms. The gels were stained with Coomassie Brilliant Blue G250. Tm, tropomyosin; TnTe1, TnTe2, TnTe3, embryonic troponin T isoforms; TnTf1, adult white (fast) muscle troponin T isoform; TnIe1, embryonic troponin I isoform; TnI_{l1}, TnI_{l2}, TnI_{l3}, TnI_{l4}, larval troponin I isoforms; TnI_{f1}, adult white (fast) muscle troponin I isoform; IEF, iso-electric focusing.


Developmental stage
60 Somite
Hatching
11mm
13mm
15mm
17mm
H+ IEF → OH-
Temperature (0C)
5
8
12
15
SDS
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
TnTe1 e2 e3
TnIe1
TnIf1
TnTf
TnIi2
TnIi1
TnIi3
TnIf2
TnIi4

TnI_{I4}. TnIe was no longer expressed (Fig. 3.16B, H, L, P). Also at hatching, TnI_{I2} and TnI_{f1} were the major isoforms present, and the proportion of TnI_{I2} was greater at 12 °C and 15 °C (Fig. 3.16L, P) than at 5 °C and 8 °C (Fig. 3.16B, H). TnI_{f2} was present as a minor component at 12 °C and 15 °C (Fig. 3.16L, P), but not at lower temperatures (Fig. 3.16B, H).

By 11 mm TL there were major variations in TnI expression at the different temperatures. The adult pattern of TnI expression was already established at this body size in 15 °C larvae (Fig. 3.16Q). In 12 °C larvae, there was an increase in TnI_{f2} compared with the level at hatching, and this isoform was now expressed at a similar level to TnI_{I2} (Fig. 3.16M). Also at 11 mm TL, TnI_{f2} started to be expressed at lower temperatures, but was more abundant at 8 °C than at 5 °C (Fig. 3.16C, I). TnI_{I4} was present at hatching at 12 °C and 15 °C (Fig. 3.16L, P) but not until 11 mm TL at 5 °C and 8 °C (Fig. 3.16C, I).

Larvae reared at 5 °C expressed TnI_{I2}, TnI_{f1}, TnI_{f2} and a trace of TnI_{I3} and TnI_{I4} at 13 mm TL (Fig. 3.16D). The adult pattern of TnI expression was established at 13 mm TL at 8 °C and 12 °C (Fig. 3.16J, N). Some expression of larval isoforms still occurred in 15 mm TL larvae reared at 5 °C (Fig. 3.16E). Only at 17 mm TL was the adult pattern of TnI expression established in larvae reared at 5 °C (Fig. 3.16F).

3.4 Discussion

The results of this study show that there is a significant difference in the rate of embryonic development with respect to rearing temperature. This was illustrated by first somite formation; as the rearing temperature increased, the time for first somite formation decreased (see Table 3.1). In addition, the number of days to hatch increased as the rearing temperature decreased. This study used the same species of fish as Johnston *et al.* (1995), who showed that the rate of somite formation was greater at higher incubation temperatures; embryos reared at 15 °C formed a somite every 52 minutes, whereas embryos reared at 5 °C formed a somite every 178 minutes. Johnston *et al.* (1995) also showed that somitogenesis occurred relatively earlier at lower temperatures when somitogenesis was expressed as percentage developmental time. For example, at 32 % developmental time, there were 42 somites at 5 °C and only 25 somites at 15 °C. With the exception of 15 °C, the major differences in embryonic development rates occurred at developmental temperatures well within those that herring embryos would normally experience, as a result of climatic variability (Jones and Jeffs, 1991).

This study revealed distinct isoforms of myosin heavy chain (MHC), myosin light chain (MLC), tropomyosin (Tm), troponin T (TnT) and troponin I (TnI) in red and white muscle of adult herring, clearly demonstrating that red and white muscle contains distinct fibre types. These results are consistent with the findings of other authors (Crockford and Johnston, 1993; Johnston and Horne, 1994) and a number of other fish species (Rowlerson *et al.*, 1985; Scapolo and Rowlerson, 1987; Karasinski and Kilarski, 1989; Chanoine *et al.*, 1992; Martinez and Christiansen, 1994; Mascarello *et al.*, 1995).

In this present study, inner muscle of larvae contained isoforms of Tm, LC1, LC3 which were not distinguishable from those of adult fast muscle. However, the inner muscle fibres of embryos and larvae were found to contain MHC, LC2, TnT and TnI developmental isoforms which were not present in either adult white or red muscle fibres, as was also found by Crockford and Johnston (1993). The expression of these isoforms varied with rearing temperature. The expression of embryonic isoforms was switched off at progressively shorter body lengths as rearing temperature was increased. The transition from the embryonic MHC isoform to the adult white muscle MHC isoform occurred between 20 mm and 25 mm TL in larvae, regardless of rearing temperature (Fig. 3.14A-C).

Previously, developmental patterns of myofibrillar isoforms have been related to the size, development and locomotory capacity of fish larvae (Waterman, 1969; Van Raamsdonk *et al.*, 1977; Veggetti *et al.*, 1990; Johnston, 1993; Brooks and Johnston, 1995). Brooks and Johnston (1995) showed that a developmental transition in the composition of myofibrillar proteins occurred in the plaice. This has been shown in other teleosts (Scapolo *et al*, 1988; Focant *et al.*, 1992) which presumably reflects changes in contractile function as the fish grows. The sequential expression of developmental protein isoforms has also been shown in birds and mammals (Bandman *et al.*, 1982; Crow and Stockdale, 1986; Briggs *et al.*, 1990).

Protein isoforms can result from the differential expression of multigene families or from multiple transcripts from a single gene *via* alternative splicing (Bandman, 1992). Different combinations of isoforms are expressed in developmental stage and muscle fibre type specific patterns, producing fibres with different contractile properties (see Schiaffino and Reggiani, 1996, for review). Herring myofibrillar proteins have previously been shown to vary with rearing temperature in 1 day and 7 day old larvae,

particularly with respect to TnT isoforms (Crockford and Johnston, 1993). This study has examined myofibrillar isoform composition with rearing temperature of embryos, newly hatched larvae through to metamorphosed juveniles.

Isoforms of MHC have figured prominently in studies of determinants of contractile properties in muscle. The composition of MHC and MLC is a major determinant of muscle contraction speed (Schiaffino and Reggiani, 1996). Lannergen (1987), Reiser *et al.* (1988a), and Pette and Starron (1990) showed that MHC was the major determinant of V_{max} in amphibian and mammalian skeletal muscle. MHC is encoded by a multigene family in vertebrates (Nguyen *et al.*, 1982; Buckingham, 1985; Robbins *et al.*, 1986). In this study, red and white muscle fibres contained distinctly different isoforms of MHC (Fig 3.7A-D), as has been reported for other fish species (Rowlerson *et al.*, 1985; Scapolo and Rowlerson, 1987; Karasinski and Kilarski, 1989). The embryonic white muscle fibres in herring also contained a distinct isoform(s) of MHC from that found in red and white muscles (Fig. 3.13A-D, lanes 1, 6, 7).

LC2 is located at the head-rod junction of the myosin molecule and is thought to be an important determinant of the kinetics of cross-bridge cycling (Lowey *et al.*, 1993; Szczesna *et al.*, 1996). LC2 isoforms are thought to be encoded by a single gene and to arise by alternative transcription (Bandman, 1992). Adult herring white muscle contains one major and one minor LC2 isoform (Fig. 3.8A). This was reported in the plaice (*Pleuronectes platessa* L.) (Brooks and Johnston, 1993). The minor isoform (LC2e) is the only isoform present in the presumptive white muscle of 60-somite stage herring embryos (Fig. 3.15A, F, J, N). The presence of a trace amount of LC2e in adult stages may indicate that embryonic isoforms are expressed in newly recruited muscle fibres, as has been reported for MHCs in fish (Enion

et al. 1995). At hatching, larvae reared at 5 °C only expressed LC2e (Fig. 3.15A). At higher temperatures, the proportion of LC2f increased in the series 8 °C > 12 °C > 15 °C (Fig. 3.15B, G, K, O). The amount of LC2e decreased with increasing body size until the adult pattern was established at 11 mm at 15 °C, 13 mm at 8 °C and 15 mm at 5 °C (Fig. 3.15P, I, E). Several other developmental isoforms of LC2 were identified in plaice white muscle, with complex changes in expression patterns occurring at metamorphosis and during the first year of the juvenile stage (Brooks and Johnston, 1993).

TnT is a key protein in the regulation of skeletal muscle contraction by calcium (Greaser and Gergely, 1973). It is a component of the troponin complex which also contains TnI and TnC. The troponin complex is situated on the thin filament. Interactions between Tm and the troponin complex mediate the Ca^{2+} sensitivity of actomyosin ATPase activity. Stage specific isoforms of TnT have also been shown to be sequentially expressed with development in both birds and mammals (Dhoot and Perry, 1980; Breitbart *et al.*, 1985; Saggin *et al.*, 1990; Briggs *et al.*, 1990; Briggs and Schachat, 1996). Developmental isoforms of TnT have been identified in teleosts (Crockford and Johnston, 1993). In this study, two dimensional gels showed three embryonic isoforms of TnT (Fig. 3.16A, G, K, O). As body length increased there was a gradual increase in the proportion of TnT_f and a decrease in proportion of embryonic TnT isoforms, with the adult pattern being established at 15 mm at 5 °C, 13 mm at 8 °C and at 11 mm at 15 °C (Fig. 3.16E, J, Q). Isoforms of TnT have been shown to arise from alternate transcription of a single gene (Brietbart *et al.*, 1985). Changes in TnT expression during development are correlated with an increased sensitivity to Ca^{2+} (Briggs *et al.*, 1990). Direct correlations between the pCa^{2+} tension relationship of single skinned fibres and their content of TnT isoforms have

been demonstrated for rabbit muscles (Schachat, 1987; Greaser *et al.*, 1988).

TnI is the inhibitory component of the troponin complex. Separate genes have been identified for TnI in fast and slow skeletal and cardiac muscle in mammals (Koppe *et al.*, 1989). Embryonic and neonatal forms of TnI have not been described in birds or mammals, although in mice the fast skeletal muscle isoform is a developmental isoform in the early embryonic heart (Zhu *et al.*, 1995). However, a mixture of adult fast and slow skeletal muscle isoforms are present in the muscles of embryonic birds and mammals. The expression of the inappropriate isoform is gradually switched off as development proceeds (Dhoot and Perry, 1980). In contrast to this, the results in this study show that herring were found to express multiple developmental isoforms of TnI. At the 60- somite stage, there was a minor embryonic TnI isoform (TNI_e) expressed in addition to the two adult isoforms (TnI_{f1}) (Fig. 3.16A, G, K, O). Three out of the four larval TnI (TnI_{l1}, TnI_{l2}, TnI_{l3}) isoforms were identified in 1-day old larvae (Fig. 3.16B, H, L, P). The larval TnI_{l4} isoform was not apparent in the inner muscle of 5 °C and 8 °C reared larvae until they had reached 11 mm TL (Fig. 3.16C, I). There were complex changes in the expression of TnI isoforms with development. The rate of loss of TnI larval isoforms and the appearance of the adult fast muscle isoforms with respect to body size were inversely related to rearing temperature (Fig. 3.16C-F, I, J, L-N, P, Q). The adult pattern of TnI expression was established at 17 mm at 5 °C (Fig. 3.16F), 13 mm at 8 °C (Fig. 3.16J) and 11 mm at 15 °C (Fig. 3.16Q). Whether the developmental isoforms of TnI represent distinct genes or post-translational modification products remains to be determined. Given the lack of similar developmental isoforms in mammals, it is interesting to note that sequencing studies have shown that herring TnIf lies outside the tetrapod TnI gene family, showing significant variation in the actin/TnC-binding sequence (Hodgson *et al.*, 1996).

The expression of individual isoforms of LC2, TnT and TnI was independently regulated, resulting in unique combinations of myofibrillar proteins at different temperatures (Fig. 3.15, 3.16). The relative persistence of embryonic isoforms in larvae at low temperatures may be some function of the later start of myofibril synthesis with respect to somite stage shown by Johnston *et al.* (1997).

In this study, temperature influenced many aspects of herring development over the length range 12-25 mm. Many of these factors are related to swimming style. This size range is where major developments in the fins and changes in swimming style occur (Blaxter, 1988). The myofibrillar protein composition and muscle innervation patterns varied with respect to fish length at different temperatures. At the same body length the development of the anal fin rays and pelvic fins was relatively retarded at high temperatures (Johnston *et al.*, 1997). Alterations in fins with rearing temperature has also be shown in larvae of the Atlantic cod (*Gadus morhua*) (Hunt von Herbling *et al.*, 1996). These phenotypic variations may affect the swimming style and performance of larval fish, and hence prey capture and predator avoidance, and thus survival.

At hatching, most fish larvae express fast isoforms in the red muscle fibres (Johnston and Horne, 1994; Focant *et al.*, 1992) and muscle metabolism of red and white muscle is predominately aerobic. As development proceeds, the gills develop and there is a change to anaerobic metabolism in the inner white muscle fibres (El-Fiky *et al.*, 1987; Goolish, 1991) accompanied by an increased expression of slow myosin isoforms in the red muscles (Johnston and Horne, 1994; Focant *et al.*, 1992). In addition, there is a marked change in swimming style (Batty, 1984; Johnston *et al.* 1996). In hatched and early larvae, the effects of frictional drag result in the predominance of viscous forces. Therefore, early larvae swim using an

anguilliform mode, in which the amplitude of body movements is a linear function of body length and the lateral acceleration of water along the body is constant. As body length increases, swimming speed increases, but maximum tail beat frequency, contraction duration and the shortening speed of the muscle fibres decrease (James *et al.*, 1997). Larger fish have a higher Reynolds number (*Re*), which is a function of length and swimming speed. Therefore, as fish length increases reactive forces predominate (Weihs, 1980). The unpaired fins form, the primordial fin is lost and a subcarangiform swimming style is adopted where the amplitude of the trunk increases towards the tail (Batty, 1984). During these changes in body form and swimming style, different functional demands are placed on the muscles. The changes in isoform expression found in this study are likely to be a reflection of these demands since myofibrillar isoforms are the major determinants of contractile properties (Moss *et al.*, 1995; Schiaffino and Reggianni, 1996).

Mechanisms of larval locomotion are fundamental to the success of larvae both as predators and prey (Morley and Batty, 1996). Prey capture and predator avoidance has been shown to be the most important factor determining the year class strength of fish (Heath, 1992). The results of this study highlight a potential mechanism whereby early thermal experience could affect the survival of larvae and hence juvenile recruitment, and the strength of particular year classes.

The life histories of cod, plaice and herring undergo the highest rates of mortality at the egg and larval stage. The larval stages of herring are subject to massive mortalities of the order of 5-20 % per day, primarily as a result of starvation and predation (Heath and Maclachlan, 1987). In contrast, adult mortality is less than 10 % per year, although it increases with age (Woodhead, 1979). Larval locomotion, and consequently muscle

performance are fundamental to herring larvae in determining their performance as both predators and prey.

The recruitment of fish into particular year classes is a highly complex process. Less than 0.1 % are thought to be recruited into the adult population (Heath and Maclachlan, 1987). Recruitment is determined by a panoply of abiotic and biotic factors (Pitcher and Hart, 1982). Factors include variations in the abundance and patchiness of predators and prey (Gotceitas *et al.*, 1996; Hinkley *et al.*, 1996). Most studies of variation in year class strength have examined the alterations in year class numbers rather than the effects upon the individuals themselves. Sakuramoto *et al.* (1997) analysed data for variations in year class strength in the sandfish (*Arctoscopus japonicus*) and demonstrated correlations with seawater temperature. Dickson *et al.* (1974) also correlated year class strength of Atlantic cod in an area in the central North Sea to sea temperature. Abundance fluctuations in the southern North Sea plaice have been related to seawater temperature and water current speed. The planktonic eggs and larvae of these plaice are affected by the temperature and the speed of the North-East-flowing residual current (Cushing, 1974; Talbot, 1978; Harding *et al.*, 1978). The sea temperature determines food availability and the length of time the larvae are exposed to predation (Cushing, 1974). Plaice need to metamorphose at the right time in order to sink into onshore currents which carry them to nursery grounds. If the current flow is too fast and/or the sea temperature too low, plaice are unable to leave the current due to insufficient growth and development, and therefore mortality for that year will be high.

In various tropical fish larvae, rearing temperature has been shown to influence the relative timing at which the jaws, which are essential for capture and consumption of prey, become functional in relation to yolk depletion (Fukuhura, 1990). In cod, rearing temperature has been found to

alter the morphology of the gastrointestinal system, which may affect digestion rates and efficiency (Hunt von Herbling *et al.*, 1996). In addition, Gibson and Johnston (1995) showed that the appearance of cephalic spines was temperature-dependent in the turbot (*Scophthalmus maximus*), thus potentially affecting the success of predator avoidance.

In response to fluctuations in environmental conditions, different herring stocks have evolved different reproductive strategies. North-east Atlantic herring populations living in relatively shallow areas, where the production cycle is more predictable than in deeper waters, have shorter life spans and greater reproductive effort per occasion than the Atlanto-Scandian stock which lives in deeper water (Mann and Mills, 1979). Laprise and Pepin (1995) correlated sea temperature with egg and larval abundance. Southward *et* al. (1988) related the relative abundance of herring and sardine stocks around Devon and Cornwall over the last 400 years to climatic variations. Small variations in the sea temperature altered the competitive advantage between the stocks, which occupy similar niches. It is possible that the alterations in phenotypic traits indicated in this study and others may contribute to the abundance and variation of fish species and stocks.

Chapter 4

The effect of body size on the myofibrillar protein composition of fast muscle fibres in the short-horn sculpin (*Myoxocephalus scorpius* L.)

4.1 Introduction

The maximum swimming speed (U_{max}) of fish is related to their body length. Wardle and He (1988) found length-specific swimming speed in fish, to decrease with increasing body length, such that $U_{max} = aL^{-0.85}$ where L was body length and a is a constant which varies with species examined. Therefore, smaller fish can achieve higher length-specific speeds than larger individuals (Bainbridge, 1958; Blaxter and Dickenson, 1959; Brett and Glass, 1973; Wardle, 1975; Videler and Wardle, 1991; Gibson and Johnston, 1995; Temple and Johnston, 1997). Bainbridge (1958) showed that for any particular tail beat frequency, speed increased with length for goldfish (*Carassius auratus*), trout (*Salmo gairdneri*) and dace (*Leuciscus leuciscus*). This has also been found during escape responses in the rainbow trout (Webb, 1976), and juvenile turbot (*Scophthalmus maximus*) (Gibson and Johnston, 1995), although this has not been demonstrated for the angelfish (*Pterophyllum eimekei*) (Domenici and Blake, 1993). In this latter study, however, size related differences in the kinematics of swimming performance were found. Small fish employed double bend escape responses, whereas larger fish performed mostly single bend escapes. Furthermore, the duration of escape responses increased with size. Hunter and Zweifel (1971) found tail beat amplitude in jack mackerel and other fishes, to be a constant proportion of length, at all swimming speeds observed. Maximum tailbeat frequency and the length-specific amplitude,

has been found to decrease with increasing fish length (Bainbridge, 1958; Webb, 1976; Archer and Johnston, 1989).

The decrease in tail beat frequency and length-specific swimming speed with increasing fish body size is reflected in *in vitro* muscle contraction times (Wardle, 1975; Archer *et al.*, 1990). Small fish have shorter isometric twitch contraction times and higher contraction velocities than larger fish. In work loop experiments, where muscle is phasically stimulated whilst undergoing length changes (often sinusoidal) (Josephson, 1985), the number of stimuli needed at a given cycle frequency decreases with increasing fish length (Archer and Johnston, 1990).

In large and small animals, the basic design and structural components of muscle are the same. Stress (force per unit area) is scale independent, because filament diameter, sarcomere length and filament overlap is constant in all muscles (Hill, 1950). The relative muscle shortening, or strain is also constant, being around 30 % of resting fibre length (Schmidt-Nielsen, 1986).

Size related differences in swimming performance have been correlated with increases in the length of the muscle fibres, myotomal cross-sectional area, muscle length and mass (Bainbridge, 1961; Greer-Walker, 1970; Webb, 1976; Webb and Johnstrude, 1988; Archer *et al.*, 1990). Examination of the mechanical properties of muscle fibres has shown absolute maximum power output to increase with increasing muscle mass (Anderson and Johnston, 1992; Webb and Johnstrude, 1988). Under steady state conditions the mass-specific maximum power output decreased with increasing body size, scaling to length, $L^{-0.29}$ and body mass (M_b), $M_b^{-0.1}$, in the Atlantic cod (*Gadus morhua*) (Anderson and Johnston, 1992). Altringham *et al.* (1996) examined the effects of scaling on the power output of muscle fibres of *Xenopus* during oscillatory work, and found that the cycle

frequency for maximum power output was dependent on body mass, decreasing as a function of $M_b^{-0.07}$ in fast fibres. Similarly, Altringham and Johnston's (1990) study of fish fast muscles during multiple *in vivo* contractions, found the cycle frequency for optimum power output decreased from 5 Hz in 13 cm fish to 2.5 Hz in 67 cm fish. In other words the frequency at which fish myotomal muscles contract and stretch is lower in larger fish.

Myofibrillar isoforms have been shown to be the major determinants of muscle contractile properties. Differences in maximum shortening velocity (V_{max}) have been correlated with changes in myosin heavy and light chain isoforms (see Moss *et al.*, 1996, for review). For example, studies on single muscle fibres have shown that there is a relationship between contractile properties and myofibrillar proteins in man (Larsson and Moss, 1993; Bottinelli *et al.*, 1996), rat (Bottinelli *et al.*, 1994a; Bottinelli and Reggiani, 1995) and toad (Lännergren, 1987). These authors have shown that V_{max}, maximum power output, and force are largely determined by the MHC composition of the muscle fibres. In other studies, differences in V_{max} have reflected the ratio of myosin light chains. Bottinelli and Reggiani (1995), showed in rats that V_{max} is proportional to the relative content of alkali myosin light chain 3f. Similarly, in a study using common carp, Crockford and Johnston (1990) showed that the ratio of MLC_{3f} to MLC_{1f} contributed to differences in V_{max}. Greaser *et al.* (1988) put forward evidence for LC3 of fast muscle as a modulator of contractile properties in rabbit. Pemrick (1977) showed that myosin LC2 changes the ability of myosin to interact with the thin filament in the presence of calcium and decreases the Ca^{2+} concentration required to activate the thin filament.

The aim of this study was to establish the effect of body size on the contractile proteins of fast muscles in the short-horn sculpin (*Myoxocephalus scorpius* L.), to accompany an *in vitro* assessment of scaling of muscle

performance, examined by Dr. Rob James, in this laboratory. Short-horn sculpin are marine members of the family Cottidae. They are specialist sit-and-wait, ambush predators, which can grow to over 60 cm total length (TL), although specimens over 30 cm are rarely caught (Whitehead *et al.*, 1986). They are temperate teleosts with a distribution from the Bay of Biscay to Spitzbergen (Whitehead *et al.*, 1986). Around the British Isles, adults are usually caught at a depth of between 30 and 50 m (King *et al.*, 1983; Whitehead *et al.*, 1986) and juveniles (about 45-80 mm TL) are found intertidally and in the shallow subtidal zone (Temple and Johnston, 1997). The muscle contractile proteins and myofibrillar ATPase activities were assessed in order to determine the factors influencing variation in muscle contraction characteristics with changing fish body size.

4.2 Materials and Methods

Specimen collection

Short-horn sculpin (*Myoxocephalus scorpius L.*) were caught by local fisherman in lobster creels or by trawling in the Firth of Forth from October to November in 1993 and 1994. Fish were acclimated to 10-12 °C for 5-7 weeks in re-circulating sea-water tanks (photoperiod 12 hours light: 12 hours dark). Fish were fed twice a week on live shrimps (*Crangon crangon*) and chopped squid. Total body lengths and masses ranged from 6.2 cm, 2.6 g to 32.8 cm, 594 g.

Muscle sampling

Fish were stunned by a blow to the head and pithed. Anterior abdominal myotomes were removed on ice, in a 5 °C constant temperature room. The muscle sampled was approximately 0.35 body lengths from the snout. The contractile properties, I filament lengths and Z disk widths of the fibres were measured by Dr. R. S. James.

4.2.1 Determination of Myofibrillar ATPase Activity

Myofibrillar ATPase is the enzyme resposible for the cycling of ATP during muscle contraction. The rate at which this enzyme can hydrolyse ATP, known as the ATPase activity, can be measured and has been correlated with muscle contraction speed. The assay measures the amount of free phosphate that is produced from ATP in solution by a known quantity of myofibrils in a given time in the prescenceof calcium. By conducting the assay in the abscence of free calcium, by its removal with EGTA it is possible to determine the sensitivity to calcium of myofibrillar ATPase. The myofibrillar ATPase activity of the collected muscle from eleven individuals was determined as follows:

Figure 4.1. Photograph of the species used in this study, the short-horn sculpin (*Myoxocephalus scorpius* L.). Scale bar = 10 cm.

Preparation of myofibrils

All procedures were carried out at 0-2 °C. Rostral muscle was rapidly dissected out and macerated using an ice cold scalpel blade. The muscle was then added to 10 volumes of solution A containing (in mmol l^{-1}): imidazole, 20; KCl, 100; EDTA, 1 (pH 7.2 at 0 °C), and homogenised for 15 seconds using a Polytron blender. The homogenate was centrifuged at 2000 g for 5 minutes and the pellet re-suspended in 10 volumes of solution B containing (in mmol l^{-1}): imidazole, 20; KCl, 100 (pH 7.2 at 0 °C), and centrifuged at 2000 g for 5 minutes. The pellet was resuspended once more and spun at 400 g for 5 minutes and the supernatent discarded. This loose pellet contained washed myofibrils. The protein content was determined using the microbiuret method of Itzhaki and Gill, (1964) and adjusted to 2 mg ml^{-1} with solution B.

The ATPase reaction

The ATPase activity of the myofibrils was then determined by incubating them at 12 °C with assay medium containing (in mmol l^{-1}): KCl, 50; imidazole, 40; $MgCl_2$, 7; $CaCl_2$, 5. ATPase activity was measured in the presence of 1 mM EGTA to remove Ca^{2+} by chelation. The reaction was started by the addition of ATP (to give a final concentration of 5 mM) and allowed to proceed for 10 minutes at 12 °C. The reaction was stopped by the addition of an equal volume of 10 % (m/v) trichloroacetic acid (TCA). In controls, TCA was added before the addition of ATP in order to determine background levels of phosphate.

Phosphate assay

The stopped reaction mixture was centrifuged at 5000 g to precipitate any solids which would interfere with the phosphate assay. The phosphate concentration was determined using the method of Bers (1979). The ATPase

activity was calculated in μmol Pi produced per minute per mg of myofibrils. Each assay was replicated in triplicate.

4.2.2 Electrophoretic Analysis of Myofibrillar Proteins

Preparation of myofibrils

Myofibrils were prepared for electrophoresis from the anterior abdominal muscle collected as described in Chapter 2. Myofibrils of known protein concentration were added to electrophoresis sample buffers for IEF or SDS, and electrophoresis was carried out as described in Chapter 2. Alkali-urea gel electrophoresis was carried out in the presence and absence of Ca^{2+} in order to identify troponin I (TnI). This is possible since TnI binds to troponin C (TnC) in the presence of calcium and migrates into the gels, whereas in the absence of calcium, TnI and TnC do not migrate together, resulting in a difference between gels. The resolved bands were then cut out and analysed on SDS PAGE gels to confirm the identity of TnI (Crockford *et al.*, 1991). The methodology for this procedure is described below.

Alkali-urea polyacrylamide gels (AU-PAGE)

Alkali-urea polyacrylamide gel electrophoresis (AU-PAGE) was performed as described by Focant and Huriax (1976) with the modifications given by Crockford (1989). Myofibrils were thoroughly mixed with 20 mM glycine, pH 8.9 using 1 M Trisma base, 12 M urea, 1 mM DTT, 0.001 % BPB, and either 10 mM EGTA or 5 mM $CaCl_2$ to give a final protein concentration of 2 mg/ml. Samples were warmed at 30 °C for 1 h prior to use. The resolving gel contained 8 M urea, 100 mM glycine, pH 8.9 using 1 M Tris base, and 10 % total acrylamide of which 2.67 % was bis crosslinker. The gels were polymerised by the addition of 1 μl of TEMED per ml of gel and 0.5 ml of ammonium persulphate per ml of gel. The electrode buffer was 100 mM glycine, pH 8.9 with 1M Tris base. Before samples were applied, the gels

were pre-run at 400 V for 1 hour to remove unpolymerised acrylamide and urea impurities which would interfere with the running of the samples. Samples were initially run at 50 volts until the samples had entered the gel. The gels were then run at 400 V until the dye front reached the bottom of the gel. The first dimension alkali urea gels were first rapidly stained with a solution of 0.01 % (w/v) Coomassie blue R250. The bands to be analysed were cut out and placed in separate Eppendorf tubes containing 0.5 ml of double concentration SDS PAGE sample buffer as described in chapter 2. The gel pieces were left to equilibrate for 30 minutes. The pieces were placed into the sample wells of a 1 mm thick SDS PAGE gel, and overlaid with 5 ml of SDS PAGE sample buffer. Gels were run at 50 V until the dye front had entered the gel, and then at 250 V until the dye front ran off the bottom of the gel. Gels were acid fixed and stained in Coomassie Brilliant Blue G as described in Chapter 2.

Peptide mapping

Peptide mapping was carried out as previously described in Chapter 2. The enzymes used to digest the electrophoretically purified myosin heavy chain (MHC) were either trypsin from bovine pancreas, endoproteinase Glu-C from *Straphylococcus aureus* V8, elastase from porcine pancreas, α-chymotrypsin from bovine pancreas, papain from papaya latex, thermolysin from *Bacillus thermoproteolyticus rokko*, clostropain from *Clostridium histolyticum*, or ficin from fig tree latex.

Western blotting

Western Blotting was carried out as described in Chapter 2. Monoclonal antibodies to rabbit skeletal muscle actin (clone ACT-471, Sigma Ltd), rabbit skeletal muscle troponin-T (clone JLT-12, Sigma Ltd) and

monoclonal antibodies to mouse skeletal muscle troponin-I (clone-C5, Advanced Immunochemical Inc. CA, USA) were used.

Analysis of the protein bands

Analysis of the protein bands by densitometry was performed using Scan One scanner system on the Apple Macintosh in combination with NIH image gel electrophoresis analysis software.

4.3 Results

4.3.1 Myofibrillar ATPase Activity

Myofibrillar ATPase activity measured in μmoles phosphate released per mg of protein per minute, decreased with increasing fish total length (Fig. 4.2). The ATPase activity scaled to 2.51 $\mu mol\ mg^{-1}\ min^{-1}\ TL^{-0.28}$ (Fig. 4.2). ATPase activity in the presence of EGTA was independent of fish body length with mean values of 70±0.05 $\mu mol\ mg^{-1}\ min^{-1}$.

4.3.2 Myofibrillar Protein Isoforms

One dimensional SDS PAGE gels of myofibrils from fish of 6.2 cm to 32.6 cm contained the myofibrillar proteins myosin heavy chain (MHC), actin (Act), tropomyosin (Tm), troponin T (TnT), light chain 1 (LC1), light chain 2 (LC2), light chain 3 (LC3) and troponin I (TnI) (Fig. 4.3). Troponin C (TnC) was only visible by staining with 'stains-all' (Fig. 4.5). The proteins were identified by their characteristic migration and relative molecular masses, and also by comparison with previous analysis of sculpin myofibrillar proteins by Ball and Johnston (1996).

One dimensional SDS PAGE gels showed no difference with fish total length in the proteins MHC, Act, Tm, TnT, LC1, LC2, LC3 (Fig. 4.3) and TnC (Fig. 4.5). However, one dimensional gels did show a difference in TnI with fish total length (Fig. 4.3). Myofibrils from fish over 28 cm TL contained only a single isoform of TnI with a relative molecular mass of 17 x 10^3 Da. This isoform of TnI has been designated TnI_{f1} (Fig. 4.3, lanes 1-3). Myofibrils from fish 19 cm TL and less contained an additional two TnI protein isoforms of molecular masses of 22 x 10^3 (TnI_{f2}) and 23 x 10^3 (TnI_{f3}) Daltons, respectively. The myofibrillar proteins of 28.5, 29.5 and 32.8 cm TL fish contained only the TnI_{f3} isoform. Myofibrillar proteins of 19 and 15 cm fish

Figure 4.2. Myofibrillar ATPase activity (μmol phosphate released mg^{-1} min^{-1}) for fish of differing body lengths. The lines represent a first order polynomial fitted to the log-log data using a least squares regression, and 95 % confidence limits. The log-log data represent mean ± SEM, 4 samples from individual fish were used in each case.

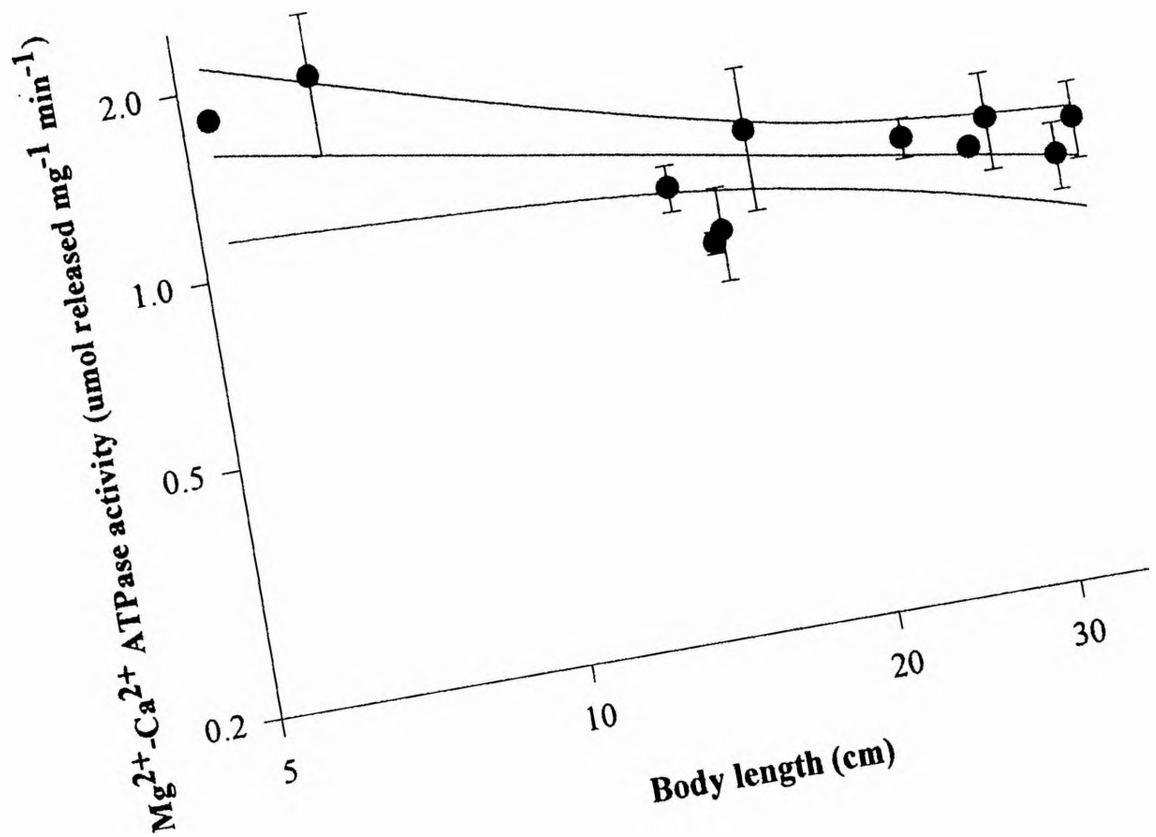
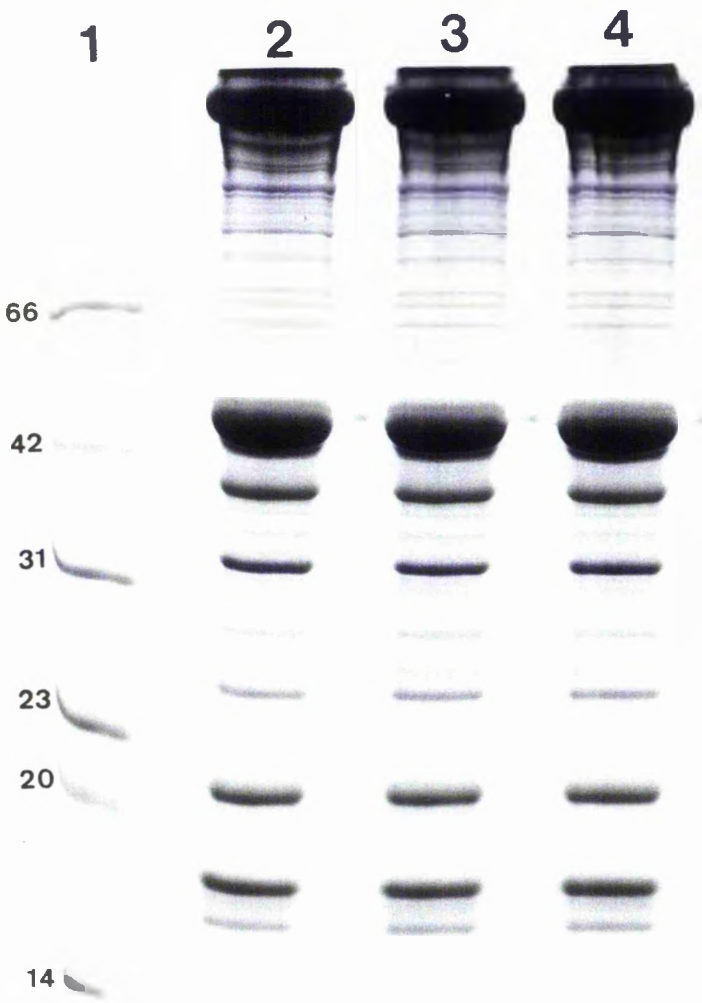

2.0
1.0
0.5
0.2
5
10
20
30
Body length (cm)
Mg2+-Ca2+ ATPase activity (umol released mg-1 min-1)

Figure 4.3. One dimensional 12 % acrylamide SDS-PAGE gel of myofibrillar proteins from short-horn sculpin; lane 1, molecular mass marker (relative molecular masses given in KDa); lane 2, myofibrils from sculpin 32.8 cm TL ; lane 3, 29.5 cm TL; lane 4, 28.5 cm TL; lane 5, 19.0 cm TL; lane 6, 15.0 cm TL; lane 7, 8.8 cm TL; lane 8, 6.9 cm TL; lane 9, 6.2 cm TL. MHC, myosin heavy chain; Act, actin; Tm, tropomyosin; TnT, troponin-T; LC1, light chain 1; TnI_{f3}, troponin I (23 KDa isoform); TnI_{f2}, troponin I (22 KDa isoform); LC2, light chain 2; TnI_{f1}, troponin I (17 KDa isoform); LC3, light chain 3. The gel was stained with Coomasie Brilliant Blue G-250.


1
2
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66
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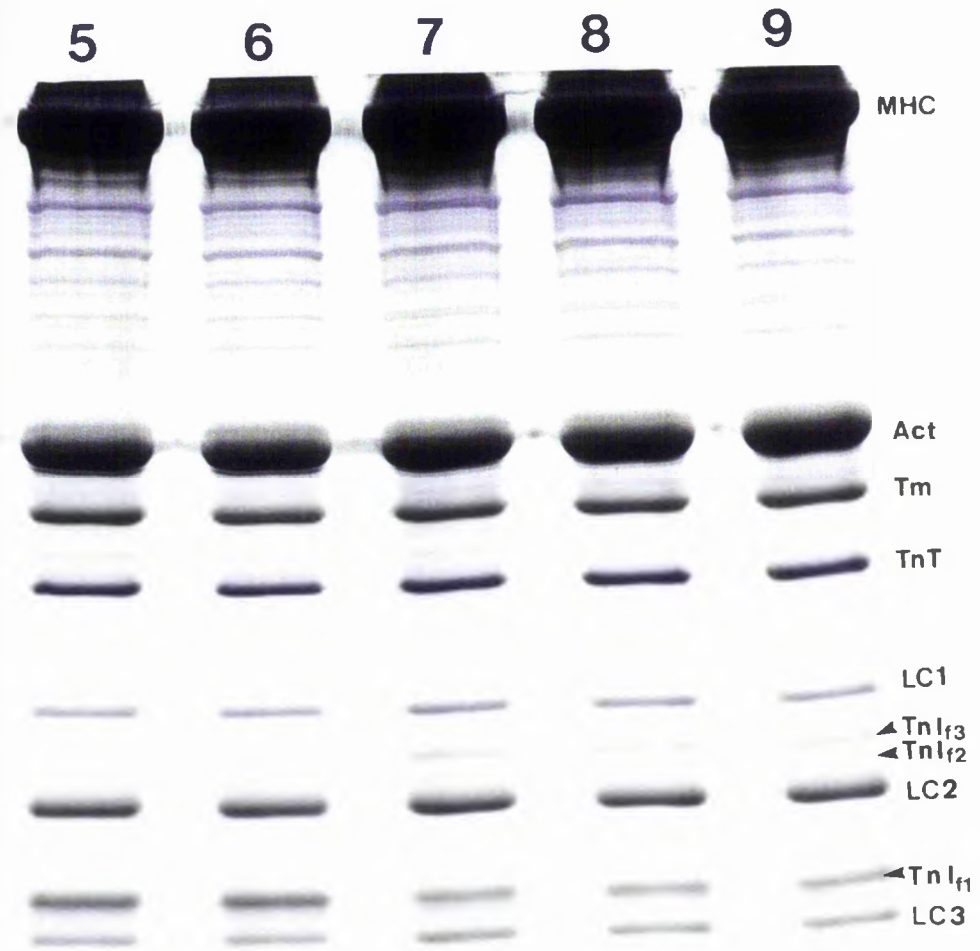

5
6
7
8
9
MHC
Act
Tm
TnT
LC1
TnI$_{f3}$
TnI$_{f2}$
LC2
TnI$_{f1}$
LC3

Figure 4.4. Densometric scans of from one dimensional 12 % SDS PAGE gel stained with Coomassie Brilliant Blue G250. (A) Densometric scan of resolved myofibrils from 32.8 cm TL sculpin, (lane 1 of Figure 4), (B) 15.0 cm TL sculpin, (lane 5 of Figure 4), (C) 6.2 cm TL sculpin, (lane 9 of Figure 4). MHC, myosin heavy chain; Act, actin; Tm, tropomyosin; TnT, troponin-T; LC1, light chain 1; TnI_{f3}, troponin I (23 KDa isoform); TnI_{f2}, troponin I (22 KDa isoform); LC2, light chain 2; TnI_{f1}, troponin I (17 KDa isoform); LC3, light chain 3.

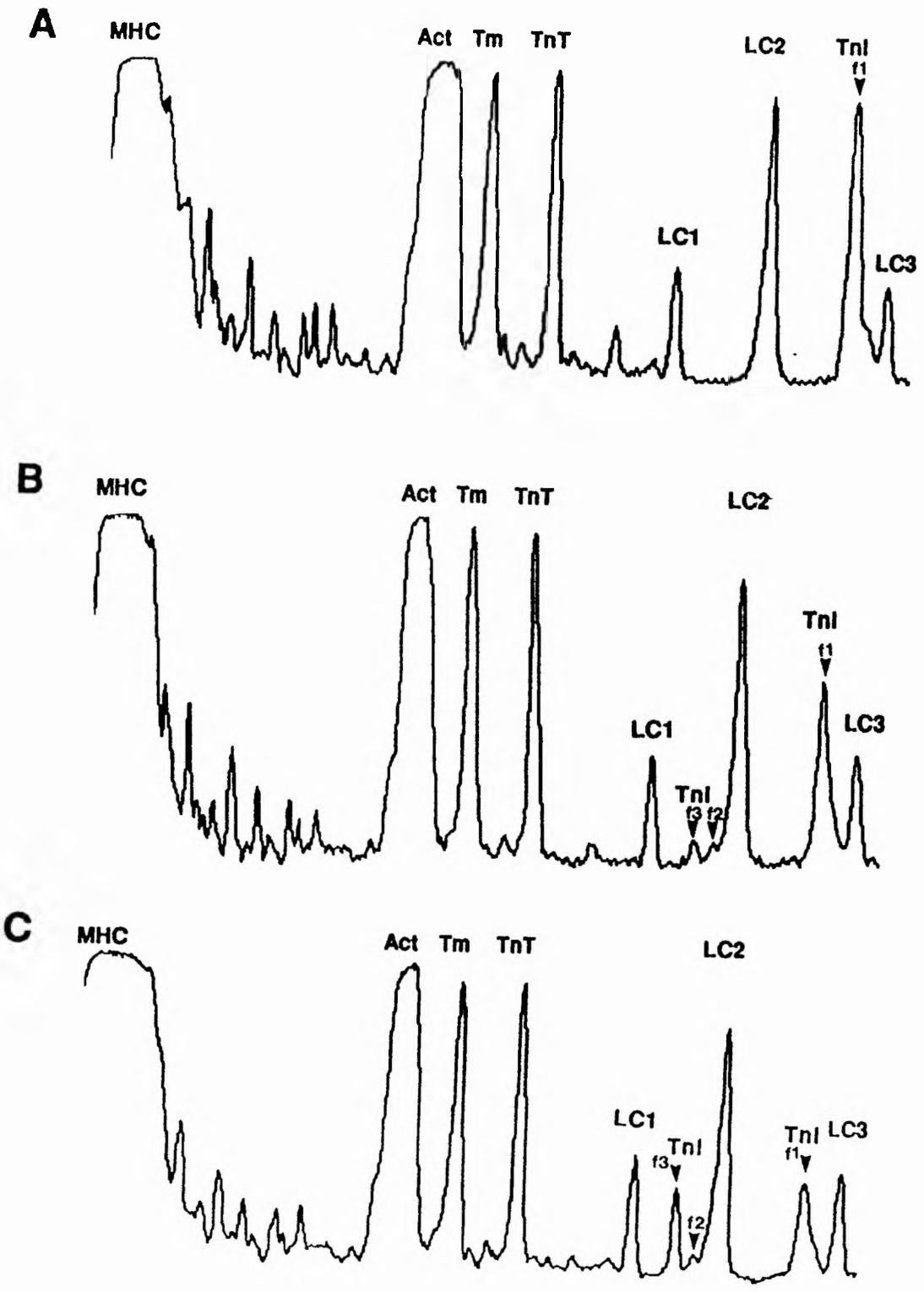
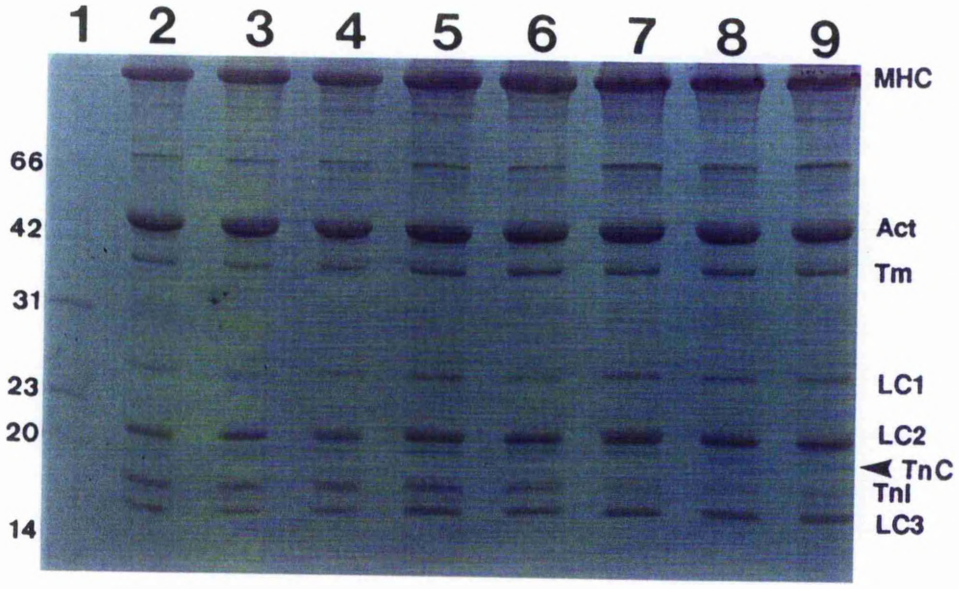

A
MHC
Act
Tm
TnT
LC2
TnI
f1
LC1
LC3
B
MHC
Act
Tm
TnT
LC2
TnI
f1
LC1
LC3
TnI
f3
f2
C
MHC
Act
Tm
TnT
LC2
LC1
TnI
f3
f2
TnI
f1
LC3

Figure 4.5. One dimensional 13 % acrylamide SDS-PAGE gel of myofibrillar proteins from short-horn sculpin. The gel was stained with 'Stains-All'; lane 1, molecular mass marker; lane 2, short-horn sculpin 32.8 cm TL; lane 3, 29.5 cm TL; lane 4, 28.5 cm TL; lane 5, 19.0 cm TL; lane 6, 15.0 cm TL; lane 7, 8.8 cm TL; lane 8, 6.9 cm TL; lane 9, 6.2 cm TL. MHC, myosin heavy chain; Act, actin; Tm, tropomyosin; TnT, troponin-T; LC1, light chain 1; TnI, troponin I (17 KDa isoform); LC2, light chain 2; TnC, Troponin C; LC3, light chain 3.


1
2
3
4
5
6
7
8
9
66
42
31
23
20
14
MHC
Act
Tm
LC1
LC2
TnC
TnI
LC3

contained relatively less of the TnI_{f3} and a relatively small amount of both the TnI_{f1} and TnI_{f2} isoforms. Myofibrils from fish 6.2, 6.9 and 8.8 cm TL, contained all three TnI isoforms, but there was a relative increase in the amount of TnI_{f3} present and a relative reduction in the TnI_{f1} isoform. Densitometric analysis (Fig. 4.4) reflected these changes in TnI isoforms with fish size. Quantitative analysis showed that myofibrils from fish of 15 and 19 cm TL contained a ratio of TnI_{f1}:TnI_{f2}:TnI_{f3} equivalent to 10: 1: 1 whereas TnI isoforms from myofibrils from fish of 9 cm and less were in the ratio 4:1:2.

The molecular masses of each protein are given in Table 4.1.

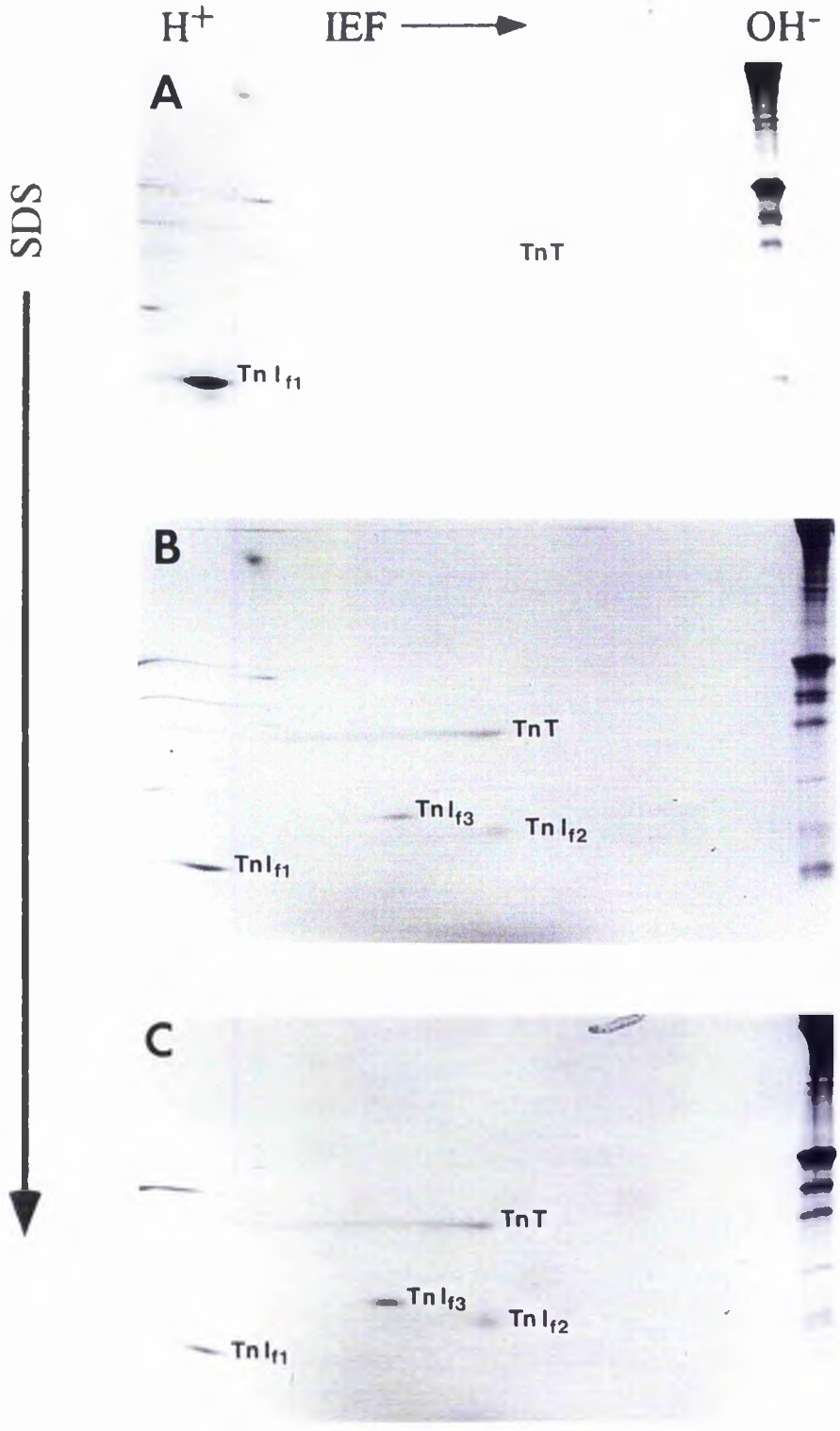
Separation of the basic proteins from the myofibrils was possible using iso-electric focusing with an extended iso-electric gradient between 9 and 11, followed by SDS PAGE. This technique has previously been shown to enable the separation of TnT and TnI (Rowlerson *et al.*, 1985; Crockford and Johnston, 1993; Johnston and Ball, 1996). TnT and TnI were clearly separated from the other myofibrillar proteins, but were distinguishable from each other because of their difference in relative molecular mass (Fig. 4.6). The difference in TnI isoform expression is clearly apparent from these gels. Myofibrils from the 32.8 cm fish (Fig. 4.6A) contained only TnI_{f1} (17×10^3 Da), whereas the 15 cm TL fish myofibrils (Fig. 4.6B) and the 6.2 cm TL fish myofibrils (Fig 4.6C) contained all three TnI isoforms.

Additional confirmation that TnI_{f1} is a TnI isoform was shown by the AU-PAGE of myofibrillar proteins (Fig. 4.7). The proteins TnI and TnC form a stable complex in the presence of 8 M urea and calcium; in the absence of calcium these proteins separate. This enables TnI and TnC to be identified by their calcium dependant migrations in AU-PAGE gels. In these gels, TnI and TnC migrate together when calcium is present but when calcium is absent, TnI does not enter the gels and TnC migration is very different. The other proteins are unaffected by the difference in calcium. Myofibrils from a

Table 4.1. Table which shows the apparent relative molecular masses of the myofibrillar components of short-horn sculpin myofibrils.

PROTEIN	Mr (KDa)
MHC	200
Act	45
Tm	38
TnT	32
LC1	26
TnI_1	23
TnI_2	22
LC2	21
TnC	20
TnI_3	17
LC3	16

Figure 4.6. Two dimensional gels (IEF in the first dimension, SDS PAGE in the second) of basic myofibrillar proteins from the myofibrils of the short-horn sculpin. (A) myofibrils from a 32.8 cm TL sculpin, (B) 15.0 cm TL sculpin, (C) 6.2 cm TL sculpin. TnT, troponin T; TnI_{f1}, troponin I (17 KDa isoform); TnI_{f2}, troponin I (22 KDa isoform); TnI_{f3}, troponin I (23 KDa isoform) . Gels were stained with Coomassie Blue G-250.


H+
IEF
OH-
A
SDS
TnT
TnI f1
B
TnT
TnI f3
TnI f2
TnI f1
C
TnT
TnI f3
TnI f2
TnI f1

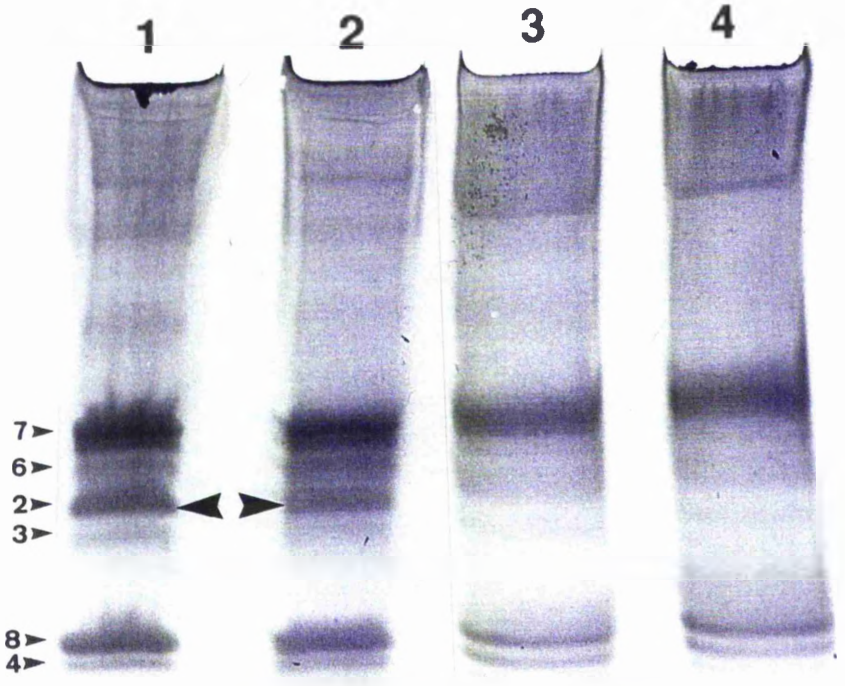
32.8 cm TL sculpin and a 6.2 cm TL sculpin in the presence of calcium (Fig. 4.7A, lanes 1-2) possessed an additional protein band (indicated by the larger arrowheads). This band was absent in the presence of EGTA (Fig. 4.7A, lanes 3-4). The additional band was cut out from the myofibrils of the 32.8 cm TL fish (Fig. 4.7A, lane 1) and run in a 1D SDS PAGE gel (Fig 4.7B, lane 2). The band migrated with the same relative molecular mass as TnI_{f1} from complete myofibrils (Fig. 4.7B, lanes 1-2).

Western blotting was carried out in order to further confirm that the different isoforms found were troponin I. Antibodies against Act and TnT were used as controls to confirm the validity of the technique. Western blotting using antibodies against Act (Fig. 4.8A), TnT (Fig. 4.8B) and TnI (Fig 4.8C) identified the respective proteins. Each blot contained some background cross reactivity but this was the same for each antibody used. The background cross reactivity is probably a result of the technique, for example, insufficient washing between steps. The actin antibody highlighted Act relatively more strongly than any of the background bands (Fig. 4.8A). The TnT antibody detected the position of TnT, again there was some relatively less intense background reaction (Fig. 4.8B). Blots developed with TnI antibody (Fig. 4.8C) had the same low level of background banding as the previous blots and there was also high reactivity with TnT and a protein of molecular mass approximately 100 x 10^3 Daltons. However, the antibody did pick out the three proteins TnI_{f1}, TnI_{f2}, TnI_{f3} with a greater intensity than the background banding (Fig. 4.8C, lanes 1-8).

The proteins Tm, LC1, LC2 and LC3 were separated by iso-electric focusing in the first dimension followed by SDS PAGE in the second dimension (Fig. 4.9A-C). These gels showed that in each fish there was a single isoform of Tm, LC1 and LC3. Figure 4.10A-C shows myofibrils from different sizes of fish mixed together, resolved by IEF followed by SDS

Figure 4.7. (A) One dimensional Alkali Urea polyacrylamide gel electrophoresis (AU-PAGE) gel of myofibrillar proteins from sculpin. Lanes 1-2 were run in the presence of free calcium, and lanes 3-4 in the presence of EGTA and therefore in the absence of free calcium; lane 1, myofibrils from short-horn sculpin 32.8 cm TL ($+Ca^{2+}$); lane 2, 6.2 cm TL ($+Ca^{2+}$); lane 3, 32.8 cm TL ($-Ca^{2+}$); lane 4, 6.2 cm TL ($-Ca^{2+}$). The larger arrowheads indicate the additional protein bands present in lanes 1 and 2, which are absent in lanes 3 and 4. The arrows adjacent to lane 1, indicate the bands which were cut out and run on the SDS PAGE gel shown in Fig. 4.7B. The numbers indicate the lane of the SDS PAGE gel shown in Fig. 4.7B that the bands were run in. The gels were stained with Coomassie Brilliant Blue G-250. (B) One dimensional, 12 % total acrylamide, SDS PAGE gel of sculpin myofibrils and bands cut from AU-PAGE gel shown in Fig. 4.7A; lane 1, myofibrils from a 32.6 cm TL sculpin; lane 2, band 2 from Fig. 4.7A lane 1; lane 3, band 3 from Fig. 4.7A lane 1; lane 4, band 4 from Fig. 4.7A lane 1; lane 5, myofibrils from a 32;6 cm TL sculpin; lane 6, band 6 from Fig. 4.7A lane 1; lane 7, band 7 from Fig. 4.7A lane 1; lane 8, band 8 from Fig. 4.7A lane 1; lane 9, molecular mass marker. Relative molecular masses given in KDa. MHC, myosin heavy chain; Act, actin; Tm, tropomyosin; TnT, troponin-T; LC1; light chain 1; TnI, troponin I (17 KDa isoform); LC2, light chain 2; LC3, light chain 3. The gel was stained with Coomasie Brilliant Blue G-250.

A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6
2
3
8
4

B

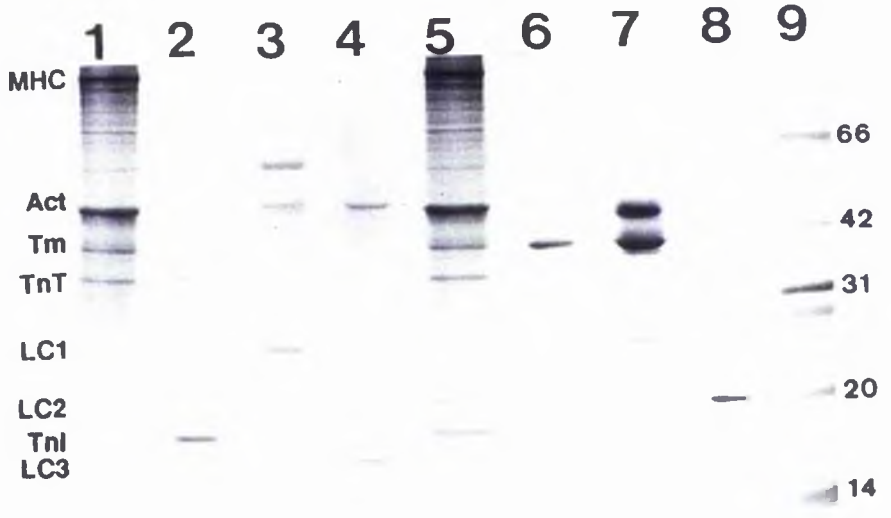
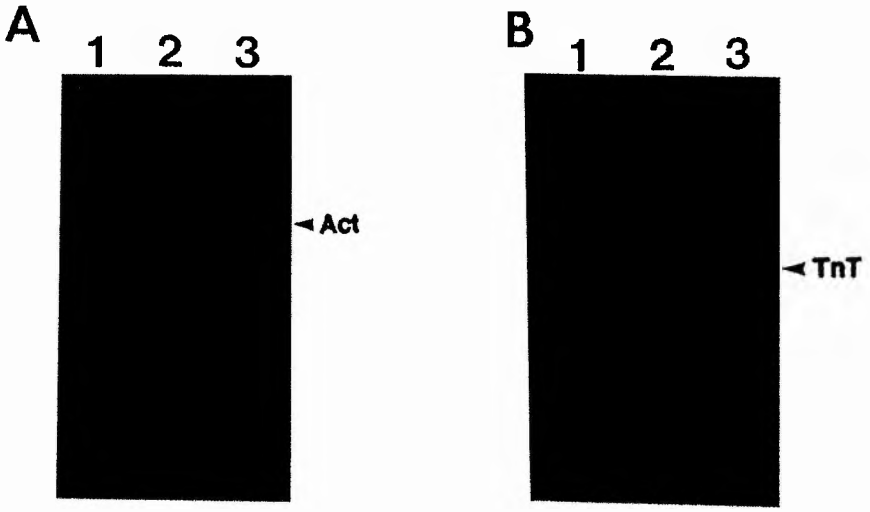

1
2
3
4
5
6
7
8
9
MHC
Act
Tm
TnT
LC1
LC2
TnI
LC3
66
42
31
20
14

Figure 4.8. Western blots of 12 % total acrylamide, one dimensional SDS PAGE gel of myofibrils from sculpin. Blots were developed using chemiluminescence onto X-ray film. (A) Blot detected with actin antibody (Clone ACT-471; Sigma, U.K.); lane 1, myofibrils from a 32.8 cm TL sculpin; lane 2, 15 cm TL sculpin; lane 3, 6.2 cm TL sculpin. Act, actin. (B) Blot detected with Troponin T antibody (Clone JLT-12; Sigma U.K.); lane 1, myofibrils from a 32.8 cm TL sculpin ; lane 2, 15 cm TL sculpin; lane 3, 6.2 cm TL sculpin. TnT, troponin-T. (C) Blot detected with Troponin I antibody (Clone C5; Zymed Laboratories, Cambridge, U.K.); lane 1, myofibrils from a 32.8 cm TL sculpin; lane 2, 29.5 cm TL sculpin; lane 3, 28.5 cm TL sculpin; lane 4, 19 cm TL sculpin; lane 5, 15 cm TL sculpin; lane 6, 8.8 cm TL sculpin; lane 7, 6.9 cm TL sculpin; lane 8, 6.2 cm TL sculpin. TnT, troponin-T; TnI_{f3}, troponin I (23 KDa isoform); TnI_{f2}, troponin I (22 KDa isoform); TnI_{f1}, troponin I (17 KDa isoform).


A
1 2 3
Act
B
1 2 3
TnT

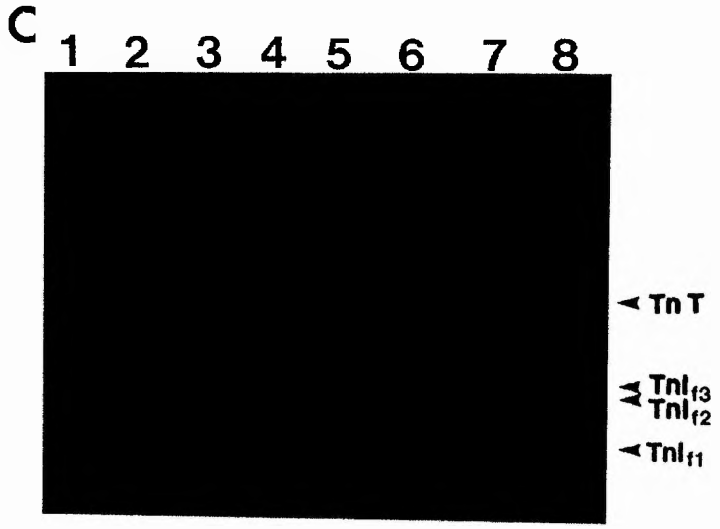

C
1 2 3 4 5 6 7 8
Tn T
Tnl f3
Tnl f2
Tnl f1

Figure 4.9. Two dimensional gels (IEF in the first dimension, SDS PAGE in the second) of acidic myofibrillar proteins from the myofibrils of sculpin. (A) myofibrils from a 32.8 cm TL sculpin, (B) 15.0 cm TL sculpin, (C) 6.2 cm TL sculpin. Tm, tropomyosin; LC1, light chain 1; $LC2_{f1}$, light chain 2 basic pI isoform; $LC2_{f2}$, light chain 2 acidic pI isoform; LC3, light chain 3. The gels were stained with Coomassie Brilliant Blue G-250.

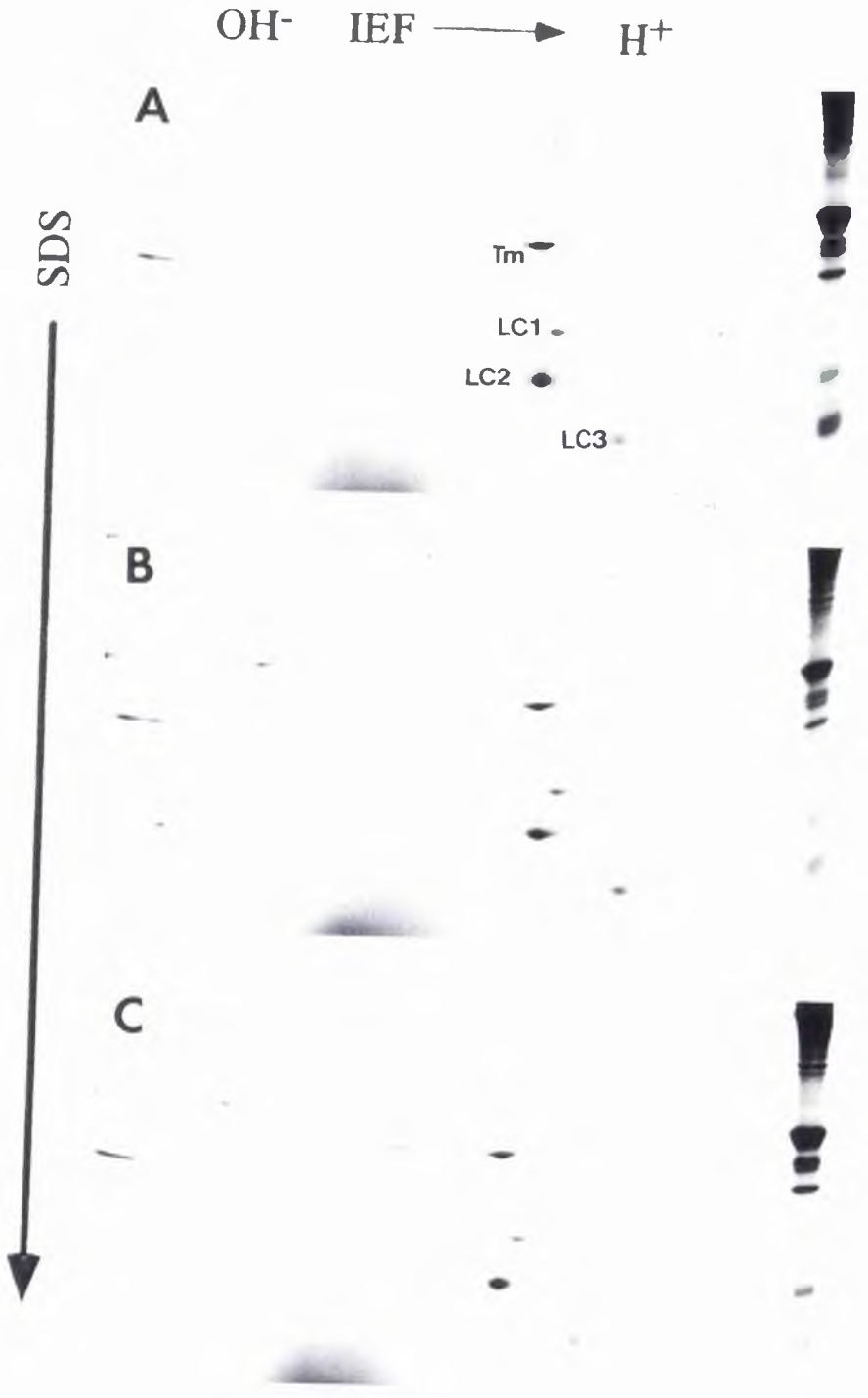
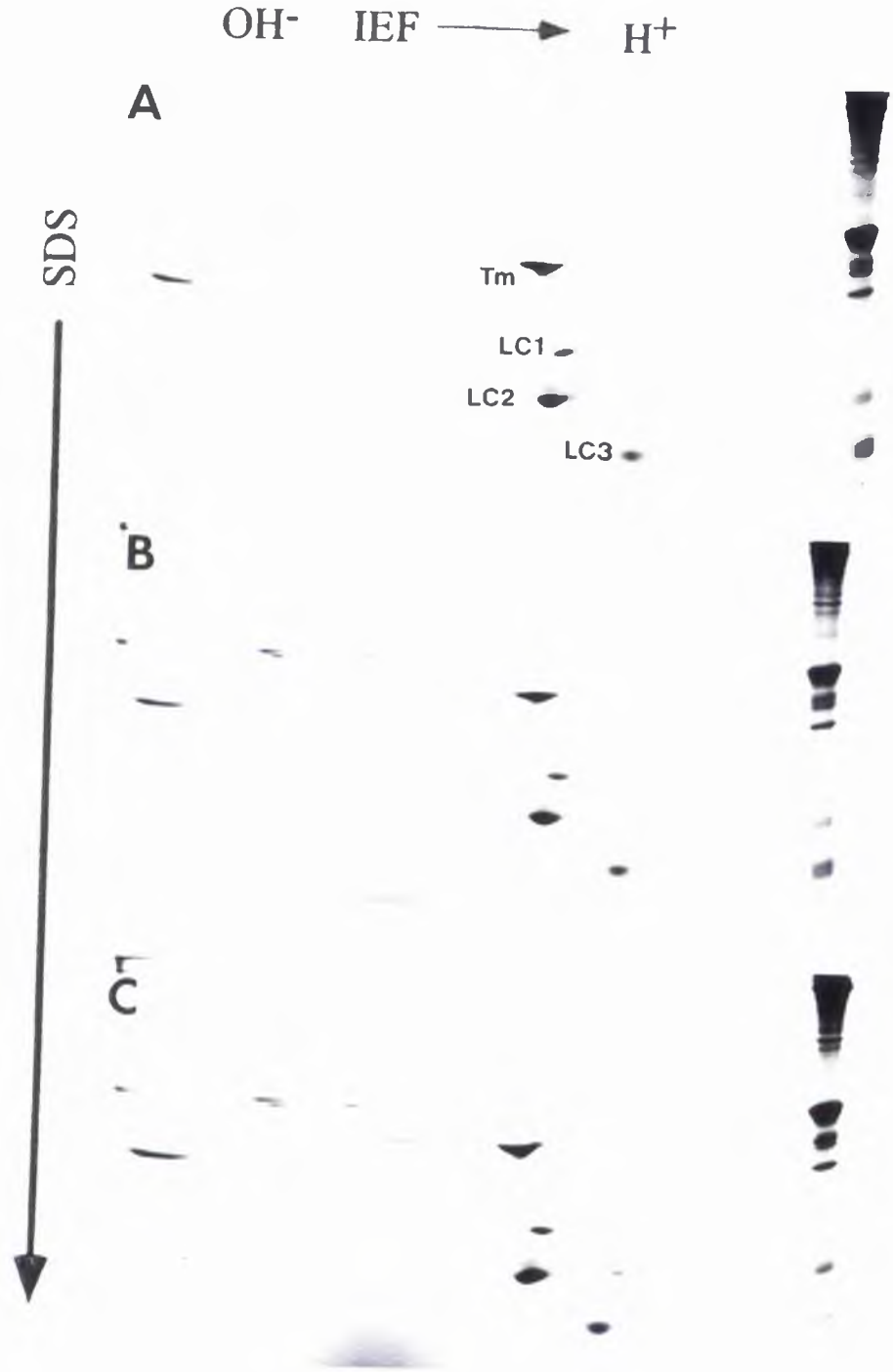

OH-
IEF
H+
A
SDS
Tm
LC1
LC2
LC3
B
C

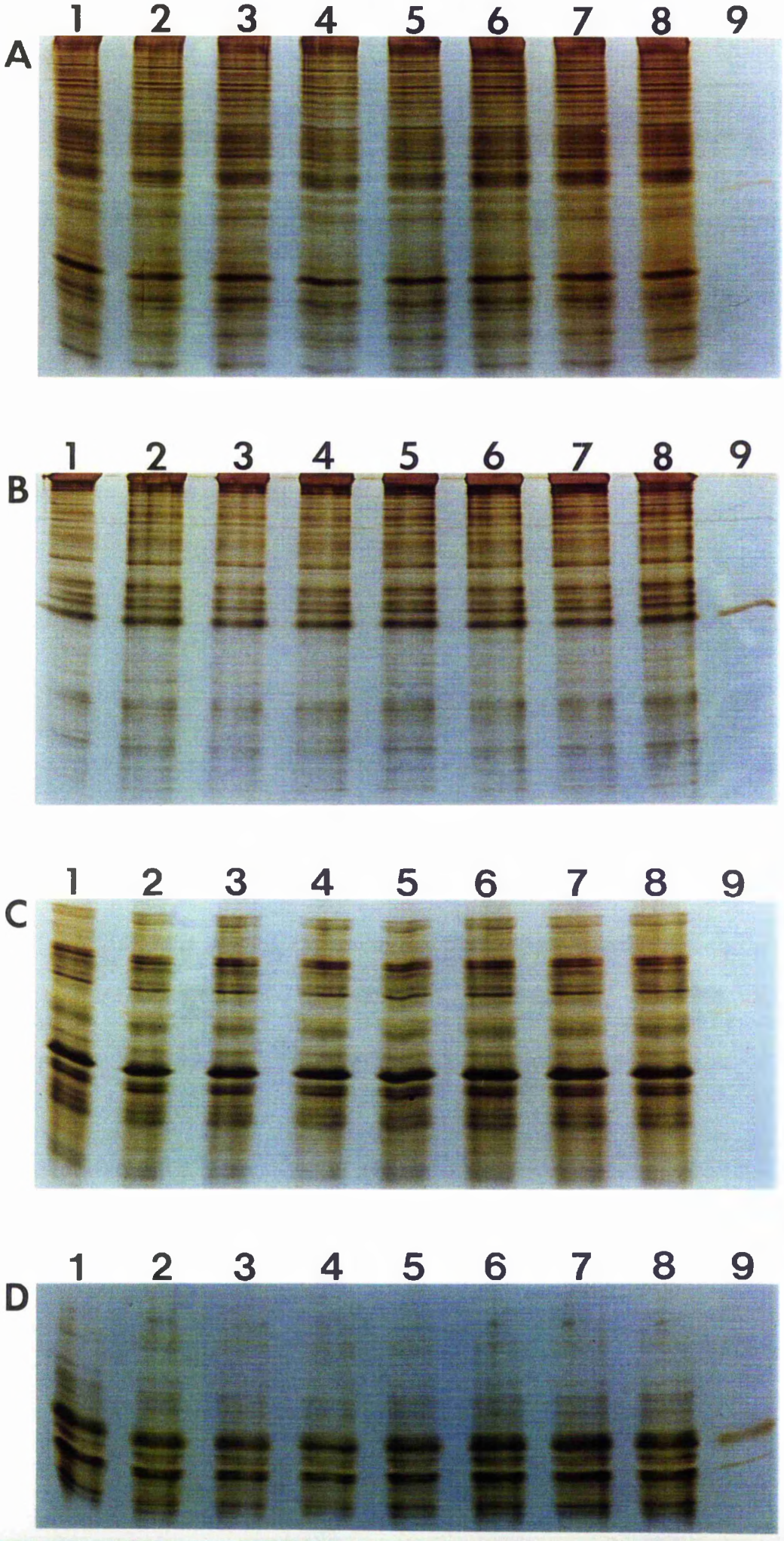
Figure 4.10. Two dimensional gels (IEF in the first dimension, SDS PAGE in the second) of acidic myofibrillar proteins from the myofibrils of sculpin. (A) Mixture of myofibrils from a 32.8 cm and 15 cm TL sculpin, (B) mixture of myofibrils from a 32.8 cm and 6.2 cm TL sculpin, (C) mixture of myofibrils from a 15 cm and 6.2 cm TL sculpin. Tm, tropomyosin; LC1, light chain 1; $LC2_{f1}$, light chain 2 basic pI isoform; $LC2_{f2}$, light chain 2 acidic pI isoform; LC3, light chain 3. The gels were stained with Coomassie Brilliant Blue G-250.

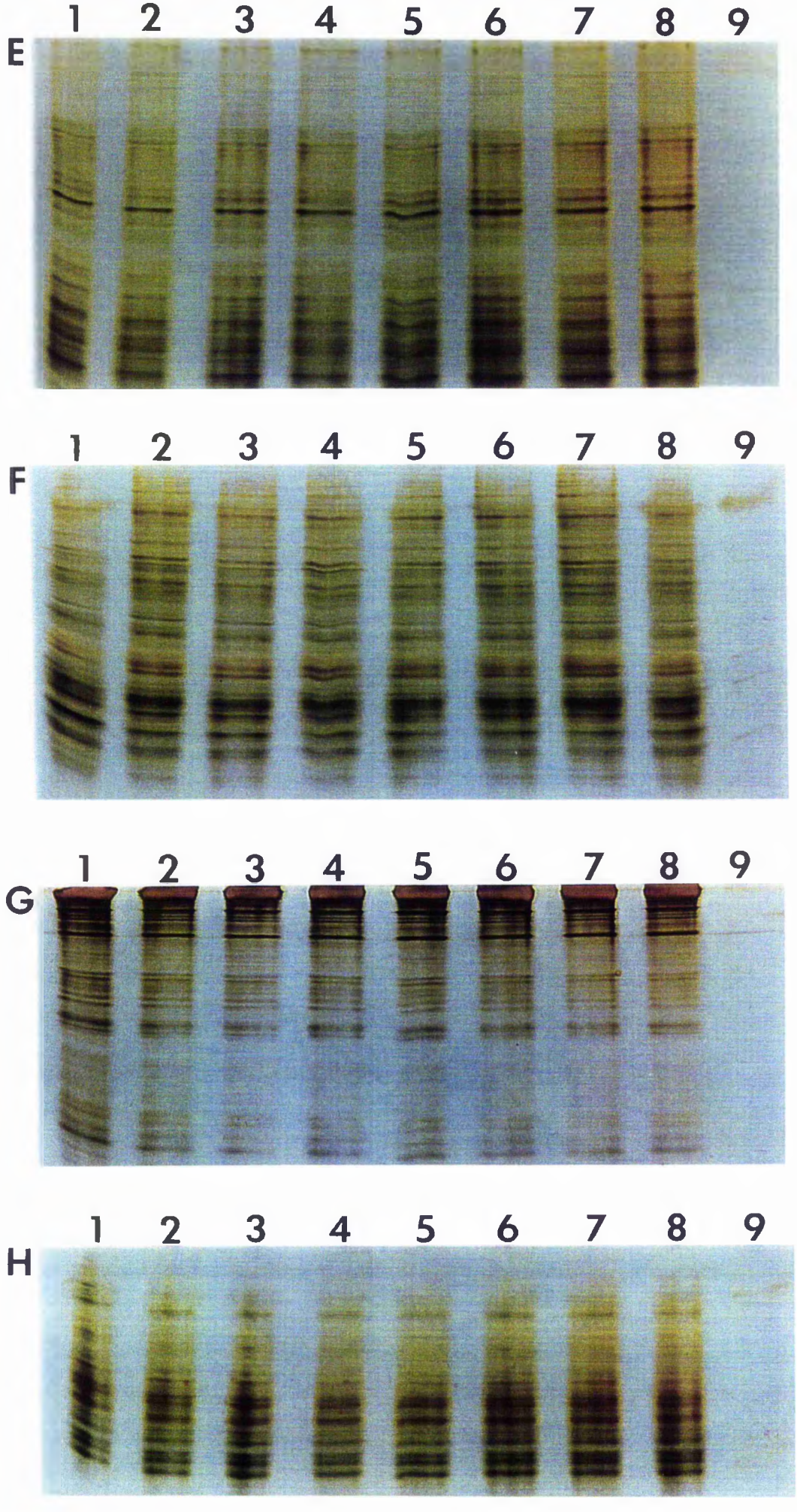

OH⁻ IEF H⁺
A
SDS
Tm
LC1
LC2
LC3
B
C

PAGE. The acidic proteins from fish of different sizes showed no differences in relative molecular masses of iso-electric points.

There was no observable difference in MHC with increasing fish length. Electrophoretically purified MHC digested by trypsin and resolved on a 15 % acrylamide gel, produced the same banding pattern for each fish length (Fig. 4.11A-H). Eight enzymes were used in total. Each enzyme produced a different banding pattern when digesting MHC (Fig. 4.11A-H). In each case there was no difference in banding pattern with increased fish length. (Fig 4.11A-H, lanes 1-8). Lane 9 in each gel contained the enzyme only, showing that the banding pattern observed was caused by the digestion of MHC.

Figure 4.11. Peptide digests of electrophoretically purified sculpin myosin heavy chains from one dimensional SDS PAGE gels, resolved on 15 % total acrylamide SDS PAGE gels. The gels are stained with silver using the Plus One silver stain kit (Pharmacia Biotech). The enzymes used were (A) trypsin from bovine pancreas, (B) endoproteinase Glu-C from *Straphylococcus aureus* V8, (C) elastase from porcine pancreas, (D) α-chymotrypsin from bovine pancreas, (E) papain from papaya latex, (F) thermolysin from *Bacillus thermoproteolyticus rokko*, (G) clostropain from *Clostridium histolyticum*, (H) ficin from fig tree latex; lane 1, digested MHC from 32.8 cm TL sculpin; lane 2, 29.5 cm TL sculpin; lane 3, 28.5 cm TL sculpin; lane 4, 19.0 cm TL sculpin; lane 5, 15.0 cm TL sculpin; lane 6, 8.8 cm TL sculpin; lane 7, 6.9 cm TL sculpin; lane 8, 6.2 cm TL sculpin; lane 9, enzyme only.


1 2 3 4 5 6 7 8 9
A
1 2 3 4 5 6 7 8 9
B
1 2 3 4 5 6 7 8 9
C
1 2 3 4 5 6 7 8 9
D


1 2 3 4 5 6 7 8 9
E
1 2 3 4 5 6 7 8 9
F
1 2 3 4 5 6 7 8 9
G
1 2 3 4 5 6 7 8 9
H

4.4 Discussion

Myofibrillar ATPase activity was found to decrease with increasing fish body length, such that the ATPase activity scaled to 2.51 μmol mg^{-1} min^{-1} $L^{-0.28}$. Previously, Witthames and Greer-Walker (1982) examined the ATPase of five species of marine teleosts in relation to their age and hence body size. They also found that ATPase activity declined with fish length in all five species. They related the reduction in ATPase activity to a decrease in the *in vitro* muscle contraction rate, but did not analyse myofibrillar proteins of the fish examined. The general decrease in ATPase activity with increasing fish length correlates with the decreased length-specific muscle contraction velocities observed in fish swimming.

The molecular basis of alterations in ATPase activity in cyprinid fish species (Johnston *et al.*, 1975; Heap *et al.*, 1986; Johnson and Bennett, 1995) has been attributed to altered expression of myosin heavy chain (MHC) genes (Gerlach *et al.*, 1990; Hwang *et al.*, 1991). Bottinelli *et al.* (1994b, 1996) and Steinen *et al.* (1996) showed that MHC composition is a major factor in determining the myofibrillar ATPase activity of muscle during isometric contractions. Hwang *et al.* (1990) also attributed changes in carp myosin ATPase induced by temperature acclimation to alterations in myosin heavy chain (MLC) composition. However, Wagner and Weeds (1977) indicated that the activation of myosin ATPase by actin is regulated by the alkali light chains which have been found to be essential for ATPase activity (Weeds and Pope, 1971). In contrast, more recently Lowey *et al.* (1993) used an *in vitro* motility assay that demonstrated that myosin ATPase activity was not affected by the removal of the light chain subunits. Ball and Johnston (1996) found a variation in the ratio of myosin alkali light chain contents but no variation in MHC expression, with temperature acclimation in the fast muscle of the short-horn sculpin. In their study the myofibrillar ATPase

activity was not altered, again indicating that MLCs do not influence ATPase activity in this species.

In this study of the fast muscle of the short-horn sculpin, there was no variation in MHC, MLCs, tropomyosin (Tm), troponin T (TnT) or troponin C (TnC) isoform content with increasing fish length. Therefore changes in ATPase activity cannot be attributed to the alteration in MHC or MLC composition in this study. It is possible that peptide mapping was not sufficiently sensitive to detect changes in the amino acid structure of the MHCs. For example, the MHCs may have differed by only a few amino acids and/or the distinct amino acids in the sequence were not affected by the particular enzymes used. Since the MHCs were digested with 8 separate enzymes the differences between MHCs would have to be slight to remain undetected.

The major finding was a difference in TnI isoform expression. Large fish (>28 cm TL) contained a single TnI isoform whereas the small fish examined (<9 cm TL) contained three TnI isoforms. TnI is the inhibitory sub-unit of the troponin complex (TnI, TnT, and TnC), which is associated with the thin filament and controls the calcium-mediated contraction of striated muscle (see Zot and Potter, 1987, for review). This is the first time that differences in TnI expression with altered body size have been found, with the exception of the data in Chapter 3 which reports complex developmental changes in TnI expression in the early larva of the Atlantic herring, *Clupea harengus*.

In mammals three TnI isoforms have been identified and each is encoded by a separate gene (Zhu *et al.*, 1995). Slow skeletal and fast skeletal TnI are differentially expressed in slow twitch and fast twitch muscle fibres, respectively (Dhoot and Perry, 1979; Koppe *at al.*, 1989). The cardiac TnI isoform is exclusively expressed in the adult heart (Sabry and Doot,

1989). At birth the developing heart contains predominately the slow skeletal isoform but this eventually disappears as development proceeds (Murphy *et al.*, 1991; Sasse *et al.*, 1993; Schiaffino *et al.*, 1993). Two forms of TnI have been identified in bovine fast *sternomandibularis* muscle fibres, a ventral neck muscle used for lowering the head (Young and Davey, 1981). Each had a different electrophoretic mobility from the characteristic forms found in slow fibres. This is the only report to date of adult mammals with multiple TnI isoforms in the same muscle fibre.

TnI inhibits the Ca^{2+} induced myosin ATPase activity and thus contraction (Weber and Murray, 1973). Although TnI isoform expression has never been correlated with myofibrillar ATPase activity, there have been a number of studies linking the members of the troponin complex to ATPase activity. Horwitz *et al.* (1979) measured ATPase activity of rabbit muscle with the troponin complex removed and found that without the troponin complex, there was no calcium sensitivity, and there was a 62.5 % inhibition of ATPase activity when troponin complex was restored. Perry (1985) suggested that TnI isoforms may differentially alter the Ca^{2+} binding characteristics of troponin C, and Hartshorne *et al.* (1972) showed that the binding of Ca^{2+} to its receptor site on troponin C caused a discontinuity in Arrhenius plots of natural rabbit actomyosin $Mg^{2+}Ca^{2+}$ ATPase at 15-20 °C. When the Ca^{2+} regulatory protein was removed the plots were linear over the same temperature range (Hartshorne *et al., 1972*; Fuchs *et al.*, 1975).

Johnston (1979) concluded that in goldfish the regulatory proteins can influence the kinetics of ATPase activity. The calcium regulatory proteins appear to adapt following thermal acclimation causing a modification of actin-myosin interactions in addition to their more familiar role in excitation contraction coupling. Penney and Goldspink (1980) also reported that the regulatory proteins are responsible for the differences in heat tolerance of

ATPase activity from warm- and cold-acclimated fish. These differences disappeared when the regulatory proteins were removed. These authors concluded that on acclimation, the actomyosin system remains unchanged but its properties are in some way modified by the regulatory proteins. More recently, Guo and Watabe (1992) failed to find a temperature dependent effect of regulatory proteins on the activity or thermostability of actomyosin ATPase in carp.

During escape responses in the short-horn sculpin, James and Johnston (1997) found that maximum muscle mass-specific power output during the first propulsive stroke was independent of body length, despite the fact that the kinematics change with size. Power output remained constant as a result of alterations in twitch and tetanus times and maximum shortening velocity (V_{max}). As body length increased, twitch and tetanus activation and relaxation times increased and V_{max} decreased. V_{max} scaled to body length with values similar to those found for mammalian fast muscle fibres (Rome *et al.*, 1990; Seow and Ford, 1991). The alterations in the twitch and tetanus contraction times may be partially related to the changes in TnI expression with body length. In previous studies, decreases in isometric muscle activation and relaxation time due to an increase in calcium sensitivity, have been correlated with alterations in TnT isoforms in both the dragonfly (Fitzburgh and Marden, 1997) and chicken (Reiser *et al.*, 1992). The muscles used in this study were examined under the electron microscope and no variations in sarcomere I filament lengths or Z line width were observed. Therefore the changes in contractile properties, such as V_{max}, could not be related to structural differences between the muscles of different sized fish.

The findings of Dr. James are consistent with previous studies on mammals (Rome *et al.*, 1990) and amphibians (Altringham *et al.*, 1996) in

that, as body size increased there was a relatively greater alteration in relaxation times than activation times and V_{max}. Muscle activation times and V_{max} are thought to remain fairly constant over a range of body sizes to maintain the necessary speed of movement required for burst swimming (Rome *et al.*, 1990; Altringham *et al.*, 1996). Rapid relaxation times are of relatively less importance for burst swimming than the rate of muscle activation and so alter with body size, possibly as a means of avoiding the large increases in energetic costs of calcium pumping involved with decreases in relaxation time.

In conclusion, TnI was the only protein to be expressed in a size-dependent manner and no variation in muscle filament lengths was observed in different sized fish. It appears most likely that the differential expression of TnI with body size contributes to observed differences in contractile properties found in this study, since TnI is an integral part of the troponin complex. An alteration in any component of the molecular motor is likely to have some effect on the interaction of the proteins, and therefore effect contractile properties. For example, a change in the components of the troponin complex is likely to alter its interaction with Tm. This in turn would alter the cyclic interaction of myosin and actin, and the hydrolysis of ATP, contributing to the observed differences in ATPase activity and contractile properties. It may one day be possible to test this hypothesis by substituting the TnI isoforms found in muscle fibres from different sized fish *in vitro*, and determining the subsequent alterations in contractile properties.

Chapter 5

A comparison of the myofibrillar proteins of Antarctic and sub-Antarctic fish

5.1 Introduction

Studies on the denaturation kinetics of myofibrillar ATPase activity have correlated normal body temperature with variations in myosin structure and function. Johnston *et al.* (1975) showed that myosin ATPase activity was much higher at low temperature (0-10 °C) in Antarctic, than in tropical species. At higher temperatures, Antarctic fish ATPase activity decreased significantly. They suggested this was due to an evolutionary response to attain high catalytic efficiency at low temperatures.

The myosins of Antarctic fish are thus highly specialised for function at low temperature (Johnston *et al.* 1975; Johnston and Walesby, 1977). Antarctic fish myosins are unstable, aggregate on storage, and myofibrillar ATPase rapidly denatures (Connel, 1961; Hamoir *et al.*, 1960; Richards *et al.*, 1967; Syrovy *et al.*, 1970). This instability is thought to reflect changes in myosin tertiary structure (Johnston and Walesby, 1977). Antarctic fish muscles have been found to be the most susceptible to tryptic digestion and titration of SH groups with sulphydryl blocking agents (Connell, 1961; Johnston *et al.*, 1975), which has been used as evidence to suggest that the tertiary structure of myosins from Antarctic fish is more 'open' than that of warm water species. Antarctic myosin may be a more loosely folded protein, stabilised by fewer weak bonds, and therefore be capable of more energetically favourable conformational changes at low temperatures (Johnston and Altringham, 1988). The cold adapted structure of the myosin molecule has been implicated as the cause of high levels of force production

at 0 °C during both isometric contractions (Johnston and Brill, 1984; Johnston and Altringham, 1985) and cyclical contractions (Franklin and Johnston, 1997). The cold adapted tertiary structure of myosin may have important implications in limiting the geographical distributions of polar species.

In Antarctic fish, some of the structural and contractile properties of fast and slow muscle fibres are similar to those of temperate species (Johnston, 1989). However, in contrast to other teleosts studied, histochemical methods for measuring myosin ATPase activity have been unable to distinguish between different fibre types in Antarctic and sub-Antarctic species (Davidson and MacDonald, 1985; Harrison *et al.*, 1987; Dunn *et al.*, 1989).

The relative proportions and distributions of slow and fast fibres in pectoral fin muscles have been correlated with activity levels of teleost species. For example, the Antarctic cryopelagic species, *P. borchgrevinki* , is a low density fish that relies completely on labriform locomotion for slow cruising. The pectoral fin adductor muscle is a simple muscle consisting of a single class of relatively small (49 μm) aerobic fibres (Davidson and Macdonald, 1985). In contrast, the pectoral fin muscles of the Antarctic *T. bernacchii,* have a mosaic portion containing a high proportion of larger diameter white fibres, which may provide a few fast strokes to lift this more dense, demersal species off the substrate to capture prey (Davidson and Macdonald, 1985).

The first aim of this study was to determine if the proteins present in Antarctic fish were unique to this low temperature environment. This was achieved by characterising the myofibrillar proteins from the myotomal fast muscles and the *m. adductor profundis* muscle of Antarctic and sub-Antarctic fish species. The second aim of this study was to determine if the two muscle

types contained distinct protein isoforms, characteristic of fast and slow muscles. This was accomplished by characterising and comparing the myofibrillar protein isoforms present in myotomal fast fibres with the *m. adductor profundis* slow fibres of each species. The third aim of this study was to determine if the myofibrillar protein isoforms present in the red muscle could be related to the activities of the pectoral fins during locomotion.

The Antarctic species studied were all members of the sub-order Notothenioidei and family Nototheniidae. This is the most diverse family of Antarctic fish in terms of body size, form, habitat and distribution. The fish used included four species of the genus *Trematomus* which occupy different sub-habitats (DeVries and Eastman, 1981): *Trematomus pennelli* (Regan, 1914) is benthic and sedentary; *Trematomus bernacchii* (Boulenger, 1902) is epibenthic and active; *Trematomus hansoni* (Boulenger, 1902) is benthic and active; *Trematomus newnesi* (Boulenger, 1902) is semipelagic and active. The fifth Antarctic species examined was *Pagothenia borchgrevinki* (Boulenger, 1902), a cryopelagic Nototheniid which lives under the surface of the sea ice and exhibits substantial morphological specialisations for life in the water column (Eastman and DeVries, 1985). This species was originally described as a Trematomid, but was later placed in *Pagothenia* by Andriashev and Jakubowski (1971).

The sub-Antarctic species examined occur in the Beagle Channel, Tierra del Fuego, Argentina. Four of the species were of the sub-order Notothenioidei and family Nototheniidae. *Paranotothenia magellanica* (Forster, in Bloch and Schneider, 1801) is a semipelagic species (DeWitt *et al.*, 1990). *Eleginops maclovinus* (Cuvier, in Cuvier and Valenciennes, 1830) is one of only 2 euryhaline species in the sub-order. It has a non-Antarctic distribution and has been found to routinely breed in estuaries. It lives in coastal waters around the Falklands, estuaries and rivers along South

America as far north as Uruguay (35 °S), and the East coast of Chile (37 °S). Eastman (1993) suggests that the distribution of *Eleginops* reflects an original distribution pattern on the South American component of the Gondwanan shelf. *Eleginops* did not become associated with the margins of the Antarctic plate and hence its subsequent evolution has been little influenced by the Tertiary cooling of the Southern Ocean. *Eleginops* has a heavy skeleton, little cartilage, no significant lipid deposits, a streamlined body and no substrate contact adaptations (Eastman, 1993). With the exception of the pectoral muscles and the thin *lateralis superficialis* down the lateral line, the body musculature consists of white fibres. *Eleginops* is a subcarangiform burst swimmer. The two remaining Nototheniids were *Patagonotothen cornucola* (Richardson, 1844) and *Patagonotothen tessellata* (Richardson, 1844). These species are labriform swimmers, endemic to Patagonia. The non-Notothenioid fish was *Austrolycos depressiceps* from the sub-Order Zoarcoidei and Family Zoarcidae. Zoarcidae are elongated fish, with confluent dorsal, anal and caudal fins. They are generally sluggish, benthic, epi- or infaunal predators, living at low temperatures (Anderson, 1984).

5.2 Materials and Methods

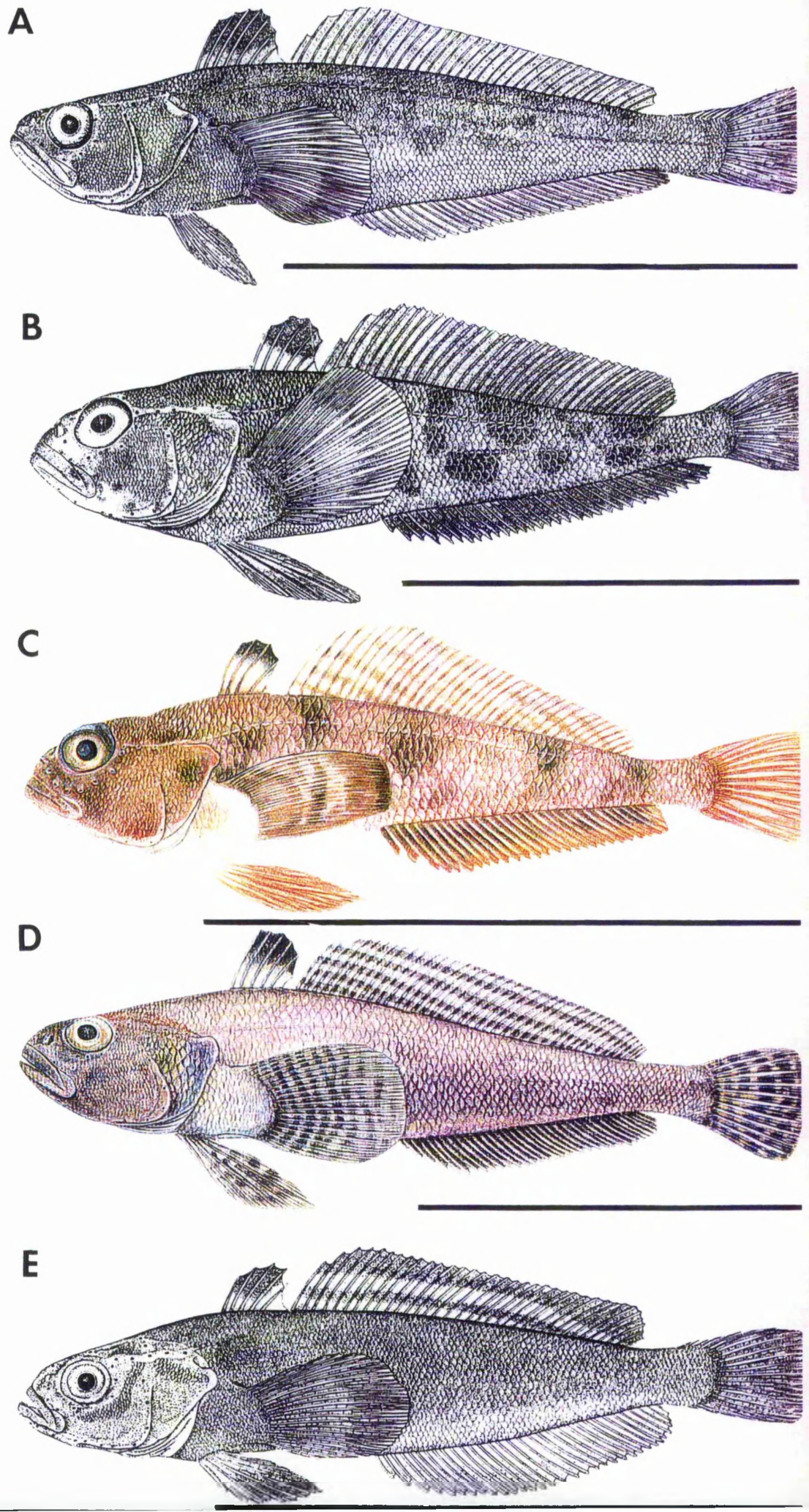
Antarctic fish were collected from Ross Island, Antarctica (76 ° 81 'S, 174 ° 28 'E) in January 1996. Fish were captured by baited trap or fishing lines dropped through ice holes in the Ross Ice Shelf. The sea water temperature beneath the ice shelf was -1.83 ± 0.1 °C. Sub-Antarctic fish were collected in January 1997, in baited traps placed in the Beagle Channel, Tierra del Fuego, Argentina (55 ° 39 'S, 68 ° 59 'W). The sea water temperature in the Beagle Channel was 8.5 ± 0.4 °C at the time of sampling.

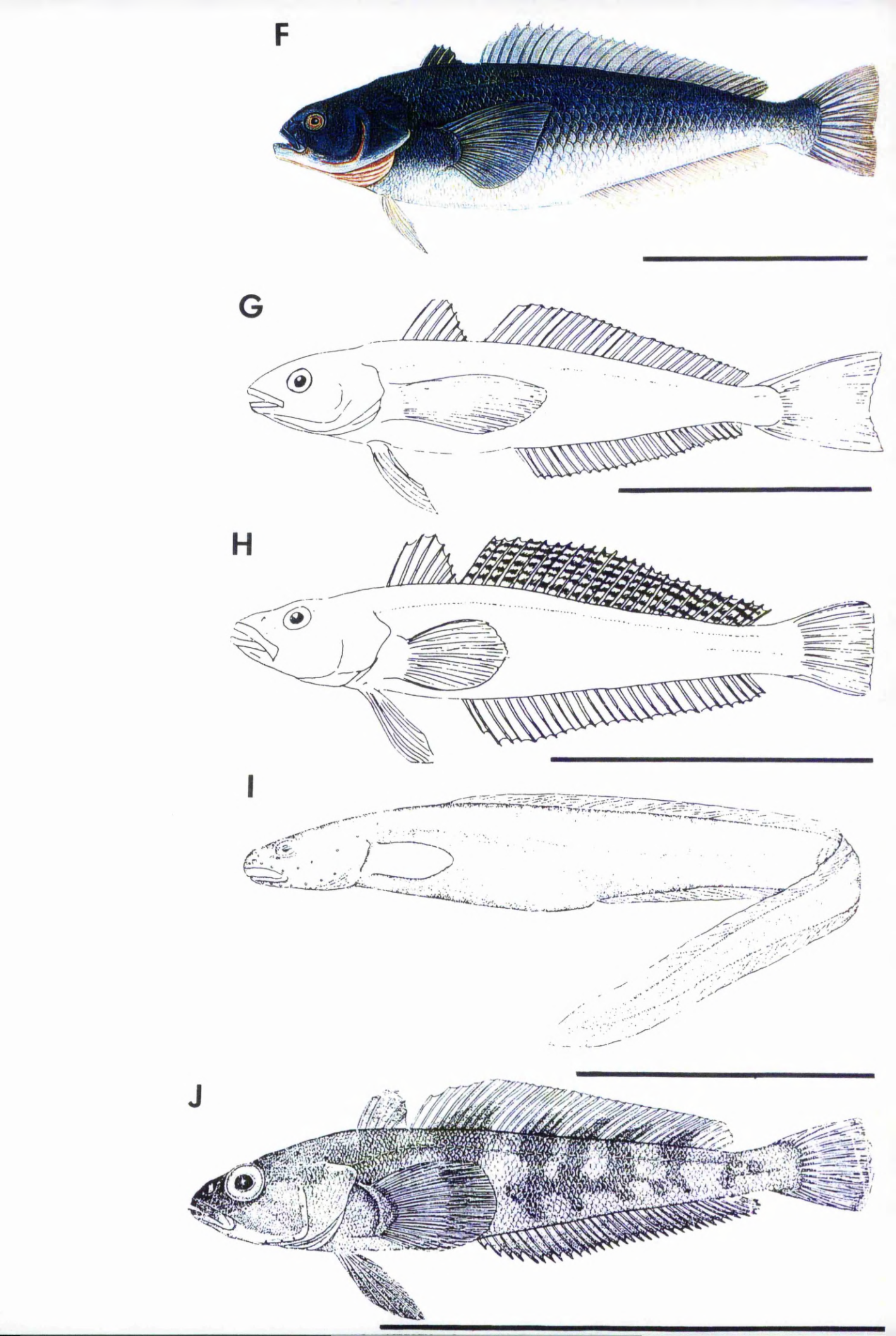
The Antarctic species total length and masses were: *Trematomus newnesi* (104-148 mm, 84-106 g), *Trematomus bernacchii* (154-179 mm, 102-122 g), *Trematomus pennelli* (78-117 mm, 56-83 g), *Trematomus hansoni* (145-166 mm, 87-115 g), and *Pagothenia borchgrevinki* (113-143 mm, 64-88 g). These species are illustrated in Figure 5.1A-E.

The Sub-Antarctic species total length and masses were: *Paranotothenia magellanica* (250-280 mm, 232-267 g) *Eleginops maclovinus* (307-358 mm, 354-469 g), *Patagonotothen tessellata* (190 mm, 96-128 g) *Austrolycos depressiceps* (314-412 mm, 235-283 g), and *Patagonotothen cornucola.* (91-112 mm, 67-89 g). These species are illustrated in Figure 5.1F-J.

Four individuals of each species were collected and returned live to the laboratory cold room and killed by a blow to the head followed by decapitation. On ice, approximately 2 g of white muscle was dissected from the myotomes level with the dorsal fin, approximately 0.35 body lengths from the snout. The dissection was carefully performed in order to not contaminate the sample with red muscle fibres which follow the lateral line, immediately beneath the skin. Red muscle was collected from *m. adductor profundis* muscle from each species. This muscle is the largest by mass of

Figure 5.1A-J. Fish used in the experiments. Antarctic species (A-E), sub-Antarctic species (F-J). The illustrations are taken from Gon, O. & P. C. Heemstra (eds.) 1990. *Fishes of the Southern Ocean*. J. L. B. Smith Institute of Ichthyology, Grahamstown, 462 pp. 12 pls. Scale bar represents 100 mm. (A) *Trematomus newnesi*, (B) *Trematomus bernacchii*, (C) *Trematomus pennelli*, (D) *Trematomus hansoni*, (E) *Pagothenia borchgrevinki*, (F) *Paranotothenia magellanica*, (G) *Eleginops maclovinus*, (H) *Patagonotothen tessellata*, (I) *Austrolycos depressiceps*, (J) *Patagonotothen cornucola*.


A
B
C
D
E


F
G
H
I
J

the pectoral fin adductor muscle assemblage. It is situated at the anterior region of the cleithrum around the border of the pelvic girdle. The pale tissue and tendons which ran along the length of the muscle were carefully removed and discarded and a bundle of red fibres of total mass 1 g was collected. Separate samples were prepared from each individual.

Washed myofibrils were prepared from each sample as described in Chapter 2. The myofibrils were homogenised in SDS PAGE, and IEF PAGE buffer and stored frozen (-20 °C) ready for electrophoresis as described in Chapter 2.

5.3 Results

The electrophoretic techniques used in this study enabled myosin heavy chain (MHC), actin (Act), tropomyosin (Tm), troponin T (TnT), alkali light chains 1 and 3 (LC1, LC3), and regulatory light chain 2 (LC2), from each species to be separated and characterised. Troponin I (TnI) from myotomal white muscle was also separated and identified by electrophoresis. From this point onwards, the myofibrils of myotomal white muscle will be referred to as 'white' and myofibrils of red fibres from the pectoral fin adductor muscle will be referred to as 'red' for clarity.

5.3.1 Myosin Heavy Chains

Myosin heavy chain (MHC) migrated as a single band, with a relative molecular mass (Mr) of 205 kDa, in both white and red muscle of all the species examined when resolved in 1D-SDS PAGE gels. (Fig. 5.2 and 5.3).

One dimensional electrophoresis alone was not sufficient to distinguish between isoforms of MHC. Therefore peptide mapping was used. The banding patterns produced by each digest were then compared by calculating the estimate of difference (D) (method as described in chapter 2).

MHCs were electrophoretically purified by 1D-electrophoresis from white and red myofibrils of the Antarctic and sub-Antarctic fish and digestion with papain (Fig. 5.4A), thermolysin (Fig. 5.4B), elastase (Fig. 5.4C), trypsin (Fig. 5.4D), endoproteinase Glu-C (Fig. 5.4E) and α-chymotrypsin (Fig. 5.4F). Each enzyme produced a unique banding pattern for the same species and muscle type, for example, compare lane 3 of Figure 5.4A-F.

Antarctic species

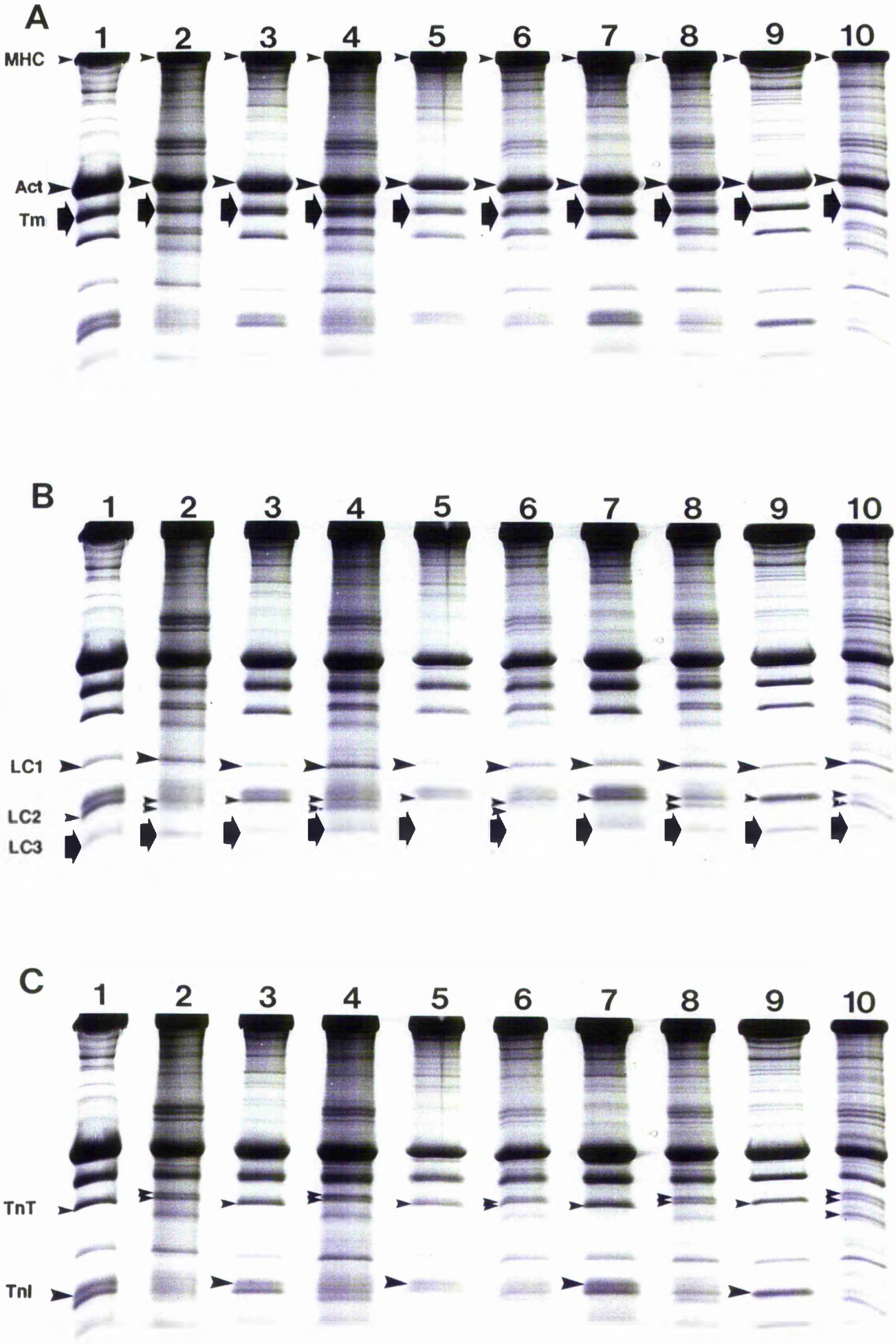
MHC digests from the Antarctic fish myofibrils are shown in Fig. 5.4A-F, lanes 1 to 10. There were no differences in MHC digests between the white muscle of the four Trematomus species analysed (D = 0) (Fig. 5.4A-F, lanes 1, 3, 5, 7). In addition, the red muscle from all the *Trematomus* species produced the same banding pattern (D = 0) (Fig. 5.4A-F, lanes 2, 4, 6, 8). The MHC digests of white muscle from the *Trematomus* species were different from the MHC digests of red muscle. However, there were similarities in the banding patterns (D = 0.26) (compare lanes 1 and 2 from Fig. 5.4A). The MHC digests of both white and red muscle from the other Antarctic fish, *P. borchgrevinki,* were distinct from one another (Fig. 5.4, compare lanes 9 and 10). There was some similarity in the MHC digest of white muscle of *P. borchgrevinki* and the *Trematomus* species (D = 0.42) (compare lane 9 with lanes 1, 3, 5, 7 in Fig. 5.4A-F). The MHC digest of red muscle of *P. borchgrevinki* was very different from the MHC digests of red muscle of the other Trematomus species (D = 0.75) (compare lane 10 with lanes 2, 4, 6, 8 of Fig. 5.4A-F).

Sub-Antarctic species

MHC digests from the sub-Antarctic fish myofibrils are shown in Fig. 5.4, lanes 11 to 20. Digestion of white muscle MHC of *P. Tessellata* and *P. cornucola* produced a very similar banding pattern (D = 0.27) (compare lanes 15 and 19 of Fig. 5.4A-F). A similar banding pattern was also produced by digestion of red muscle MHC from *P. tessellata* and *P. cornucola* (D = 0.48) (compare lanes 16 and 20 of Fig. 5.4A-F).

Gels of digested MHC from the white and red muscle of the other sub-Antarctic fish studied produced unique banding patterns indicating no

Figure 5.2. One dimensional 13 % total acrylamide SDS PAGE gel of myofibrillar proteins from Antarctic fish. Each photograph A-C shows the same gel. Three gels are shown to enable the proteins to be labelled unambiguously; lane 1, *Trematomus newnesi* white muscle; lane 2, *Trematomus newnesi* red muscle; lane 3, *Trematomus bernacchii* white muscle; lane 4, *Trematomus bernacchii* red muscle; lane 5, *Trematomus pennelli* white muscle; lane 6, *Trematomus pennelli* red muscle; lane 7, *Trematomus hansoni* white muscle; lane 8, *Trematomus hansoni* red muscle; lane 9, *Pagothenia borchgrevinki* white muscle; lane 10, *Pagothenia borchgrevinki* red muscle. ►indicates the position of myosin heavy chain (MHC); ►indicates the position of actin (Act); ►indicates the position of tropomyosin (Tm); ►indicates the position of myosin light chain one (LC1); ► indicates the position of myosin light chain two (LC2); ► indicates the position of myosin light chain three (LC3); ►indicates the position of troponin T (TnT); ►indicates the position of troponin I (TnI). The gels were stained with Coomassie Brilliant Blue G-250. MHC, myosin heavy chain; Act, actin; Tm, tropomyosin; TnT, troponin-T; LC1; light chain 1; TnI, troponin I; LC2, light chain 2; LC3, light chain 3.


A
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2
3
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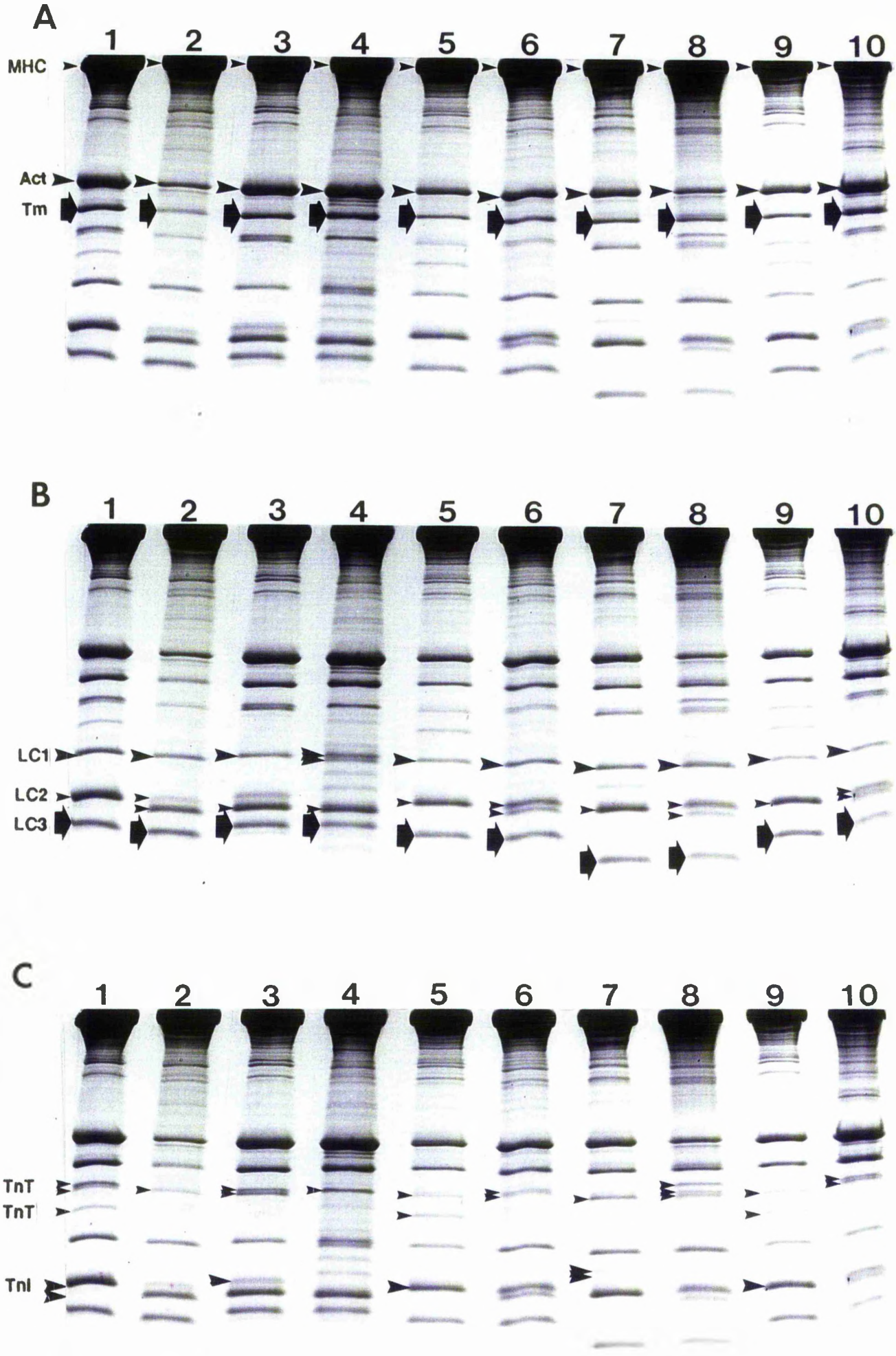
5
6
7
8
9
10
MHC
Act
Tm
B
1
2
3
4
5
6
7
8
9
10
LC1
LC2
LC3
C
1
2
3
4
5
6
7
8
9
10
TnT
TnI

Figure 5.3. One dimensional 13 % total acrylamide SDS PAGE gel of myofibrillar proteins from sub-Antarctic fish. Each photograph A-C shows the same gel. Three gels are shown to enable the proteins to be labelled unambiguously; lane 1, *Paranotothenia magellanica* white muscle; lane 2, *Paranotothenia magellanica* red muscle; lane 3, *Eleginops maclovinus* white muscle; lane 4, *Eleginops maclovinus* red muscle; lane 5, *Patagonotothen tessellata* white muscle; lane 6, *Patagonotothen tessellata* red muscle; lane 7, *Austrolycos depressiceps* white muscle; lane 8, *Austrolycos depressiceps* red muscle; lane 9, *Patagonotothen cornucola* white muscle; lane 10, *Patagonotothen cornucola* red muscle. ➤ indicates the position of myosin heavy chain (MHC); ➤ indicates the position of actin (Act); ➤ indicates the position of tropomyosin (Tm); ➤ indicates the position of myosin light chain one (LC1); ➤ indicates the position of myosin light chain two (LC2); ➤ indicates the position of myosin light chain three (LC3); ➤ indicates the position of troponin T (TnT); ➤ indicates the position of troponin I (TnI). The gels were stained with Coomassie Brilliant Blue G-250. MHC, myosin heavy chain; Act, actin; Tm, tropomyosin; TnT, troponin-T; LC1, light chain 1; TnI, troponin I; LC2, light chain 2; LC3, light chain 3.


A
1
2
3
4
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6
7
8
9
10
MHC
Act
Tm
B
1
2
3
4
5
6
7
8
9
10
LC1
LC2
LC3
C
1
2
3
4
5
6
7
8
9
10
TnT
TnT
TnI

similarity in the MHC between these species and muscle types (Fig. 5.4A-I, lanes 11-14, 17, 18. D ≥ 0.77, for each comparison Fig. 5.7F).

Comparison between the Antarctic and sub-Antarctic MHCs

In order to directly compare Antarctic and sub-Antarctic fish, digests of MHC from each species were performed in the same gel. White muscle digests (Fig. 5.5A-C) and red muscle MHC digests (Fig. 5.6A-C) were performed in separate gels. Only one *Trematomus* species was included in these gels since the previous digests showed there were no differences in banding patterns for the same muscle type between the *Trematomus* species (Fig. 5.4A-F).

There was no similarity between any of the MHC digests of Antarctic fish white muscle and sub-Antarctic fish white muscle (compare lanes 1, 3, 5, 6, 7, 8, 9, Fig. 5.5; D ≥ 0.73 for each comparison, Fig. 5.7G). Furthermore, there was no similarity between any of the MHC digests of Antarctic fish red muscle and sub-Antarctic fish red muscle (compare lanes 2, 4, 5, 6, 7, 8, 9, Fig. 5.6; D ≥ 0.79 for each comparison, Fig. 5.7H).

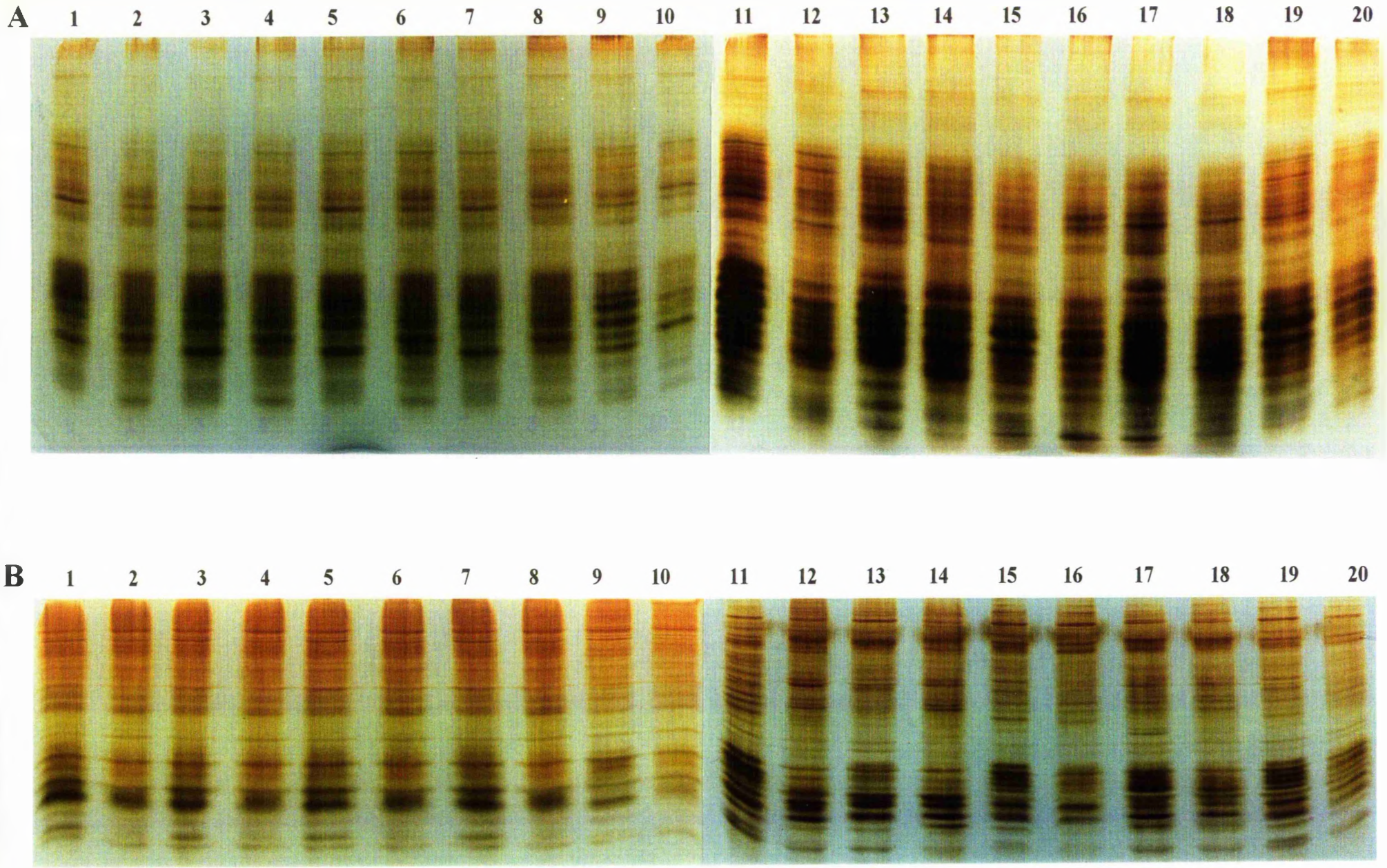
5.3.2 Myosin Light Chains

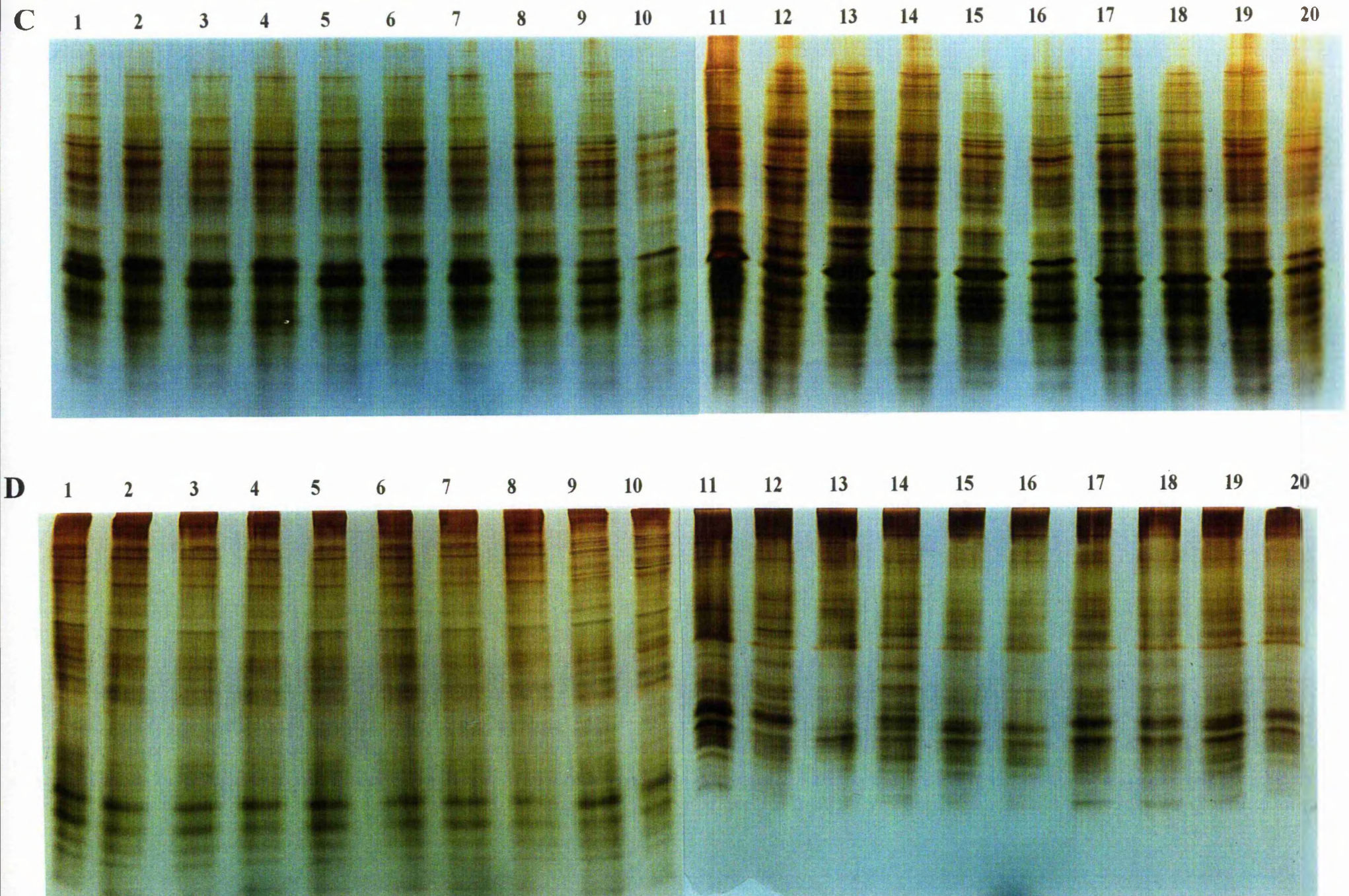
The MLCs from each species were separated and identified by their characteristic acidic migrations in IEF, followed by SDS-PAGE. The regulatory light chain (LC2) was distinguished from the alkali light chains (LC1 and LC3) by its more intense staining with Coomassie blue, and its intermediate Mr between the LC1 and LC3. The alkali light chains were numbered in order of decreasing Mr.

Myosin light chain 1

Myosin LC1 migrated as a single band of Mr 26 kDa in both the white and the red muscle of the majority of species examined (Fig. 5.2B and 5.3B),

Figure 5.4A-F. Peptide digests of electrophoretically purified myosin heavy chains from one dimensional SDS PAGE gels, resolved on 15 % total acrylamide SDS PAGE gels; lane 1, *Trematomus newnesi* white muscle; lane 2, *Trematomus newnesi* red muscle; lane 3, *Trematomus bernacchii* white muscle; lane 4, *Trematomus bernacchii* red muscle; lane 5, *Trematomus pennelli* white muscle; lane 6, *Trematomus pennelli* red muscle; lane 7, *Trematomus hansoni* white muscle; lane 8, *Trematomus hansoni* red muscle; lane 9, *Pagothenia borchgrevinki* white muscle; lane 10, *Pagothenia borchgrevinki* red muscle; lane 11, *Paranotothenia magellanica* white muscle; lane 12, *Paranotothenia magellanica* red muscle; lane 13, *Eleginops maclovinus* white muscle; lane 14, *Eleginops maclovinus* red muscle; lane 15, *Patagonotothen tessellata* white muscle; lane 16, *Patagonotothen tessellata* red muscle; lane 17, *Austrolycos depressiceps* white muscle; lane 18, *Austrolycos depressiceps* red muscle; lane 19, *Patagonotothen cornucola* white muscle; lane 20, *Patagonotothen cornucola* red muscle. (A) Papain from papaya latex, (B) thermolysin from *Bacillus thermoproteolyticus rokko*, (C) elastase from porcine pancreas, (D) trypsin from bovine pancreas, (E) endoproteinase Glu-C from *Straphylococcus aureus* V8, (F) α-chymotrypsin from bovine pancreas. The gels were stained with silver using Plus One silver stain kit (Pharmacia Biotech).


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20


C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
D
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

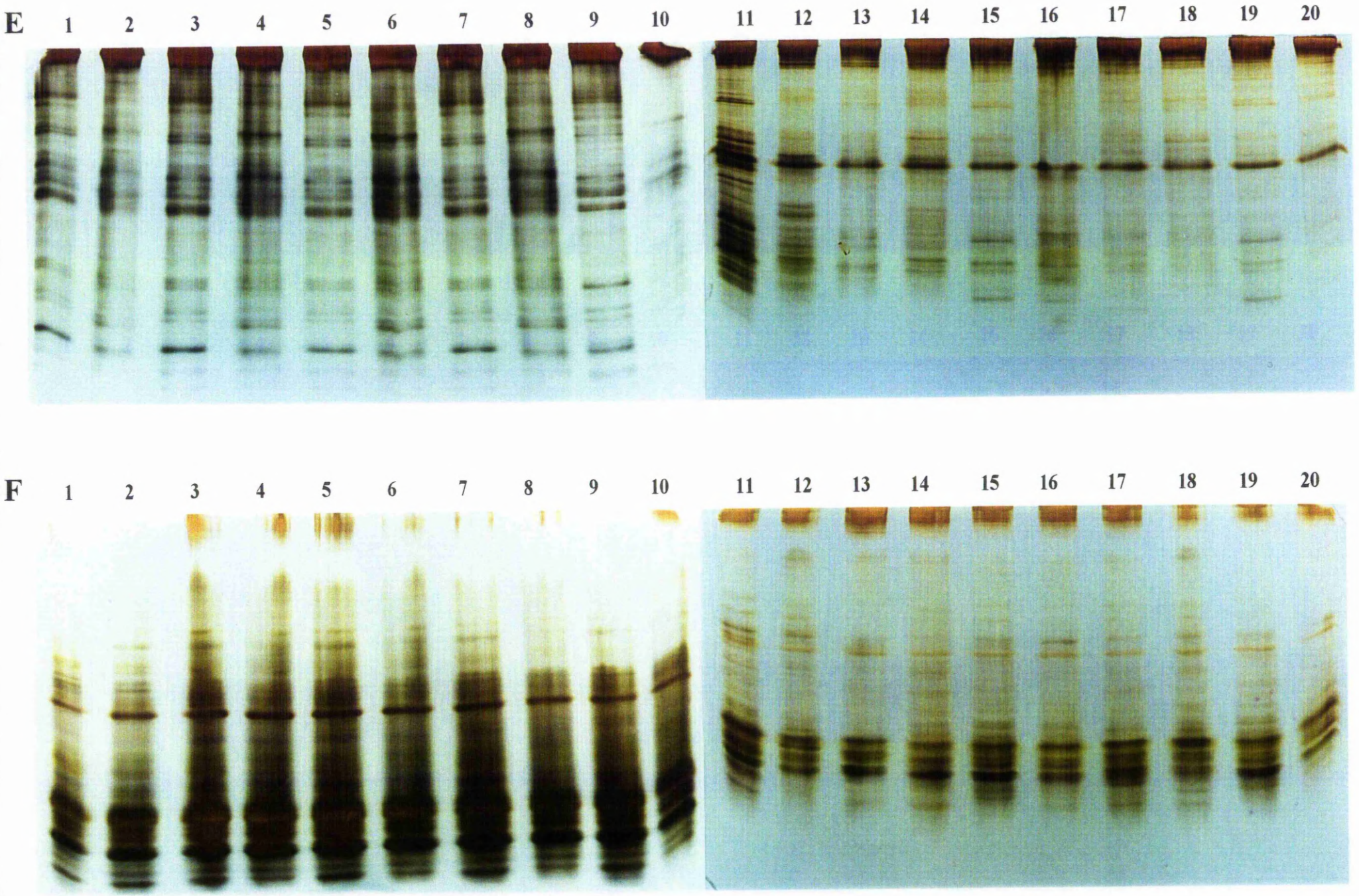

E
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
F
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Figure 5.5. Peptide digests of electrophoretically purified white muscle myosin heavy chains from one dimensional SDS PAGE gels, resolved on 15 % total acrylamide SDS PAGE gels; lane 1, *Trematomus bernacchii* white muscle; lane 2, *Trematomus bernacchii* red muscle; lane 3, *Pagothenia borchgrevinki* white muscle; lane 4, *Pagothenia borchgrevinki* red muscle; lane 5, *Paranotothenia magellanica* white muscle; lane 6, *Eleginops maclovinus* white muscle; lane 7, *Patagonotothen tessellata* white muscle; lane 8, *Austrolycos depressiceps* white muscle; lane 9, *Patagonotothen cornucola* white muscle. (A) Trypsin from bovine pancreas, (B) endoproteinase Glu-C from *Straphylococcus aureus* V8, (C) papain from papaya latex. The gels were stained with silver using Plus One silver stain kit (Pharmacia Biotech).

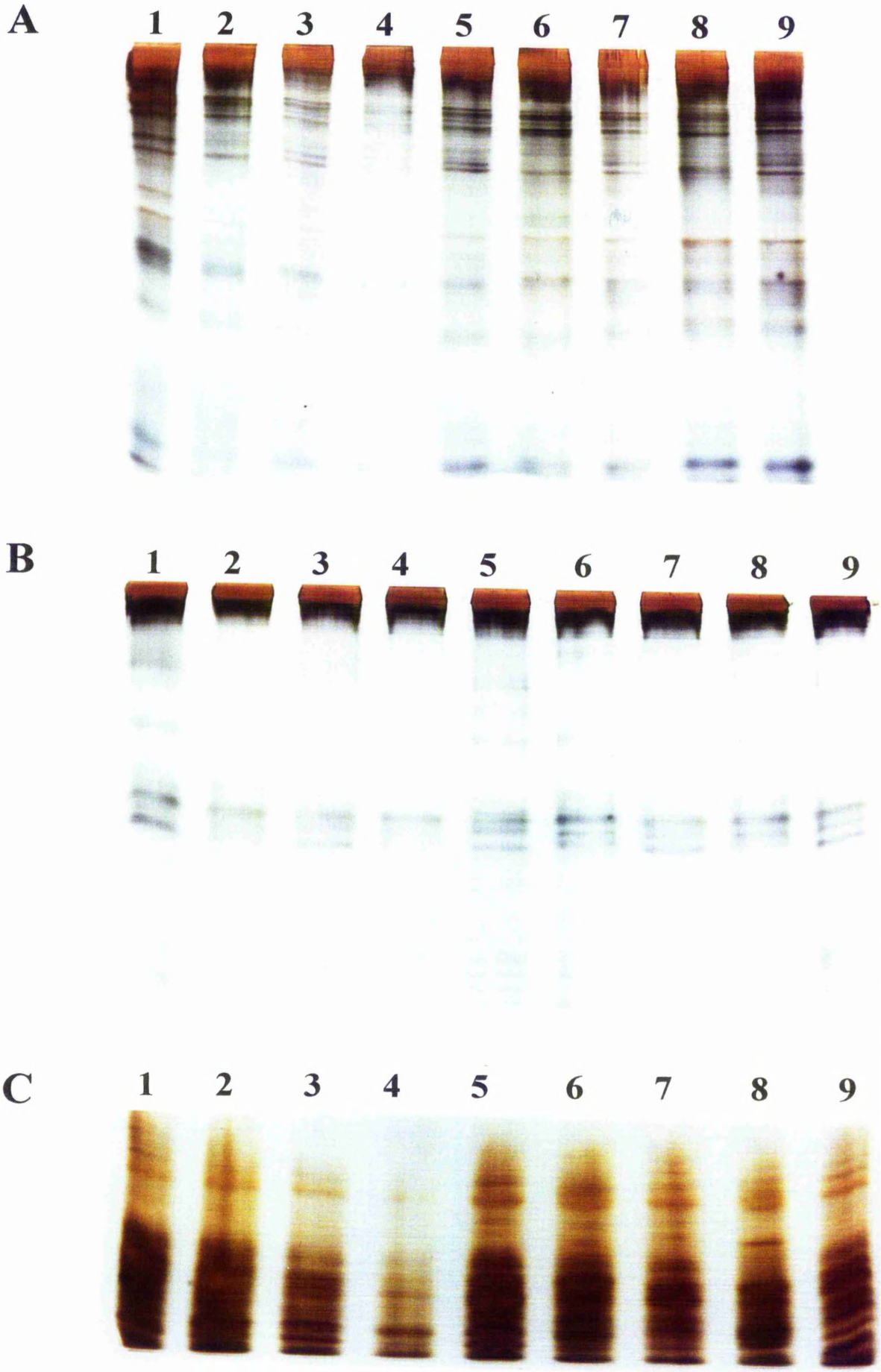

A
1 2 3 4 5 6 7 8 9
B
1 2 3 4 5 6 7 8 9
C
1 2 3 4 5 6 7 8 9

Figure 5.6. Peptide digests of electrophoretically purified red muscle myosin heavy chains from one dimensional SDS PAGE gels, resolved on 15 % total acrylamide SDS PAGE gels; lane 1, *Trematomus bernacchii* white muscle; lane 2, *Trematomus bernacchi*i red muscle; lane 3, *Pagothenia borchgrevinki* white muscle; lane 4, *Pagothenia borchgrevinki* red muscle; lane 5, *Paranotothenia magellanica* red muscle; lane 6, *Eleginops maclovinus* red muscle; lane 7, *Patagonotothen tessellata* red muscle; lane 8, *Austrolycos depressiceps* red muscle; lane 9, *Patagonotothen cornucola* red muscle. (A) Trypsin from bovine pancreas, (B) endoproteinase Glu-C from *Straphylococcus aureus* V8, (C) papain from papaya latex. The gels were stained with silver using Plus One silver stain kit (Pharmacia Biotech).

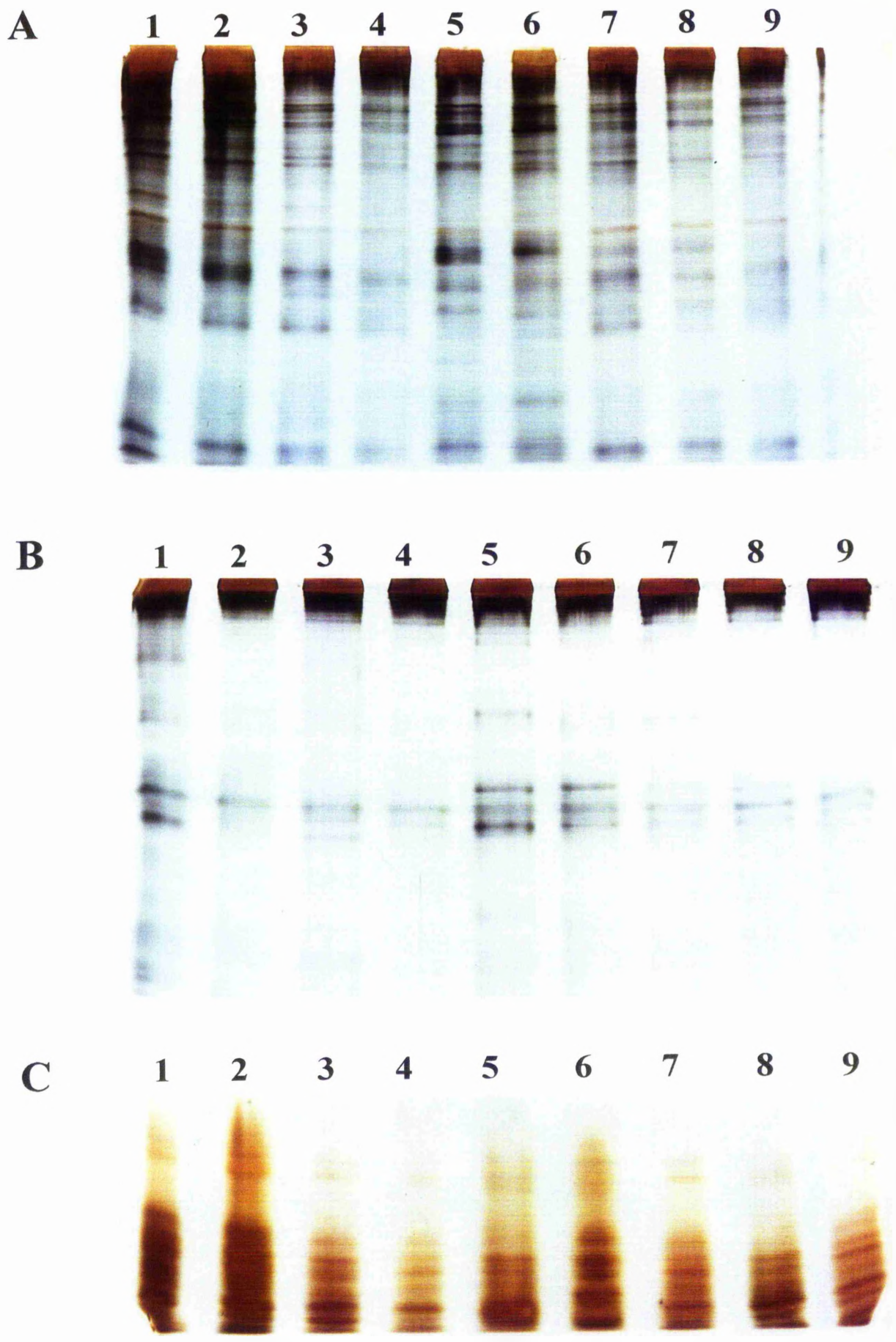

A
1 2 3 4 5 6 7 8 9
B
1 2 3 4 5 6 7 8 9
C
1 2 3 4 5 6 7 8 9

Figure 5.7. Tables which show the estimate of difference (D) between the myosin heavy chain digests of the species analysed. The values given are the mean of estimate of difference using four or more enzymes. (A) Antarctic fish myotomal white muscle, (B) Antarctic fish pectoral fin red muscle, (C) white and red muscle of Antarctic species, (D) sub-Antarctic fish white muscle, (E) sub-Antarctic fish pectoral fin red muscle, (F) white and red muscle of sub-Antarctic species, (G) Antarctic and sub-Antarctic fish white muscle, (H) Antarctic and sub-Antarctic fish red muscle. T. n, *Trematomus newnesi*; T. b, *Trematomus bernacchii*; T. p, *Trematomus pennelli*; T. h, *Trematomus hansoni*; P. b, *Pagothenia borchgrevinki*; P. m, *Paranotothenia magellanica*; E. m, *Eleginops maclovinus*; P. t, *Patagonotothen tessellata*; A. d, *Austrolycos depressiceps*; P.c, *Patagonotothen cornucola*. Low values represent a high degree of similarity between myosin heavy chains and are shown in highlighted cells.

A

WHITE	T. n	T. b	T. p	T. h
T. b	0			
T. p	0	0		
T. h	0	0	0	
P. b	.42	.42	.42	.42

B

RED	T. n	T. b	T. p	T. h
T. b	0			
T. p	0	0		
T. h	0	0	0	
P. b	.75	.75	.75	.75

C

	T. n	T. b	T. p	T. h	P. b
W v R	.26	.26	.26	.26	.67

D

WHITE	P. m	E. m	P. t	A. d
E. m	.79			
P. t	.81	.79		
A. d	.85	.80	.88	
P. c	.82	.82	.27	.87

E

RED	P. m	E. m	P. t	A. d
E. m	.80			
P. t	.87	.86		
A. d	.85	.80	.86	
P. c	.88	.88	.48	.83

F

	P. m	E. m	P. t	A. d	P. c
W v R	.79	.77	.83	.74	.77

G

WHITE	P. m	E. m	P. t	A. d	P. c
T. n	.80	.82	.89	.90	.88
P. b	.73	.78	.79	.91	.82

H

RED	P. m	E. m	P. t	A. d	P. c
T. n	.84	.91	.95	.99	.95
P. b	.92	.97	.79	.89	.86

with the exception of *Trematomus newnesi* (Fig. 5.2B, lanes 1-2), *Eleginops maclovinus* (Fig. 5.3B, lanes 3-4) and *Austrolycos depressiceps.* (Fig. 5.3B, lanes 7-8).

Both *T. newnesi* white muscle (Fig. 5.2B, lane 1; Fig. 5.8A, lane 1; Fig. 5.8B) and red muscle (Fig. 5.2B, lane 2; Fig. 5.8A, lane 2; Fig. 5.8D) contained a single LC1 isoform with a greater Mr of approximately 26.5 kDa (Fig. 5.8A, lanes 1,2), but the same iso-electric point as the LC1 isoform of the other *Trematomus* species (Fig. 5.18C). In *E. maclovinus,* the white muscle contained a single LC1 isoform (Fig. 5.3B, lane 3; Fig 14A, lane 1; Fig. 5.14B), of Mr of approximately 26.3 kDa, which migrated with a lower relative molecular mass than the LC1 of the majority of species examined. Red muscle contained two isoforms, one of which had the same approximate Mr (26.3 kDa) as the white muscle, and the other which had the same Mr (26 kDa) as the majority of other species (Fig. 5.3B, lane 4; Fig. 5.14A, lane 2; Fig. 5.14D). The red muscle isoforms also differed in iso-electric point (Fig. 5.14D). The white muscle (Fig. 5.3B, lane 7; Fig. 5.16A, lane 1; Fig. 5.16B) and red muscle (Fig. 5.3B, lane 8; Fig 16A, lane 2; Fig. 5.16D) of *A. depressiceps* contained a single LC1 isoform which migrated further than the light chain of any other species examined, with an approximate Mr of 25 kDa (Fig 5.3 Lane 7,8; Fig. 5.18H). The red and white isoforms in this species had the same iso-electric point (Fig. 5.16C).

Myosin light chain 2

In the Antarctic fish studied the LC2 isoform expression was the same in each species. White muscle contained a single isoform of approximately 21 kDa (Fig. 5.2B, lanes 1 ,3 ,5 ,7 ,9; Fig. 5.18A-D). The same LC2 expression was found in the white muscle of the sub-Antarctic species *P. magellanica, P. tessellata* and *P. cornucola* (Fig. 5.3B, lanes 1, 5, 9; Fig. 5.18E,G,I). The red muscle of each Antarctic species contained two LC2

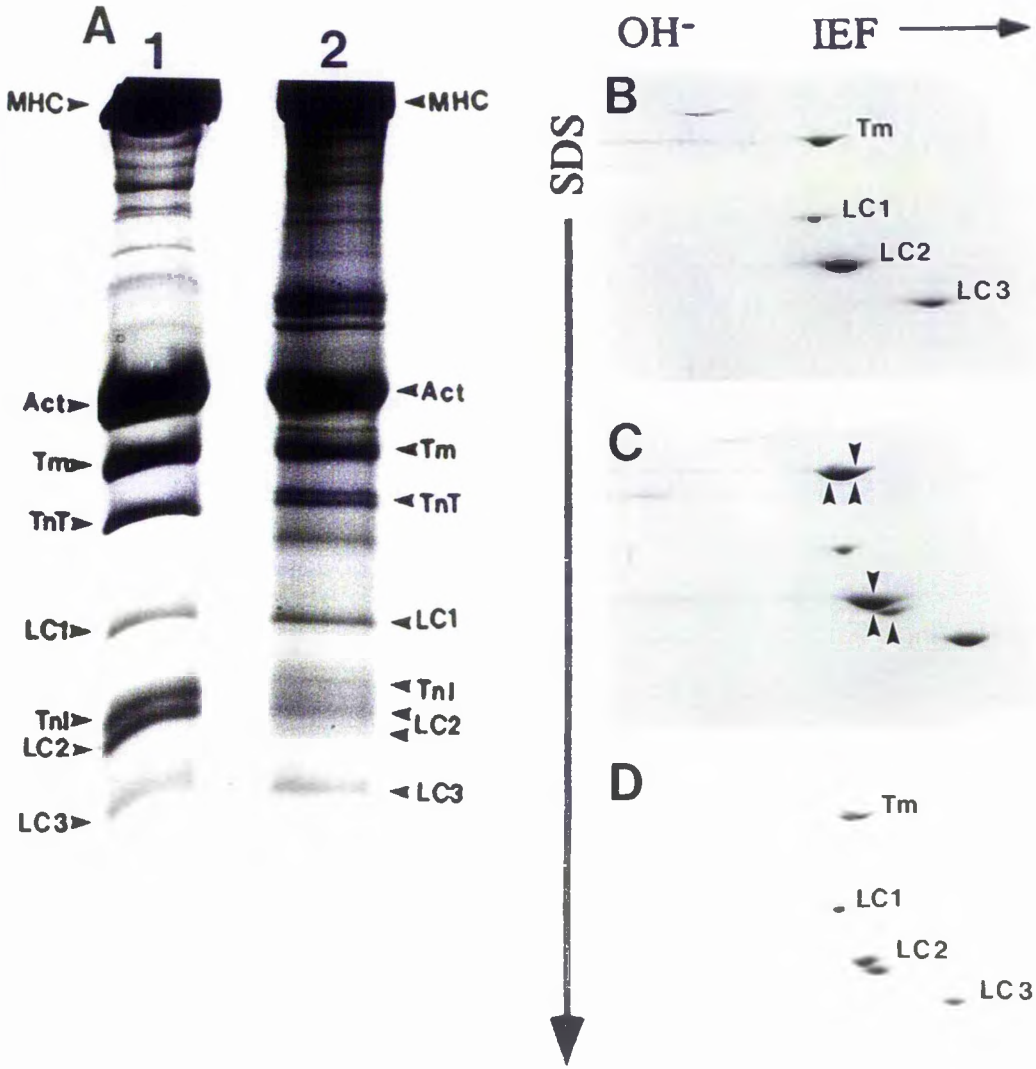
isoforms of approximately 20 and 21 kDa (Fig. 5.2B,. lanes 2, 4, 6, 8, 10). The same LC2 expression was also found in the red muscle of the sub-Antarctic species *P. tessellata* and *P. cornucola* (Fig. 5.3B, lanes 6, 10). The red muscle of *P. magellanica* contained two LC2 isoforms of approximately 20 and 21 kDa. There was relatively more of the 20 kDa isoform (Fig. 5.3B, lanes 2; Fig. 5.13A, D).

Two species contained different molecular mass LC2 isoforms from all the other species examined. These were *E. maclovinus* and *A. depressiceps*. The white muscle of *E. maclovinus* contained a LC2 isoform of approximately 20.5 kDa (Fig. 5.3B, lane 3; Fig. 5.14A, lane 1; Fig. 5.14B) as did the red muscle (Fig. 5.3B, lane 4; Fig. 5.14A, lane 2; Fig. 5.14D). However, these two isoforms had different iso-electric points (Fig. 5.14C). *A. depressiceps* white muscle also contained a LC2 isoform with a lower molecular mass than the other species of approximately 20.5 kDa (Fig. 5.3B, lane 7; Fig. 5.16A, lane 1; Fig. 5.16B), whereas red muscle contained two LC2 isoforms of approximately 20 and 20.5 kDa (Fig. 5.3B, lane 8; Fig. 5.16A, lane 2; Fig. 5.16D). These two isoforms had different iso-electric points (Fig. 5.16C).

Myosin light chain 3

E. maclovinus contained a single LC3 isoform of Mr 19 kDa in both white and red muscle (Fig. 5.3B, lanes 3,4; Fig. 5.14A, lanes 1,2; Fig. 5.14B, D). In both the white and red muscles of *A. depressiceps,* LC3 was present as a single isoform which migrated further than the light chains of any other species, with an approximate molecular mass of 17 kDa (Fig. 5.3B, lanes 7-8; Fig. 5.16A, lanes 1-2; Fig. 5.16B, D). Both isoforms had the same iso-electric point (Fig. 5.16C). In all the other species and muscle types examined there was a single LC3 isoform of 18 kDa (Fig. 5.2B; Fig 5.3B) which had the same iso-electric point in each case (Fig. 5.18A-E,G,I).

Figure 5.8. Electrophoretically analysed proteins of *Trematomus newnesi*. (A) One dimensional 13 % total acrylamide SDS PAGE gel of myofibrillar proteins; lane 1, white muscle; lane 2, red muscle. (B-D) Two dimensional gels (IEF in the first dimension, SDS PAGE in the second) of acidic myofibrillar proteins. (B) White muscle, (C) mixture of white muscle and red muscle, (D) fin red muscle. Arrowheads indicate proteins which show a difference between white and red muscle. Downward pointing arrowheads indicate proteins from white muscle. Upward pointing arrowheads indicate proteins from red muscle. (E) Two dimensional gel (IEF in the first dimension, SDS PAGE in the second) of basic myofibrillar proteins from white muscle. MHC, myosin heavy chain; Act, actin; Tm, tropomyosin; TnT, troponin-T; LC1, light chain 1; TnI, troponin I; LC2, light chain 2; LC3, light chain 3. The gels were stained with Coomasie Brilliant Blue G-250.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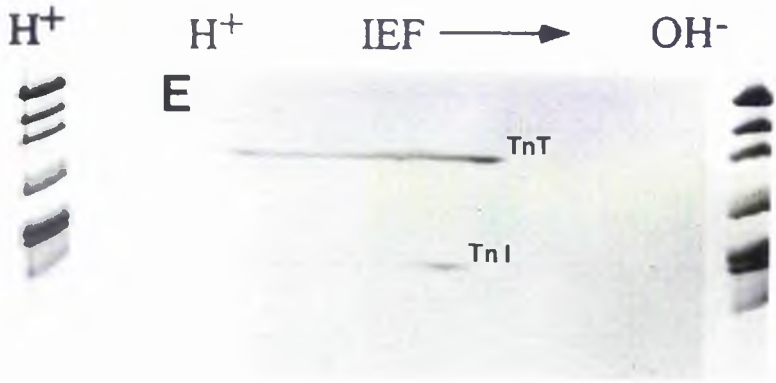


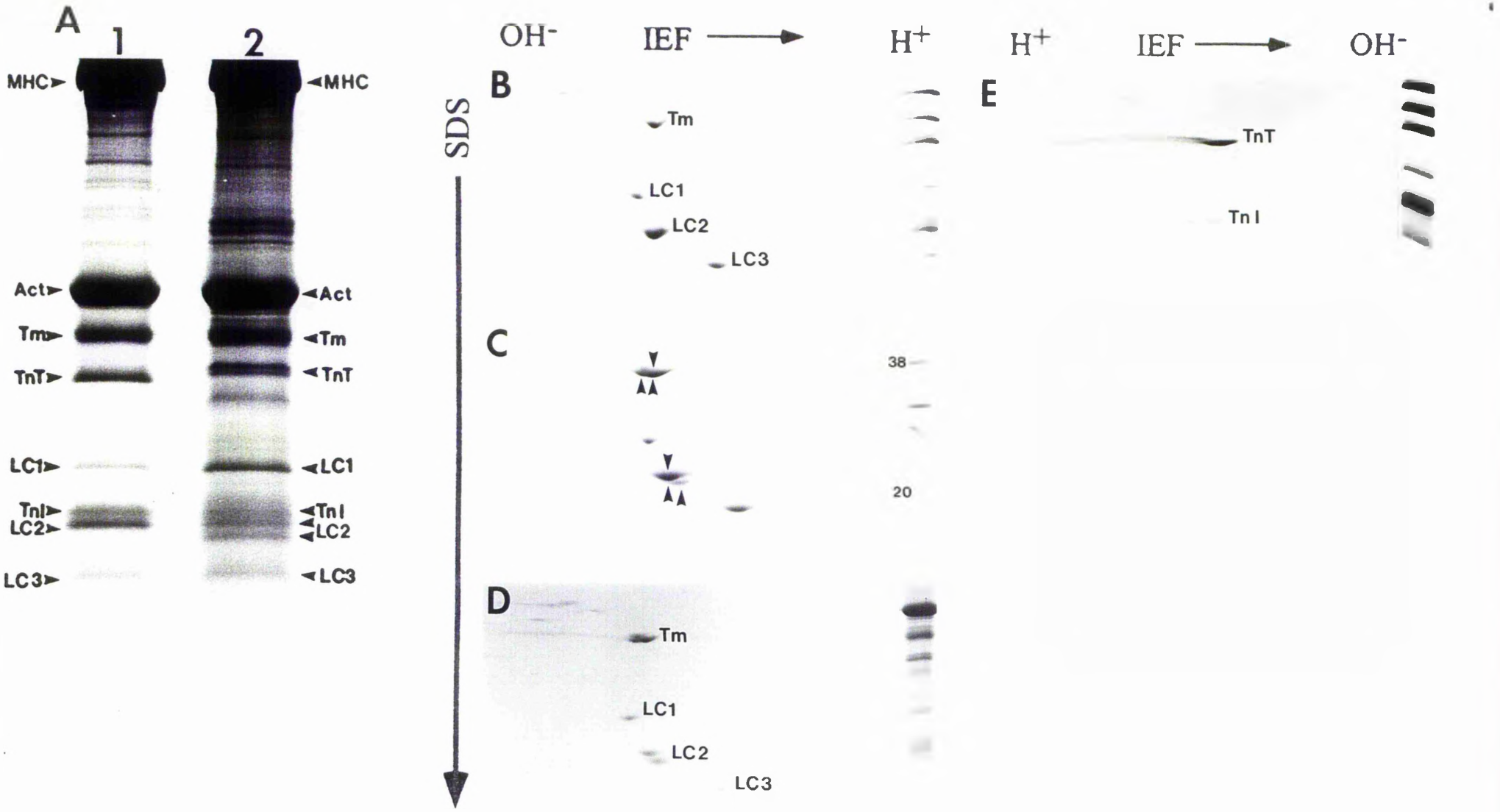
38

29

20

14

Figure 5.9. Electrophoretically analysed proteins of *Trematomus bernacchii*. (A) One dimensional 13 % total acrylamide SDS PAGE gel of myofibrillar proteins; lane 1, white muscle; lane 2, red muscle. (B-D) Two dimensional gels (IEF in the first dimension, SDS PAGE in the second) of acidic myofibrillar proteins. (B) White muscle, (C) mixture of white muscle and red muscle, (D) fin red muscle. Arrowheads indicate proteins which show a difference between white and red muscle. Downward pointing arrowheads indicate proteins from white muscle. Upward pointing arrowheads indicate proteins from red muscle. (E) Two dimensional gel (IEF in the first dimension, SDS PAGE in the second) of basic myofibrillar proteins from white muscle. MHC, myosin heavy chain; Act, actin; Tm, tropomyosin; TnT, troponin-T; LC1, light chain 1; TnI, troponin I; LC2, light chain 2; LC3, light chain 3. The gels were stained with Coomasie Brilliant Blue G-250.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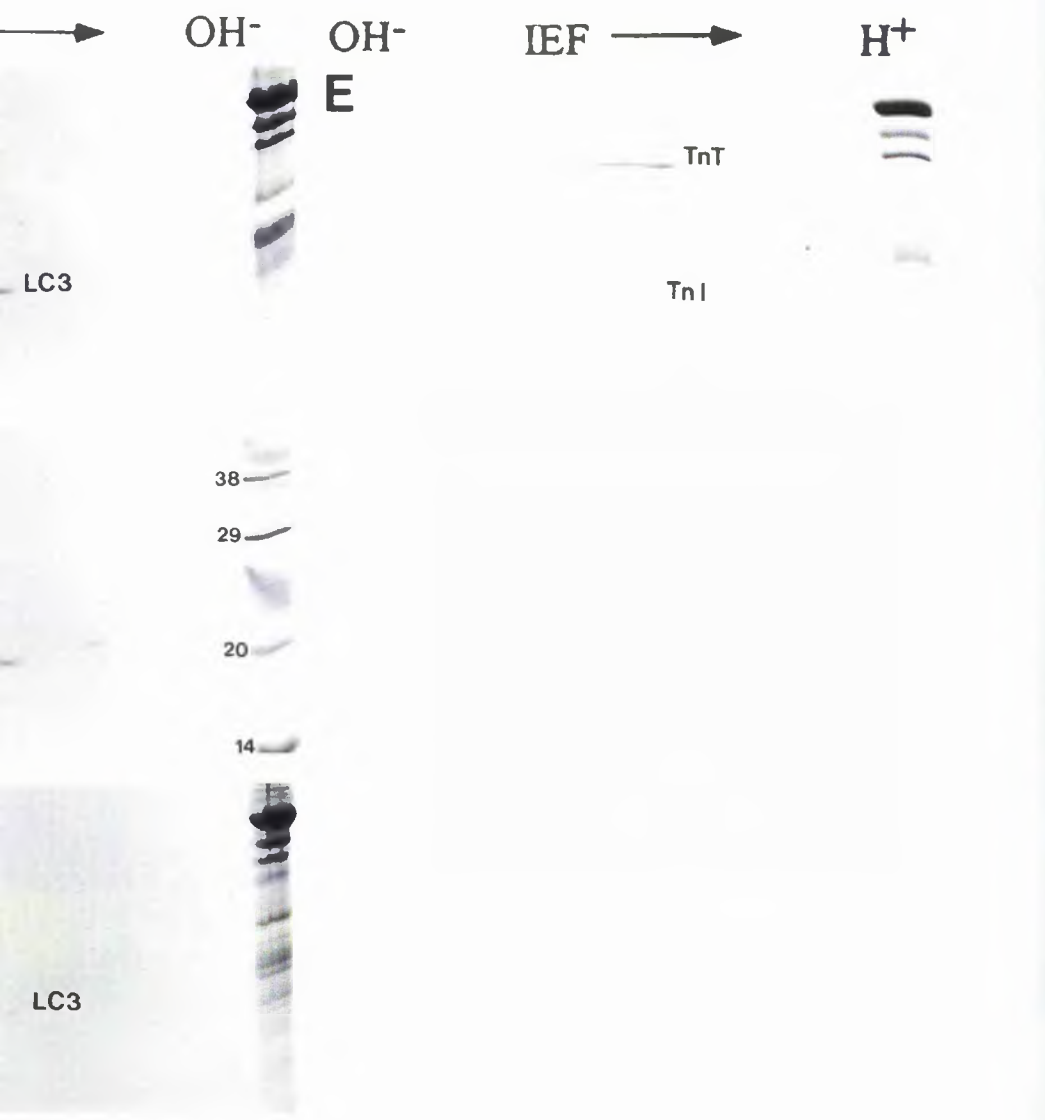
LC3
LC3
OH-
IEF
H+
SDS
B
Tm
LC1
LC2
LC3
C
38
20
D
Tm
LC1
LC2
LC3
H+
IEF
OH-
E
TnT
TnI

Figure 5.10. Electrophoretically analysed proteins of *Trematomus pennelli*. (A) One dimensional 13 % total acrylamide SDS PAGE gel of myofibrillar proteins.; lane 1, white muscle; lane 2, red muscle. (B-D) Two dimensional gels (IEF in the first dimension, SDS PAGE in the second) of acidic myofibrillar proteins. (B) White muscle, (C) mixture of white muscle and red muscle, (D) fin red muscle. Arrowheads indicate proteins which show a difference between white and red muscle. Downward pointing arrowheads indicate proteins from white muscle. Upward pointing arrowheads indicate proteins from red muscle. (E) Two dimensional gel (IEF in the first dimension, SDS PAGE in the second) of basic myofibrillar proteins from white muscle. MHC, myosin heavy chain; Act, actin; Tm, tropomyosin; TnT, troponin-T; LC1, light chain 1; TnI, troponin I; LC2, light chain 2; LC3, light chain 3. The gels were stained with Coomasie Brilliant Blue G-250.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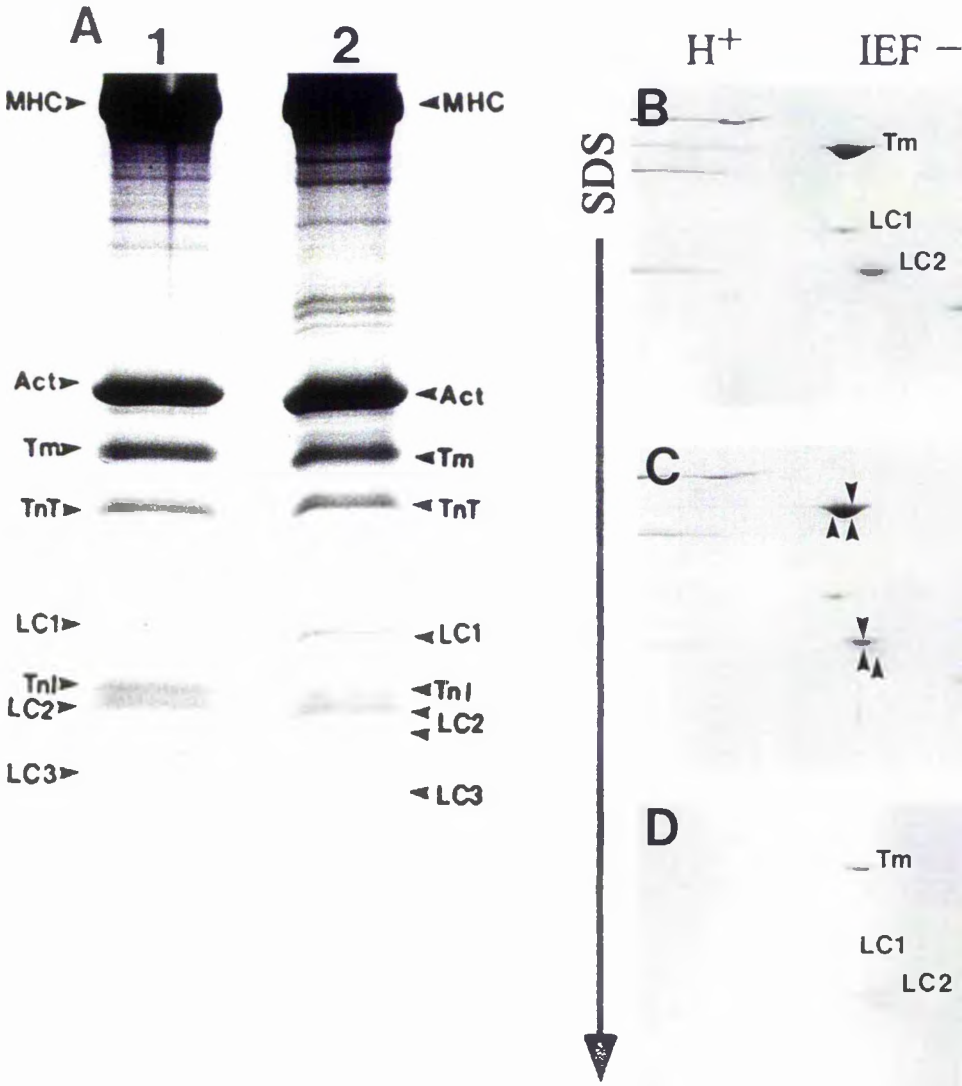
LC3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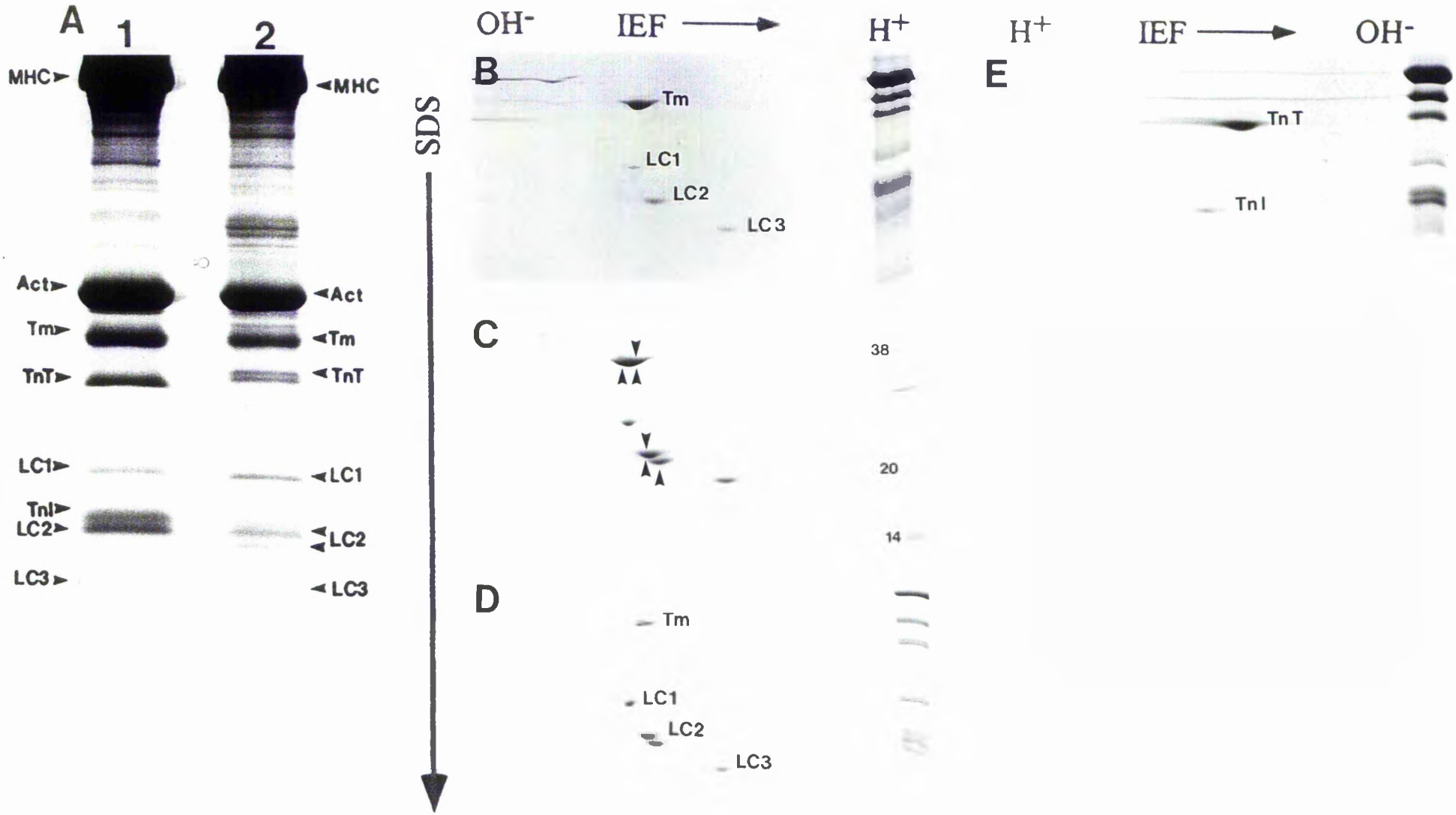
LC2
LC3
LC3
H+
IEF
SDS
B
Tm
LC1
LC2
C
D
Tm
LC1
LC2

Figure 5.11. Electrophoretically analysed proteins of *Trematomus hansoni*. (A) One dimensional 13 % total acrylamide SDS PAGE gel of myofibrillar proteins; lane 1, white muscle; lane 2, red muscle. (B-D) Two dimensional gels (IEF in the first dimension, SDS PAGE in the second) of acidic myofibrillar proteins. (B) White muscle, (C) mixture of white muscle and red muscle, (D) fin red muscle. Arrowheads indicate proteins which show a difference between white and red muscle. Downward pointing arrowheads indicate proteins from white muscle. Upward pointing arrowheads indicate proteins from red muscle. (E) Two dimensional gel (IEF in the first dimension, SDS PAGE in the second) of basic myofibrillar proteins from white muscle. MHC, myosin heavy chain; Act, actin; Tm, tropomyosin; TnT, troponin-T; LC1, light chain 1; TnI, troponin I; LC2, light chain 2; LC3, light chain 3. The gels were stained with Coomasie Brilliant Blue G-250.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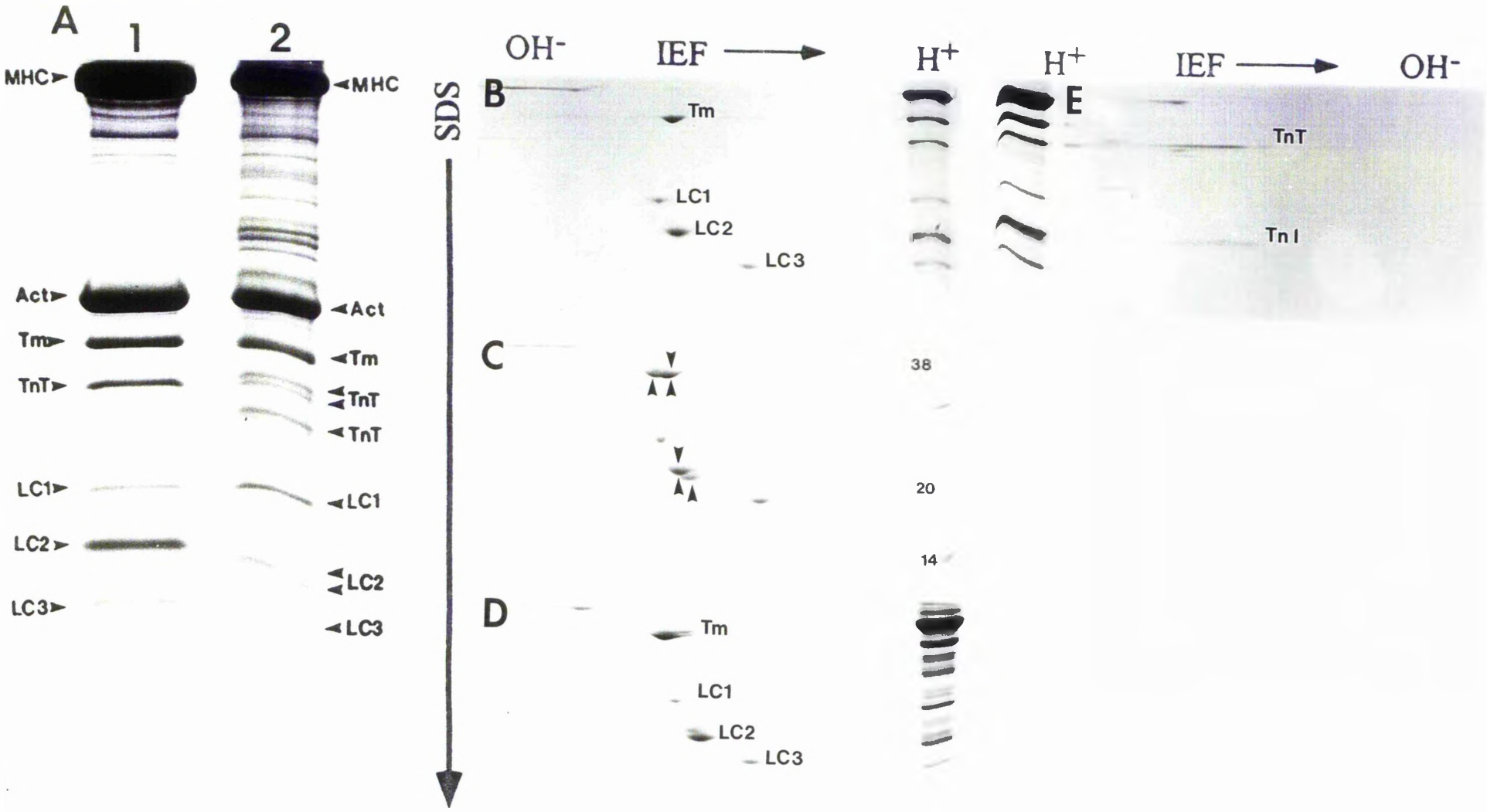
OH-
IEF
H+
B
Tm
LC1
LC2
LC3
C
38
20
14
D
Tm
LC1
LC2
LC3
H+
IEF
OH-
E
TnT
TnI

Figure 5.12. Electrophoretically analysed proteins of *Pagothenia borchgrevinki*. (A) One dimensional 13 % total acrylamide SDS PAGE gel of myofibrillar proteins; lane 1, white muscle; lane 2, red muscle. (B-D) Two dimensional gels (IEF in the first dimension, SDS PAGE in the second) of acidic myofibrillar proteins. (B) White muscle, (C) mixture of white muscle and red muscle, (D) fin red muscle. Arrowheads indicate proteins which show a difference between white and red muscle. Downward pointing arrowheads indicate proteins from white muscle. Upward pointing arrowheads indicate proteins from red muscle. (E) Two dimensional gel (IEF in the first dimension, SDS PAGE in the second) of basic myofibrillar proteins from white muscle. MHC, myosin heavy chain; Act, actin; Tm, tropomyosin; TnT, troponin-T; LC1, light chain 1; TnI, troponin I; LC2, light chain 2; LC3, light chain 3. The gels were stained with Coomasie Brilliant Blue G-250.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LC2
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SDS
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IEF
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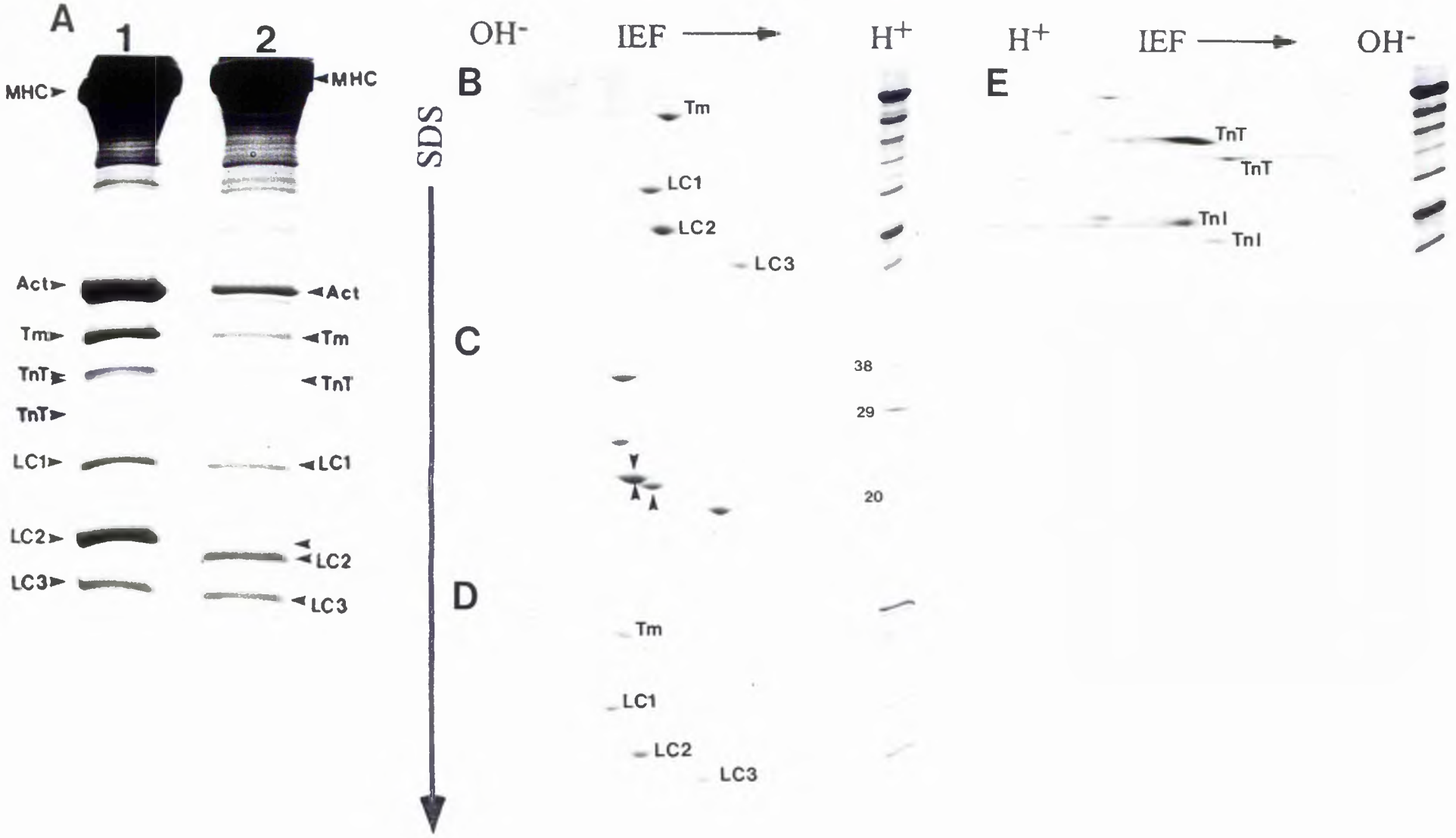
TnI

Figure 5.13. Electrophoretically analysed proteins of *Paranotothenia magellanica*. (A) One dimensional 13 % total acrylamide SDS PAGE gel of myofibrillar proteins; lane 1, white muscle; lane 2, red muscle. (B-D) Two dimensional gels (IEF in the first dimension, SDS PAGE in the second) of acidic myofibrillar proteins. (B) White muscle, (C) mixture of white muscle and red muscle, (D) fin red muscle. Arrowheads indicate proteins which show a difference between white and red muscle. Downward pointing arrowheads indicate proteins from white muscle. Upward pointing arrowheads indicate proteins from red muscle. (E) Two dimensional gel (IEF in the first dimension, SDS PAGE in the second) of basic myofibrillar proteins from white muscle. MHC, myosin heavy chain; Act, actin; Tm, tropomyosin; TnT, troponin-T; LC1, light chain 1; TnI, troponin I; LC2, light chain 2; LC3, light chain 3. The gels were stained with Coomasie Brilliant Blue G-250.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IEF
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LC2
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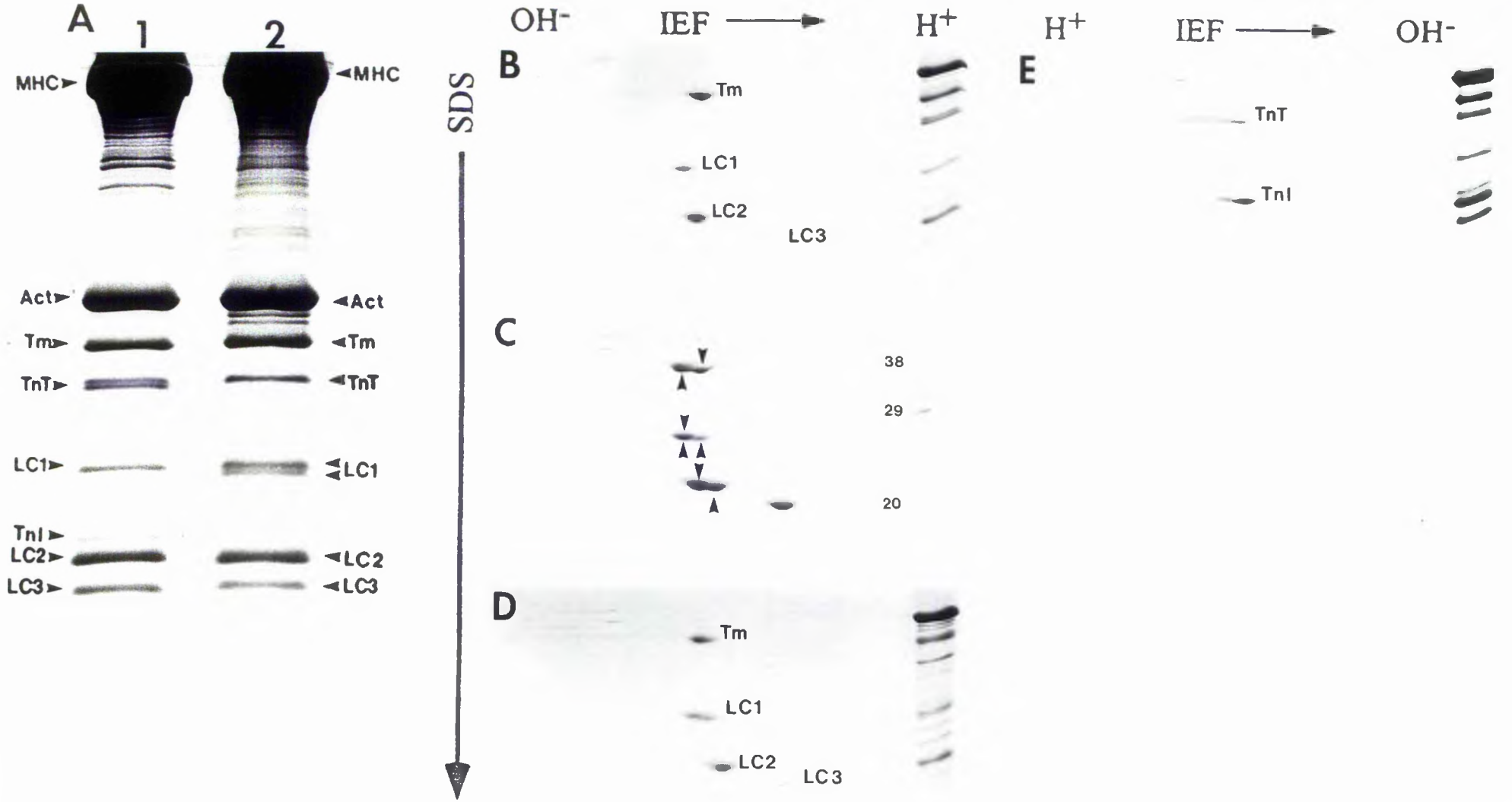
TnI

Figure 5.14. Electrophoretically analysed proteins of *Eleginops maclovinus*. (A) One dimensional 13 % total acrylamide SDS PAGE gel of myofibrillar proteins; lane 1, white muscle; lane 2, red muscle. (B-D) Two dimensional gels (IEF in the first dimension, SDS PAGE in the second) of acidic myofibrillar proteins. (B) White muscle, (C) mixture of white muscle and red muscle, (D) fin red muscle. Arrowheads indicate proteins which show a difference between white and red muscle. Downward pointing arrowheads indicate proteins from white muscle. Upward pointing arrowheads indicate proteins from red muscle. (E) Two dimensional gel (IEF in the first dimension, SDS PAGE in the second) of basic myofibrillar proteins from white muscle. MHC, myosin heavy chain; Act, actin; Tm, tropomyosin; TnT, troponin-T; LC1, light chain 1; TnI, troponin I; LC2, light chain 2; LC3, light chain 3. The gels were stained with Coomasie Brilliant Blue G-250.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LC3
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TnT
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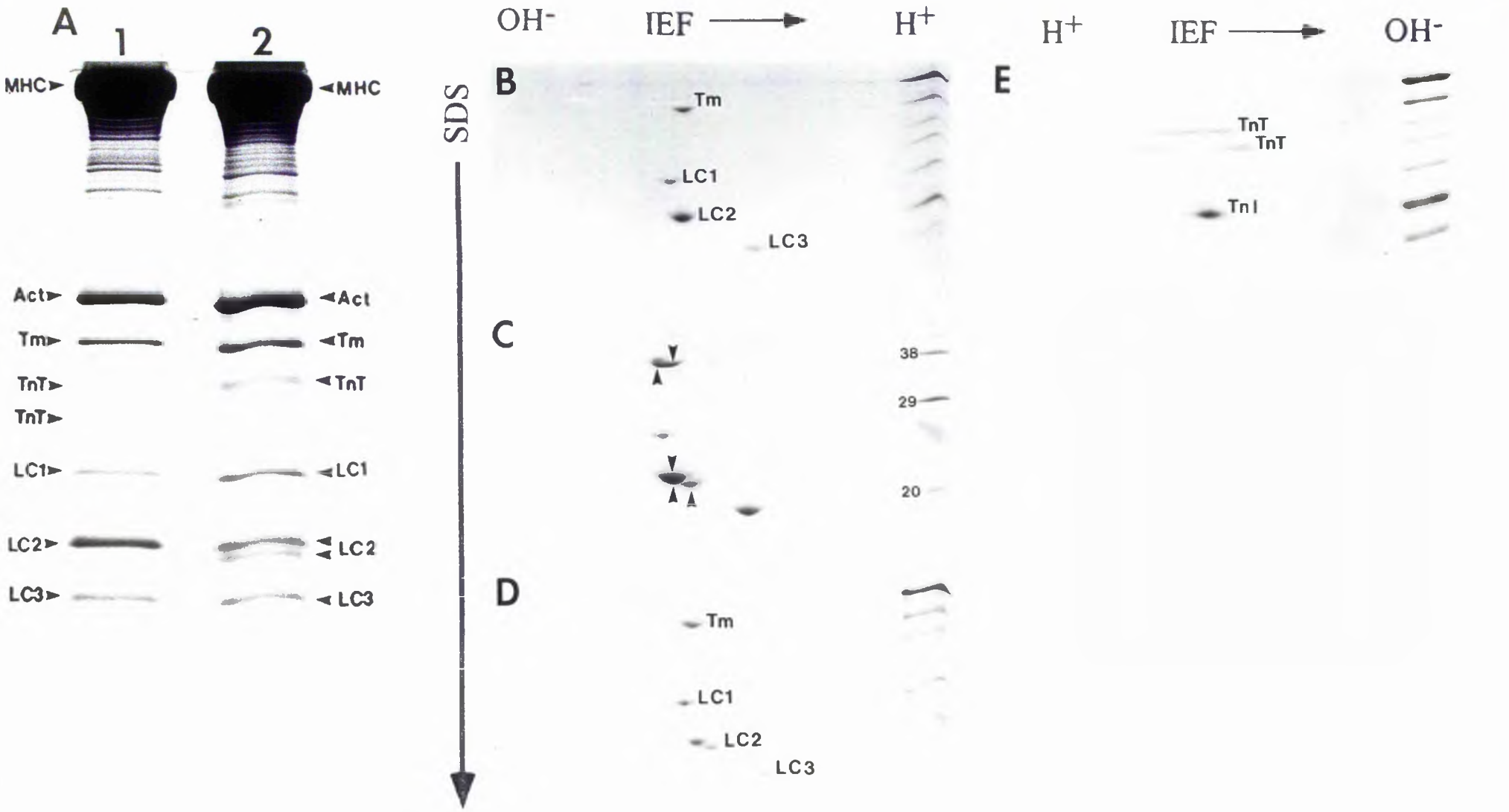
Figure 5.15. Electrophoretically analysed proteins of *Patagonotothen tessellata*. (A) One dimensional 13 % total acrylamide SDS PAGE gel of myofibrillar proteins; lane 1, white muscle; lane 2, red muscle. (B-D) Two dimensional gels (IEF in the first dimension, SDS PAGE in the second) of acidic myofibrillar proteins. (B) White muscle, (C) mixture of white muscle and red muscle, (D) fin red muscle. Arrowheads indicate proteins which show a difference between white and red muscle. Downward pointing arrowheads indicate proteins from white muscle. Upward pointing arrowheads indicate proteins from red muscle. (E) Two dimensional gel (IEF in the first dimension, SDS PAGE in the second) of basic myofibrillar proteins from white muscle. MHC, myosin heavy chain; Act, actin; Tm, tropomyosin; TnT, troponin-T; LC1, light chain 1; TnI, troponin I; LC2, light chain 2; LC3, light chain 3. The gels were stained with Coomasie Brilliant Blue G-250.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IEF
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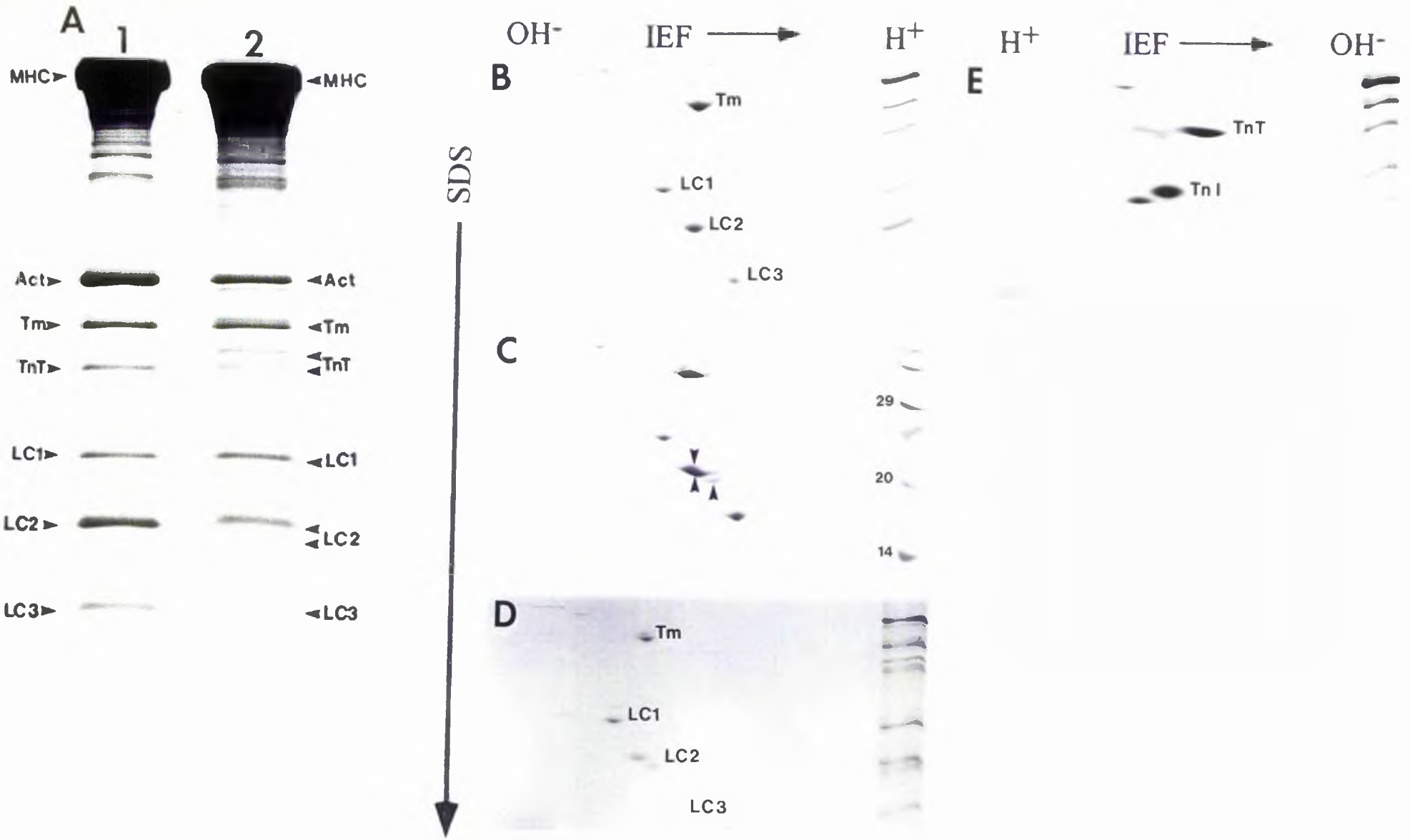
TnT
TnT
TnI

Figure 5.16. Electrophoretically analysed proteins of *Austrolycos depressiceps*. (A) One dimensional 13 % total acrylamide SDS PAGE gel of myofibrillar proteins; lane 1, white muscle; lane 2, red muscle. (B-D) Two dimensional gels (IEF in the first dimension, SDS PAGE in the second) of acidic myofibrillar proteins. (B) White muscle, (C) mixture of white muscle and red muscle, (D) fin red muscle. Arrowheads indicate proteins which show a difference between white and red muscle. Downward pointing arrowheads indicate proteins from white muscle. Upward pointing arrowheads indicate proteins from red muscle. (E) Two dimensional gel (IEF in the first dimension, SDS PAGE in the second) of basic myofibrillar proteins from white muscle. MHC, myosin heavy chain; Act, actin; Tm, tropomyosin; TnT, troponin-T; LC1, light chain 1; TnI, troponin I; LC2, light chain 2; LC3, light chain 3. The gels were stained with Coomasie Brilliant Blue G-250.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IEF
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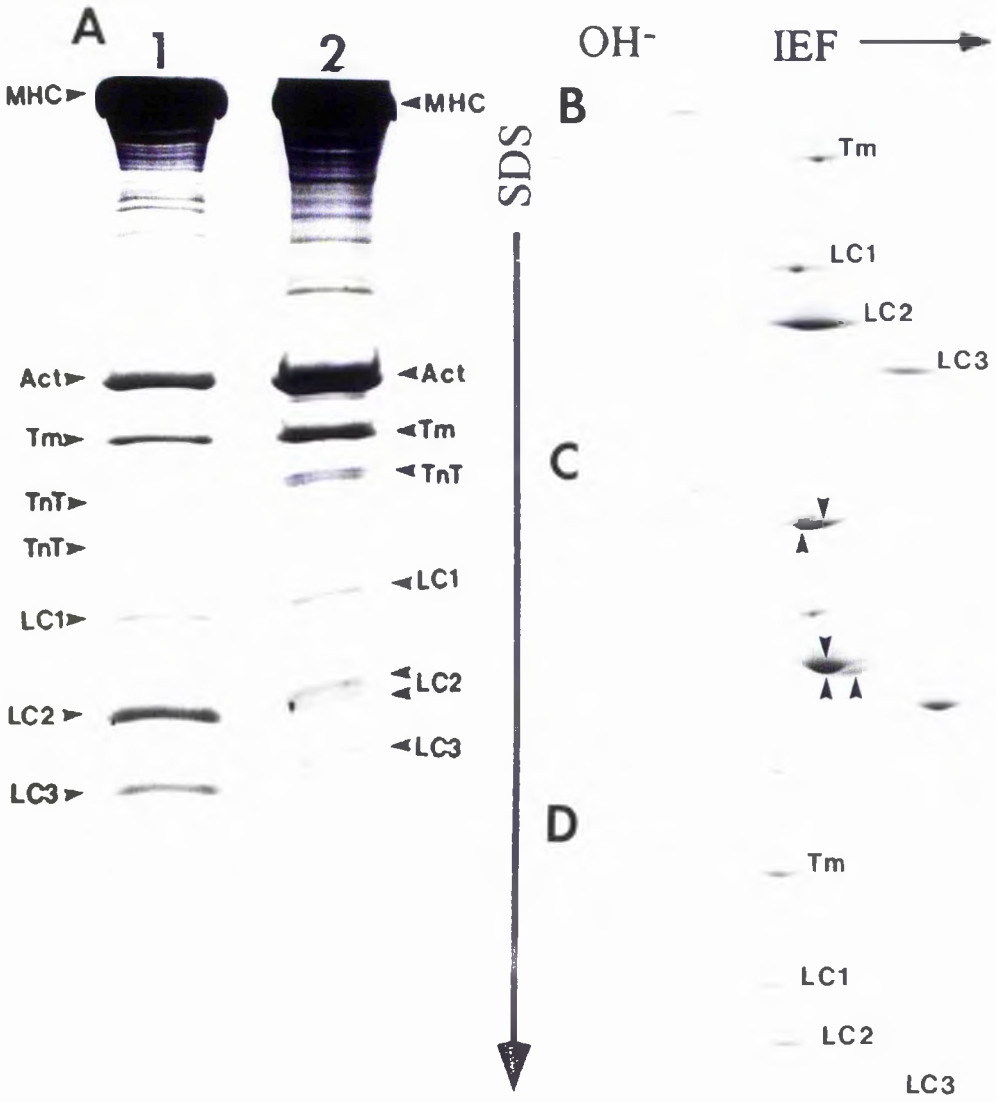
Figure 5.17. Electrophoretically analysed proteins of *Patagonotothen cornucola*. (A) One dimensional 13 % total acrylamide SDS PAGE gel of myofibrillar proteins; lane 1, white muscle; lane 2, red muscle. (B-D) Two dimensional gels (IEF in the first dimension, SDS PAGE in the second) of acidic myofibrillar proteins. (B) White muscle, (C) mixture of white muscle and red muscle, (D) fin red muscle. Arrowheads indicate proteins which show a difference between white and red muscle. Downward pointing arrowheads indicate proteins from white muscle. Upward pointing arrowheads indicate proteins from red muscle. (E) Two dimensional gel (IEF in the first dimension, SDS PAGE in the second) of basic myofibrillar proteins from white muscle. MHC, myosin heavy chain; Act, actin; Tm, tropomyosin; TnT, troponin-T; LC1, light chain 1; TnI, troponin I; LC2, light chain 2; LC3, light chain 3. The gels were stained with Coomasie Brilliant Blue G-250.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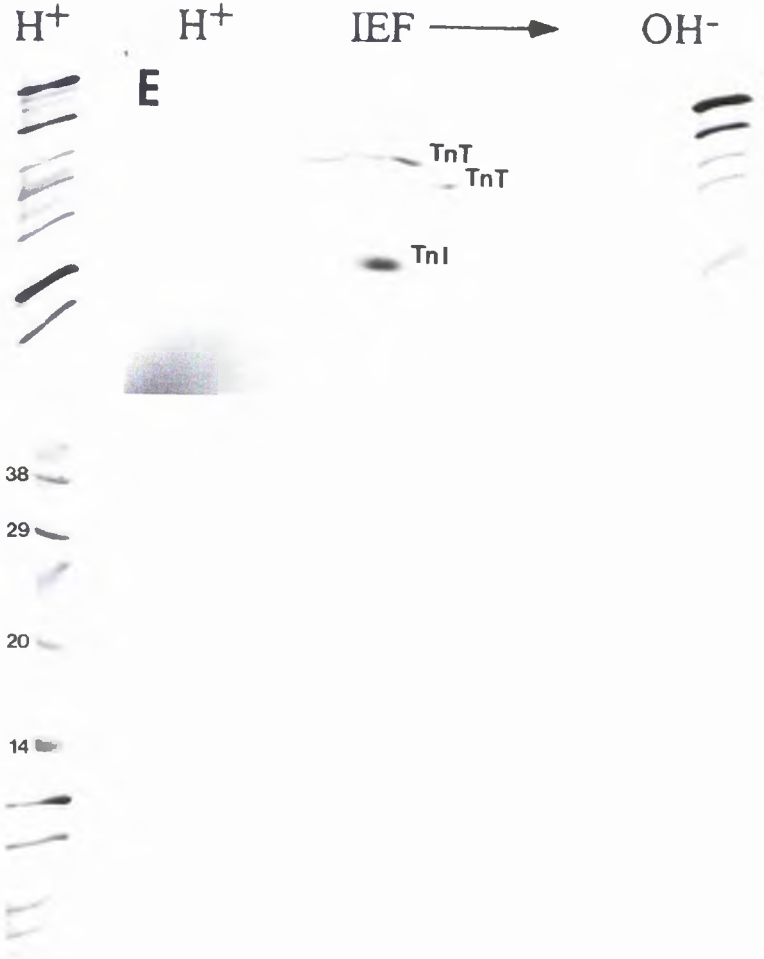
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H+
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The similarities in the Mr and pI of the light chains from each species examined are clearly shown in Figure 5.18 A-I, where white muscle samples from each species have been run together with white muscle form *T. bernacchii*. The pattern of light chain expression is the same in all species except for *T. newnesi*, *E. maclovinus*, and *A. depressiceps* (Fig. 5.18A, F and H, respectively). Only LC1 differs in *T. newnesi* (Fig. 5.18A), whereas all three light chains differ in molecular mass and iso-electric point in *E. maclovinus* (Fig. 5.18F)and *A. depressiceps* (Fig. 5.18H).

5.3.3 Other Proteins

Actin

Actin migrated as a single band, with a Mr of 44 kDa, in both white and red muscle of all the species examined when resolved in 1D-SDS PAGE gels (Fig. 5.2A and 5.3A). Actin was not investigated further.

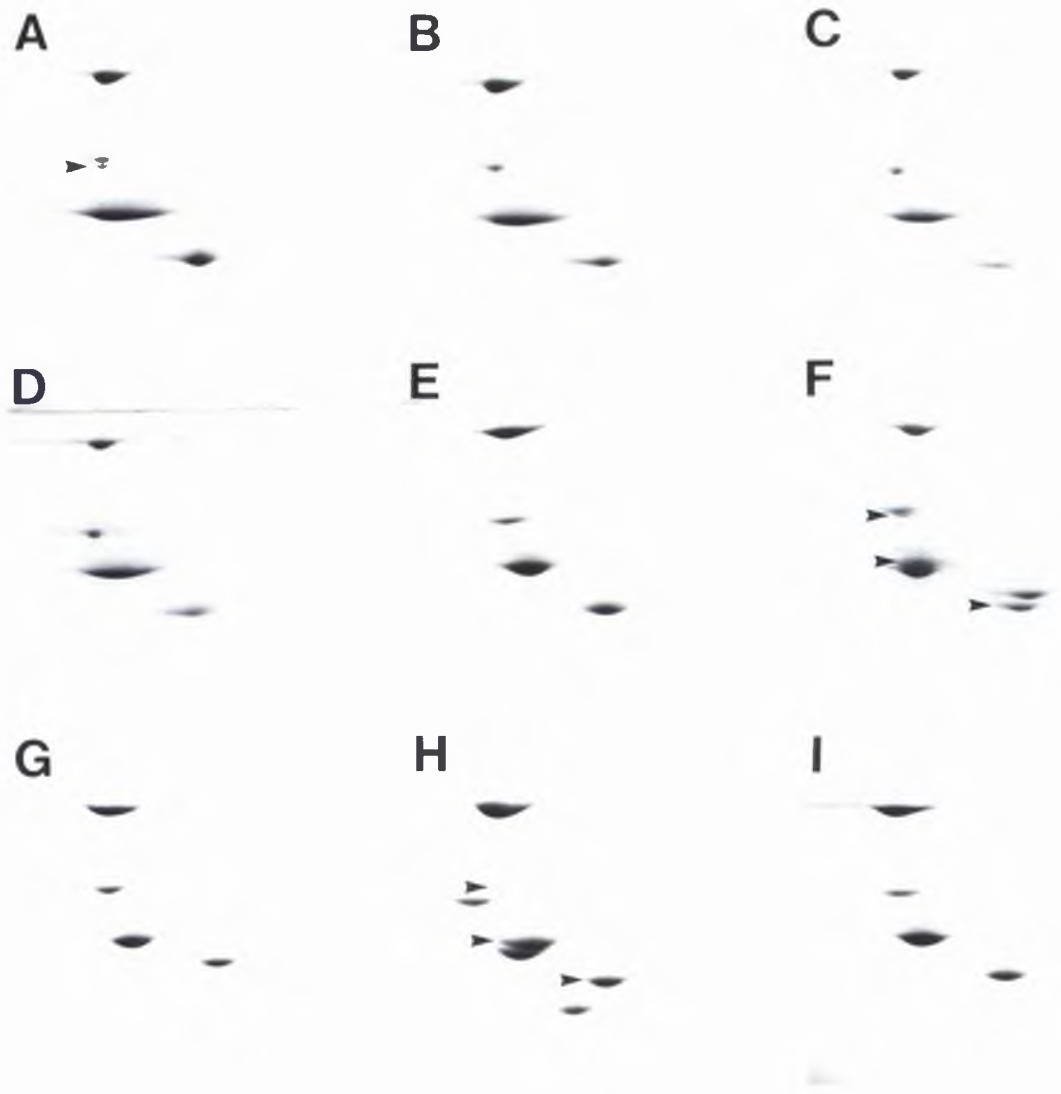
Tropomyosin

The white muscle of every species examined contained a single isoform of Tm with a Mr 38 kDa (Fig. 5.2A, Fig.5.3A). The white muscle isoforms had the same pI in each species (Fig. 5.18A-I). The Tm of red muscle of each species had a Mr of approximately 38 kDa. In *P. magellanica*, the red muscle Tm had the same pI as the white muscle Tm (Fig. 5.13C). In all the other species examined, red Tm had a different pI from white muscle Tm (Fig. 5.8C, 5.9C, 5.10C, 5.11C, 5.12C, 5.14C, 5.15C, 5.16C, 5.17C, 5.18C).

Troponin T

Troponin T was present as a single isoform in the white muscle of all the Antarctic fish species examined (Fig. 5.2C). The isoform had a Mr of 34 kDa (Fig. 5.8E, 5.9E, 5.10E, 5.11E, 5.12E). The red muscle of the

Figure 5.18. Two dimensional gels (IEF in the first dimension, SDS PAGE in the second) of acidic myofibrillar proteins. Each gel contains a mixture of white muscle proteins of *Trematomus bernacchii* with white muscle proteins from one other species. (A) *Trematomus newnesi*, (B) *Trematomus pennelli*, (C) *Trematomus hansoni*, (D) *Pagothenia borchgrevinki*, (E) *Paranotothenia magellanica*, (F) *Eleginops maclovinus*, (G) *Patagonotothen tessellata*, (H) *Austrolycos depressiceps*, (I) *Patagonotothen cornucola*. Rightward facing arrowheads indicate proteins from *Trematomus bernacchii* which are different from the equivalent protein of the other species in the mixture. The gels were stained with Coomassie Blue G-250.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H
I

Trematomus species contained two isoforms of TnT which had slightly larger molecular masses of approximately 34.5 and 35 kDa (Fig. 5.8A, 5.9A, 5.10A, 5.11A). The red muscle of *P. borchgrevinki* contained three isoforms of TnT, of approximately 32, 34.5 and 35 kDa (Fig. 5.12A, lane 2). This was confirmed by Western blotting of the Antarctic fish myofibrils against TnT antibody (Fig. 5.19).

In the sub-Antarctic species, *P. tessellata* and *P. cornucola*, the white muscle contained Mr 32 and 34.5 kDa isoforms (Fig. 5.3C, lanes 5,9; Fig. 5.15E, 5.17E). Similarly, the red muscle of these two species contained two TnT isoforms of the same molecular mass as each other, of approximately 34.5 and 35 kDa (Fig. 5.3C, lane 6,10; Fig. 5.15A, 5.17A). Therefore, these two fish had the same pattern of TnT expression in both muscle types examined. The other sub-Antarctic species examined had different patterns of TnT expression, such that no two species or muscle types had the same TnT isoform expression (Fig. 5.3C, lanes 1,2,3,4,7,8; Fig. 5.13A, 5.14A, 5.16A).

Troponin I

The Antarctic species expressed single 22 kDa isoforms of TnI in both white and red muscle (Fig. 5.2C, lanes 1-8), except for *P. borchgrevinki*, in which white muscle contained a 21 kDa isoform which co-migrated with LC2 in 1D-SDS PAGE (Fig. 5.2C, lane 9; Fig. 5.12A, lane 1; Fig. 5.8E). TnI was not identified in red muscle of *P. borchgrevinki*.

The TnI expression of *P. tessellata* and *P. cornucola* was the same in white muscle, and had an approximate Mr of 21 kDa (Fig. 5.3C, lane 5,9). It co-migrated with LC2 in 1D-SDS PAGE, but was clearly separated by IEF followed by SDS PAGE (Fig. 5.15A, lane 1, Fig. 5.15E and 5.17A, lane 1;

Figure 5.19. Western blot of Antarctic fish myofibrillar proteins developed against troponin T antibody (Clone JLT-12; Sigma, U.K.); lane 1, *Trematomus newnesi* white muscle; lane 2, *Trematomus newnesi* red muscle; lane 3, *Trematomus bernacchii* white muscle; lane 4, *Trematomus bernacchii* red muscle; lane 5, *Trematomus pennelli* white muscle; lane 6, *Trematomus pennelli* red muscle; lane 7, *Trematomus hansoni* white muscle; lane 8, *Trematomus hansoni* red muscle; lane 9, *Pagothenia borchgrevinki* white muscle; lane 10, *Pagothenia borchgrevinki* red muscle; TnT; troponin T. The blot was developed on X-ray film using chemiluminescence.

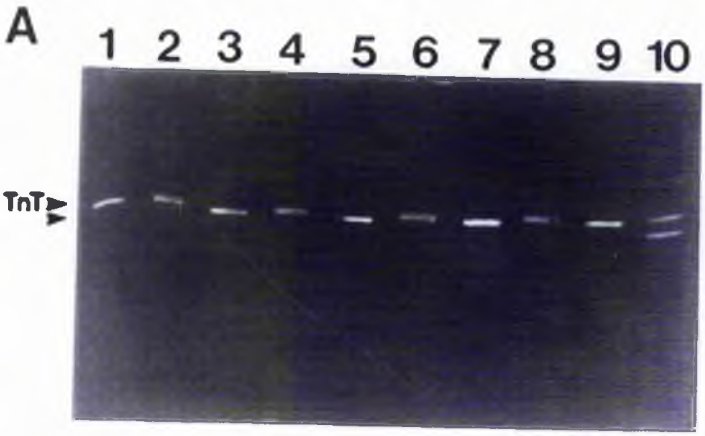
A
1 2 3 4 5 6 7 8 9 10
TnT

Fig. 5.17E). TnI was not identified in red muscle of *P. tessellata* and *P. cornucola.*

P. magellanica white muscle contained two TnI isoforms of approximately 19 and 21 kDa (Fig. 5.13A, lane 1; Fig 5.13E). *E. maclovinus* white muscle contained a single 22 kDa isoform (Fig. 5.3C, lane 3; Fig. 5.14A, E) and *A. depressiceps,* two TnI isoforms of approximately 21 and 23 kDa (Fig. 5.3C, lane 7; Fig. 5.14A, E). The red muscle TnI content of these species was not investigated.

5.4 Discussion

There was little interspecific variation in the relative molecular masses and peptide maps of certain myofibrillar proteins isoforms in Antarctic fish of the same genus. For example, there was no difference in MHC, LC2, LC3, Tm, TnT and TnI between species of *Trematomus*. On the other hand, out of the five Antarctic fish examined, two species, *Trematomus newnesi* and *Pagothenia borchgrevinki*, contained unique isoforms of LC1 and MHC, respectively. Allthough there was a large degree of similarity in the protein isoforms present in closely related species, it is clear that Antarctic fish myofibrillar proteins are not identical in each species examined but differ in molecular mass and isoelectric point. Therefore, the results suggest that divergence in the tertiary structure of myosins from these species has occurred.

The similarity in isoform expression of the *Trematomus* species may be related to their relatively recent divergence times. McDonald (1992) used molecular data to estimate divergence times. The data suggest that most of the *Trematomus* species were diverging from each other between 1.3 and 2.4 million years ago, although separation of *T. bernacchii* and *T. hansoni* is thought to have occurred only 0.4 million years ago (McDonald 1992). *Pagothenia* diverged about 13 million years ago with expansion of the ice sheet, significant sea ice and near zero water temperatures.

As found for the Antarctic fish, species of sub-Antarctic fish that were from the same genus had protein compositions that were very similar. *Patagonotothen cornucola* and *Patagonotothen tessellata* which are almost exclusively confined to the Magellanic Province (Eastman, 1993), had very similar peptide maps of white and red muscle MHCs. In addition these two species showed no interspecific variation in MLC, or TnT and TnI isoforms. Baluskin (1984) stated that the *Patagonotothen* is the most primitive genus of

the Nototheniidae, and that the dozen or so species of this genus are poorly differentiated. Presumably, species of the same genus that have similar morphologies and swimming styles, also have similar functional demands placed upon the contractile proteins.

The remaining three sub-Antarctic species examined, were each from a different genus. Each species contained different MHC isoforms. Quantitative comparisons of the protein bands produced by the digestion of MHC from these species gave high values for the Estimate of Difference. There were also differences in the alkali light chains, TnT and TnI between these species. It therefore appears that the muscle phenotype reflects the classification of these species, such that protein isoform expression is genera-specific, and thus species of the same genus contain similar protein isoforms.

The only 'fly in the ointment' to this generalisation is the different LC1 isoform of the Antarctic *T. newnesi* from other species of the genus *Trematomus*. However, examination of the literature shows that there are several characteristics of this species which differ from the other Trematomids, suggesting that its classification should be revised. Baluskin (1984) carried out a thorough study of the morphology, emphasising osteology, the cephalic and trunk lateral lines and scalation. Baluskin stated that *Trematomus newnesi* deserves "generic isolation" from the other Trematomids. His observations were based on differences between *T. newnesi* and ten other Trematomid species. *T. newnesi* showed differences in the cephalic lateral line, the caudal skeleton, the interhemals and the interneurals. He considered these differences so great that he suggested the other species should be designated *Pseudotrematomus*. Eastman (1991) described Baluskins (1988) list of 127 species of Notothenioid, and included Baluskins (1984, 1989) revision of the sub-family Trematominae, based on a

detailed study of osteology, in which he transferred all but one species of *Trematomus* (*T. newnesi*) to *Pseudotrematomus*. In contrast, DeWitt *et al.* (1990) do not recognise Baluskin's *Pseudotrematomus*, stating that since there are so many problems in classifying the Trematomids, alterations should be left until a clear revision can be made.

T. newnesi has also been differentiated from the other Trematomids in haemoglobin studies. It is the only known species to possess a Hb C, a second major and functionally different haemoglobin component (di Prisco *et al.*, 1991). Furthermore, T. *newnesi* differs from the other Notothenioids in that it lacks a choroid rete, a highly vascularised area of blood vessels which supply the brain (Eastman, 1988). The observed difference of LC1 in this study may be associated in some way, with these specific differences, further adding to the evidence for generic isolation of *T. newnesi.*

Comparisons of the MLCs from white muscle between the species studied revealed that only the Antarctic *T. newnesi,* and sub-Antarctic *E. maclovinus* and *A. depressiceps* contained MLCs which differed in Mr and pI. The MLCs of all the other species were indistinguishable with the methods used. This is contrary to previous studies which have shown that the expression patterns of the MLCs are species-specific, showing no relationships between phylogeny and isoform content. For example, Huriax and Focant (1985) isolated and compared the muscle proteins from the white muscle myosin from nine freshwater teleosts, chosen from within six families, which included species as closely related as carp and goldfish. Each species examined produced their own specific electrophoretic pattern. Huriax and Focant (1985) concluded that the variable structure of the MLCs showed no phylogenetic correlation, but reflected interspecific variations in the contractile proteins, in relation to different locomotory behaviours and modes of life. Similarly, Ochiai *et al.* (1990) examined the light chains of

sixteen fish species and rabbit muscle. Again the MLC distribution was species-specific, with LC3 showing the most specificity. In contrast, the fast myosins of birds and mammals have been shown to be comparatively more consistent between species, and frog myosins appear more related to higher vertebrates than to fish (Focant and Huriax, 1980). The reason for the similarity in MLC composition between the fish examined in this study is unclear. The composition appears to have been conserved, whereas MHC composition showed more variability. It may be that the light chain composition reflects that of a common ancestor which has not been altered by subsequent diversification, possibly because the diversification necessary for altered contractile performance has occurred in the heavy chains.

Eleginops is thought to be the primitive genus of the family Nototheniidae, which separated early from the rest of the stock (Baluskin, 1984). *Eleginops* did not become associated with the margins of the Antarctic plate and hence its subsequent evolution has been little influenced by the Tertiary cooling of the Southern Ocean. It has been suggested that the original Notothenioid stock left behind phyletically primitive elements that diverged prior to the isolation of Antarctica, and *Eleginops* was one of them (Eastman 1993). The isolated divergence and extremely distant common ancestry of *E. maclovinus* may explain its distinct MLC isoforms. The other species examined which expressed dissimilar isoforms of all the proteins examined was *A. depressiceps*. This is a species is a non-Notothenioid, from the sub-order Zoarcoidei and as so is phylogenetically, and morphologically, unrelated to the other species examined.

Most Notothenioids use large fan shaped pectoral fins in labriform swimming as the primary means of propulsion for slow sustained swimming speeds. The pectoral fins are powered by the six pectoral fin muscles, which

largely consist of oxidative red fibres (Harrison *et al.,* 1987). The largest mass pectoral muscle is the *m. adductor profundis.* This powers the adduction stroke, which produces the main forward thrust. The trunk musculature is composed of fast anaerobic muscle fibres (Dunn and Johnston, 1986), which are usually only recruited for a few tailbeats to provide occasional, short duration, burst swimming leading rapidly to fatigue (Montgommery and Macdonald, 1984).

Within the *Trematomus*, unlike the other species, there was a high degree of similarity (low Estimate of Difference) between the MHC of the red and white muscles from the same individual. The intraspecific similarity in myosin isoforms of the *Trematomus* species is probably due to the pectoral fin adductor muscle containing a mixture of red and white fibres. For example, in *T. bernacchii*, this muscle contains a high proportion of white, anaerobic fibres (Davidson and MacDonald, 1985). Therefore, the bands produced by peptide digests of pectoral fin muscle MHC in the *Trematomus* are those of both white and red fibre MHC. In contrast, a mixture of fibre types has not been shown in histological examinations of the adductor muscles of *P. borchgrevinki* (Davidson and Macdonald, 1985). Its pectoral adductor muscle consists of only one fibre type, which are relatively small (49 μm diameter) aerobic fibres, and the myotomal muscles consist almost exclusively of large diameter white muscle fibres (Davidson and Macdonald, 1985). This difference in fibre types, was reflected in the large difference in MHC digests from this species.

Despite the intraspecific differences in MHCs among the species, (other than the *Trematomids*), the MLCs of red and white muscle showed relatively little intraspecific variations in the majority of species. In these fish, the red muscles contained the same molecular mass and iso-electric point, light chain isoforms as the fast white muscles. Since isoform composition of

fibres is known to determine their contractile properties (see Moss *et al.*, 1995) the fast light chain isoforms present in the red fibres suggests that they were not true slow fibres and may have been relatively fast contracting.

In previous studies of fast and slow muscles in teleosts, the fast muscles contained three distinct MLC isoforms (Sarker *et al.*, 1971; Weeds and Pope, 1971; Sreter *et al.*, 1972; Weeds and Frank, 1972; Crockford and Johnston, 1993) and the slow muscles contained two MLC isoforms which were dissimilar from the fast isoforms (Weeds and Baker, 1968; Horvath and Gaetjens, 1972; Focant *et al.*, 1976; Crockford and Johnston, 1993). Pink (fast red) fibres from all teleosts so far studied have contained three MLCs similar to white fibres (Rowlerson *et al.*, 1985; Scapolo and Rowlerson, 1987). The red muscle in all the species in this study contained three MLCs. The red myotomal muscle fibres of plaice (*Pleuronectes platessa*) have been shown to contain three MLCs (Brooks and Johnston, 1995) and staining for mATPase suggested that the red fibres were in fact pink (fast red) fibres (Johnston, 1983). It is conceivable that the adductor muscles of fish in this study contained pink fibres. Pink fibres would not be unsuitable for pectoral fin muscles, since the fins perform both rapid adduction and slow controlled contractions during locomotion. Further biochemical analysis of the pectoral fin muscle fibres would be required to substantiate this proposal.

Chapter 6

GENERAL DISCUSSION

6.1 Present Findings

Myofibrillar protein isoform composition is one of the major determinants of muscle contractile properties (see Moss *et al.*, 1995; Schiffiano and Reggiani, 1996, for reviews). This study revealed myofibrillar isoforms to vary during ontogeny in both the Atlantic herring (*Clupea harengus*) (Chapter 3) and the short-horn sculpin (*Myoxocephalus scorpius*) (Chapter 4). In Chapter 3, rearing temperature was also found to influence isoform expression in herring. Examination of Antarctic and sub-Antarctic species revealed that myofibrillar protein composition was similar in species from the same genera.

In Chapter 3, the adult pattern of myosin LC2 expression was found to be established at shorter body lengths when reared at higher temperatures in the Atlantic herring. Similarly, there were also complex changes in the expression of the thin filament Ca^{2+} regulatory proteins, troponin I (TnI) and troponin T (TnT), with the adult isoform pattern established at shorter body lengths at higher rearing temperatures. There was also a transition in myosin heavy chain (MHC) isoforms in white muscle between 20 and 25 mm total length (TL), with respect to rearing temperature. Therefore, this study has demonstrated that different rearing temperatures produce variation in muscle phenotype over the size range 12 to 25 mm. This is the size range at which important structural changes associated with swimming take place, i.e. when fin and muscle innervation changes occur. In addition, the style of swimming changes from anguilliform to a sub-carangiform, over this size range (Batty 1984; Blaxter, 1988). It is possible that these phenotypic variations which

affect swimming style and performance, will in turn influence predator-prey interactions, larval survival and recruitment into the adult population. It would be of great interest to test this theory by experimentation in the laboratory and/or the field. However, such experiments would be extremely complex due to the number of abiotic and biotic factors which influence survival (Gotceitas *et al.*, 1996; Hinckley *et al.*, 1996).

In Chapter 4, myofibrillar ATPase activity was found to decrease with increasing body length in the short-horn sculpin. This relationship between body size and ATPase activity has also been shown in a number of freshwater and marine species (Witthames and Greer-Walker, 1982). In previous studies, variations in ATPase activity with temperature acclimation in the carp have been related to different isoforms of MHC (Gerlach *et al.*, 1990; Hwang *et al.*, 1991) and variations in the ratio of MLC (Crockford and Johnston, 1990). Ball and Johnston (1996) showed that differences in myosin ATPase activity due to temperature acclimation in the short-horn sculpin, were a result of differential expression of the alkali light chains. The striking feature of this study was that there were no differences in MHC or MLC expression with fish length, but instead differential isoforms of TnI were present and their expression varied with body size. TnI is the inhibitory component of the troponin complex and an integral part of the contractile apparatus. An alteration in the structure of this component is likely to affect the interaction between the proteins of the molecular motor, which would result in some alteration of contractile properties. No studies in birds and mammals have yet shown the presence of multiple isoforms of TnI. The exact significance of this result has yet to be determined.

The expression of myofibrillar proteins of the Antarctic and sub-Antarctic fish examined showed few differences between species of the same genera (Chapter 5). For example, the MHC was indistinct within the

four Antarctic *Trematomid* species and similar in the *Pagothenia* species. In addition, there was a high semblance in the isoform expression between the two sub-Antarctic species from the genera *Patagonotothen* . The similarities in protein phenotypes could suggest that isoform expression is unrelated to swimming behaviour and is merely a reflection of the genus. However, it is more likely that the similarity of the protein isoforms is due to swimming styles and activity levels which are alike because of the relatively recent evolutionary divergence times between species of the same genus. Closely related species therefore have comparable morphological characters, which has consequently led to their classification in the same genus. At present the phylogenetic relationships between the sub-Antarctic and Antarctic species within the sub-order Notothenioid are unclear; current classification is based entirely on morphological characteristics. It would be interesting to determine the phylogeny of the species examined, by the use of mitochondrial analysis of the cytochrome b gene (Block *et al.*, 1993). The phylogenetic relationships could then be related to the patterns of myofibrillar protein expression found. A phylogenetic map might reveal that the few exceptions to the likeness in isoform expression found between genera are in fact the result of suspected errors in the present classification.

It is conceivable that alterations in myofibrillar isoforms confer some advantage in muscle performance. The exact effect of each protein *in vivo* has yet to be fully determined, but resolving the function of these proteins *in vitro* has been aided by some ingenious techniques (see Vale, 1994 for an excellent review). *In vitro* motility assays have been used to determine the effect of manipulation upon the velocity of actin filaments sliding over myosin-coated glass slides (Lowey *et al.*, 1993; Uyeda and Spudich, 1993). The proteins of the molecular motor can be removed, altered or substituted for other isoforms during these experiments, in order to determine the effect of a particular manipulation upon the maximum shortening velocity (V_{max}).

For example, Lowey *et al.* (1993) showed that the removal of light chain subunits greatly reduces the velocity of actin filaments without affecting myosin ATPase activity. In addition, Uyeda and Spudich (1993) found a direct correlation between the reduction in actin sliding velocity and the amount of shortening of the myosin lever arm from genetically engineered slime moulds, *Dictyostelium* species.

It would be of interest to examine the genetic control of proteins and determine what turns particular proteins on and off, how and when. This would involve further research into the exact hormonal, innervation or myogenic mechanisms which regulate isoform expression.

6.2 Future Studies

The thermal environment experienced by an organism can change dramatically during ontogeny, as a result of seasonal temperature fluctuations or changes in habitat as development proceeds. Furthermore, in a number of species, the thermal tolerance and the optimum temperature for growth and locomotion have been shown to vary during ontogeny (Johnston *et al.*, 1996). It would be of great importance for the understanding of ecosystem functioning and fisheries management, to fully comprehend how each stage of the life cycle responds to different thermal environments. However, to date the majority of studies have focused exclusively on the adult stages.

Fry and Hart (1948) showed that the temperature dependence of swimming speed in goldfish (*Carassius auratus* L.) was not fixed but varied with thermal acclimation state. Swimming speed increased at low temperatures following low temperature acclimation and decreased at high temperatures. ATPase activity has been found to increase with cold acclimation, but was more readily denatured at high temperatures in many

freshwater fish, including the common carp (Johnston *et al.*, 1975; Heap *et al.*, 1985; Crockford and Johnston, 1990). In the white muscle fibres of the carp, the maximum stress and contraction velocity were found to be higher (Johnston *et al.*, 1985), and relaxation rates shorter, (Johnston *et al.* 1990) at low temperatures in cold- compared to warm-acclimated individuals. The molecular mechanisms underlying the plasticity of contractile properties involves altered expression of MLC (Crockford and Johnston, 1990) and MHC sub-units (Hwang *et al.*, 1991), and changes in sarcoplasmic reticulum ATPase activity (Fleming *et al.*, 1990). Imai *et al.* (1997) have isolated 3 cDNA's coding for MHCs that are expressed in a temperature-acclimation state dependent manner. Thus temperature specific isoforms of myosin are a key feature of the plasticity of muscle contractile properties in adult carp. Distinct developmental isoforms of myofibrillar proteins have been recognised in the embryo, larvae, juveniles and adult stages of the life cycle of several species, including Arctic Charr (Martinez *et al.*, 1991), sea bream (Mascerello *et al.*, 1995) and the Atlantic herring, as previously described in this study. By quantifying the variations in muscle contractile properties brought about by the expression of different protein isoforms, the effect of these isoforms on whole animal swimming performance may be determined. From this, the adaptive significance of this phenotypic plasticity could be assessed. This type of study would also help to clarify the significance of particular isoform changes on whole animal performance by combining examination of the protein isoforms present in larval fish with their swimming performance.

Further work could also be directed towards the investigation of alterations in temperature tolerance during development. The thermal tolerance of the common carp has previously been shown to increase during ontogeny following several weeks of cold acclimation. It would be of interest to test the hypothesis that phenotypic plasticity of swimming performance is

gradually acquired during the juvenile stage, and is accompanied by the expression of the adult myosin isoforms. This could be done by characterising myosin expression and investigating the plasticity of myofibrillar ATPase activity with temperature acclimation at different stages of the life cycle. In addition, it would be necessary to quantify swimming performance in larval, juvenile and adult stages. By combining both experimental and modelling approaches, it would be possible to estimate muscle power output *in vivo*, as well as the power requirements and hydrodynamic efficiency of fish locomotion. Such studies could be made more ecologically relevant by examining fast-start performance, employed during critical predator-prey encounters. A plausible outcome is that fast-start performance will improve at low temperatures during the juvenile stage in parallel with the seasonal cooling and accompanying expression of adult isoforms of myosin.

APPENDIX

Abbreviations, symbols, units

A	Ampere
Act	Actin
AU	Alkali-urea
Bis	N, N'-methylenebisacrylamide
D	Estimate of Difference
Da	Dalton
DTT	Dithiothreitol
DTNB	5,5'-Dithio-bis(2-Nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(b-aminoethyl ether)N,N,N',N'-tetraacetic acid
h	Hour
HRP	Horse raddish peroxidase
IEF	Isoelectric focusing
IgG	Immunoglobulin G
kDa	Kilodalton
LC1	Light chain one
LC2	Light chain two
LC3	Light chain three
MHC	Myosin heavy chain
min	Minutes
mM	Millimole
Mr	Relative electrophoretic mobility
MW	Molecular weight

PAGE	Polyacrylamide gel electrophoresis
pI	Isoelectric point
s	second
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylenediamine
Tm	Tropomyosin
Tn	Troponin
TnC	Troponin C
TnI	Troponin I
TnT	Troponin T
U_{max}	Length specific swimming speed
V	Volt
V_{max}	Maximum shortening velocity
α	alpha
β	beta
μ	micro
1D	one dimensional
2D	two dimensional

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