THE INFLUENCE OF TEMPERATURE ON MUSCLE DEVELOPMENT IN THE TELEOST 'PLEURONECTES PLATESSA L.'

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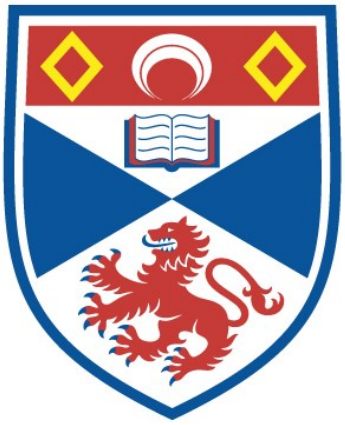

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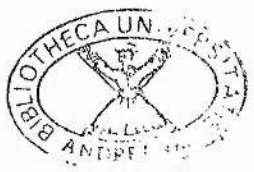

Declaration

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

The research was conducted in the School of Biological and Medical Sciences, United College of St. Salvator and St. Leonard, University of St. Andrews, under the direction of Professor I.A. Johnston.

Signed: Date: 16.8.93

Certificate

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

Signed: Date: 16/8/93

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Summary

Chapter 1

General Introduction

The general introduction begins with a brief description of the natural history of the plaice. This is followed by a review of the literature describing the development of teleost embryos and larvae. The pattern of innervation with development and the differentiation of muscle fibres are both discussed in detail, as are larval locomotion and patterns of swimming behaviour. The remainder of the general introduction concentrates upon the phenotypic plasticity of muscle and the mechanisms by which this may be achieved. Teleost muscle in particular has been found to show an extremely plastic response to changes in ambient temperature. The contractile protein composition, ultrastructure and contraction properties of fish muscle are all influenced by temperature. However most research has concentrated primarily upon adult teleosts and fewer studies have examined the effects of temperature on muscle in larval and juvenile fish. Experiments describing the effect of temperature upon muscle structure and contraction both in the larval and adult stages are discussed at the end of the Introduction.

Chapter 2

Materials and Methods

The materials and methods used throughout the thesis are described. Methods of collection, rearing and maintenance of plaice eggs, larvae and adults are all recorded in this chapter. The effects and suitability of different histological and T.E.M fixatives were tested on newly hatched larvae and the results shown here. This chapter also includes descriptions of the

histochemical stains and procedures used, methods of preparing myofibrils and purifying myosin from plaice muscles, and a description of the various electrophoretic techniques used throughout.

Chapter 3

The influence of temperature on somitogenesis and organogenesis in embryos of the plaice, *Pleuronectes platessa* L.

The development of plaice embryos was examined at two temperatures, 8°C and 12°C. Temperature influenced the rate of embryonic development but did not affect the order of appearance of morphological characters.

The rate of somitogenesis was observed in embryos reared at 5°C and 12°C. Formation of the initial somites occurred relatively later in development at high than at low temperatures. In embryos reared at 5°C, somites were observed after 25% development time from fertilisation to hatching, whereas somites were not apparent until 32% development time in embryos reared at 12°C. Spontaneous contractions of the anterior somites and a regular heart beat were observed after 47% development time at both temperatures, corresponding to the 50 somite stage at 5°C and the 44 somite stage at 12°C. Somites were added at a rate of 0.35/hour at 5°C and 0.8/hour at 12°C ($p < 0.05$). Once movement had started the rate of somitogenesis was significantly decreased. At hatching larvae reared at 5°C had 9% more somites (61 ± 1.5) than larvae reared at 12°C (56 ± 1.3) ($p < 0.05$). The total length of newly hatched larvae reared at 5°C was 9% greater (6.6 ± 0.4 mm) than larvae reared at 12°C (6.1 ± 0.2 mm).

Chapter 4

Muscle development in plaice, *Pleuronectes platessa* L.

The effects of temperature on muscle fibre ultrastructure were examined using larvae reared at 5, 8, 10, 12 and 15°C. Two types of muscle fibre could be distinguished in the newly hatched larvae; beneath the skin was a single, superficial layer of smaller diameter muscle fibres, which completely surrounded the larger diameter inner muscle fibres. Larvae reared at 15°C only survived for a few days and had significantly more myotomal muscle fibres of larger average cross-sectional area than those hatching at 5-10°C. Myofibrils occupied 61% of the volume of inner muscle fibres in 15°C larvae, compared with 35% and 36% in larvae hatching at 5°C and 10°C respectively ($p < 0.05$). The volume density (Σ mitochondria cross-sectional areas/muscle fibre cross-sectional area) of mitochondria in the superficial fibres was significantly higher at 15°C (33%) than at 5°C (24%) and 10°C (27%) ($p < 0.05$). The volume density of mitochondria in the inner fibres was not significantly different at any of the rearing temperatures, nor was the cristae density of the mitochondria.

Muscle growth and differentiation were subsequently investigated at 8°C. The yolk-sac was entirely absorbed after 8 days and by 5 weeks the trunk had increased in size both lengthwise and dorso-ventrally so that muscle fibres extended dorsal to the spinal cord. Between 1-2 weeks after hatching, well defined apical dorsal and ventral germinal zones were evident which contained numerous undifferentiated myoblasts and small diameter muscle fibres. Metamorphosis, and the change from a pelagic to a demersal lifestyle, occurred between 7 and 10 weeks post-hatching and was not associated with any change in the distribution of muscle fibre types. Thickening of the superficial muscle layer was first evident in

laboratory-reared fish 25 mm in length and in 0-group juveniles caught in June and July. On the basis of the histochemical staining reactions for myofibrillar ATPase, PAS and succinic dehydrogenase activities the myotomes of 1-group (10.4 cm long) and adult plaice (28 cm long) contained a minimum of five distinct muscle fibre types.

Chapter 5

Electrophoretic analysis of the myofibrillar components of red and white muscle fibres from adult plaice, *Pleuronectes platessa* L.

The myofibrillar proteins of the two main fibre types were identified using various electrophoretic techniques, including SDS PAGE, IEF PAGE, NEPHGE PAGE and alkali-urea gels.

The myosin light chain composition of each of the four histochemically identified fibre types was determined. Tonic, red and superficial white fibres all contained multiple isoforms of myosin light chain 2.

Plaice red muscle fibres contained all three myosin light chains. In other species of fish, red muscle has been characterised as having only two myosin light chains, myosin light chain 1 and myosin light chain 2.

Chapter 6

Developmental transitions in myosin sub-unit composition from larval inner to adult fast muscle in plaice, *Pleuronectes platessa* L.

The myosin sub-unit composition of plaice deep white muscle fibres was examined at different stages of development.

Myosin light chains 1 and 3 did not appear to alter throughout development but in newly hatched larvae two isoforms of myosin light chain 2 ($LC2_{L1}$ and $LC2_{L2}$) were present in the inner fibres which were not present in adult white fibres. The two larval isoforms of myosin light chain 2 were expressed throughout the larval stage, until metamorphosis, when the adult isoforms of light chain 2 ($LC2_{F1}$ and $LC2_{F2}$) were also observed. Larvae undergoing metamorphosis had four isoforms of light chain 2 present in the inner fibres.

Group 0 juveniles continued to express both larval and adult light chain 2 isoforms. Group 1 juvenile inner muscle fibres showed the same pattern of light chain isoforms as was observed in adult superficial white fibres.

Developmental changes in myosin heavy chain sub-unit composition of plaice inner muscle fibres were studied by peptide mapping. At 6 weeks post-hatching and at metamorphosis the inner muscle fibres had the same myosin heavy chain sub-unit composition. The myosin heavy chain component of the inner muscle fibres was, however, found to be different in group 1 juveniles and adult deep white fibres.

Chapter 7

General Discussion

The major findings of the study are discussed in relation to other experiments examining muscle development and differentiation in teleosts. The influence of temperature on the structure and development of plaice myotomal muscle is discussed, with particular reference to larval survival. Possible mechanisms by which muscle development in teleosts may be regulated are described together with suggestions for further work.

Chapter 1

General Introduction

The plaice, *Pleuronectes platessa* L., is an important commercial species, widely fished from the seas surrounding the British Isles. It has been the subject of intensive study since the beginning of the century (Johansen and Krogh 1914, Simpson 1956, Ryland 1966, Harding and Talbot 1973, Harding 1974). Adult plaice are demersal flatfish, living on or above the seabed, feeding on invertebrates such as molluscs and polychaetes. Although morphologically and behaviourally the plaice is highly adapted to life on the seabed (Arnold and Weihs 1978), they cannot be considered truly benthic because they make long migrations to breeding grounds (de Veen 1962, 1970), and may spend as much time in midwater as on the bottom (Greer Walker *et al.* 1978). By contrast the early life history of the plaice occurs in the plankton. Both the eggs and larvae are pelagic. As the larvae grow and develop they gradually sink down the water column, metamorphosing stages spending long periods on the substratum, only being found in midwater at night after losing contact with the bottom (Harding and Talbot 1973).

Plaice populations in the North Sea were very stable in the years between 1906 and 1960, which led Beverton (1962) to suggest that there was a density-dependent compensating mechanism in effect, which took place during the pelagic phase of their life history. For the majority of marine teleost species the egg and particularly the larval stages constitute the most delicate phases of the life cycle and are consequently more vulnerable to changes in their environment (Simpson 1956). The small size of plaice larvae means that they are exposed to all the environmental fluctuations inherent in the water column and are easy prey for larger predators.

Development of the teleost embryo

Teleost embryos have often formed the basis for embryological study (Ballard 1973a, 1973b, Betchaku and Trinkaus 1978, Hanneman *et al.* 1988, Timmermans 1989). The morphogenic movements of even very deep cells within the embryo are visible because of the transparent nature of the chorion and developing tissues. Sperm entry is restricted to the animal pole of the teleost egg (Roosen-Runge 1938). The sole means of entry is via the micropyle, adjacent to the cortical location of the maternal nucleus, the only part of the chorion not sperm impermeant (Devillers 1961). After fertilisation there is a dramatic reorganisation of the one celled zygote. Cytoplasm segregates from the yolk and flows towards the animal pole accompanied by cortical contractions progressing along the animal-vegetal axis (Devillers 1961, Lewis and Roosen-Runge 1942, 1943). Cleavage of the embryo then begins. The early cleavages of fish are meroblastic; the blastoderm cells remaining linked by cytoplasmic connections to the underlying yolk cell (Wilson 1889, Betchaku and Trinkaus 1978). By the time of the tenth cleavage division of the zebrafish *Brachydanio rerio* embryo, there are three types of blastoderm cells present: a monolayer of cells around the surface of the blastoderm, termed the enveloping layer (EVL); non-marginal 'deep layer' cells lying between the EVL and the yolk syncytial layer (YSL). The YSL is unique to teleosts (Long 1983), forming when cell membranes of adjacent marginal EVL cells partially fuse and cytoplasmic connections to the yolk cell enlarge, causing nuclei and cytoplasm to collapse into and become part of the yolk cell (Long 1980, Kimmel and Law 1985a). Strehlow and Gilbert (1993) simultaneously injected two very-high-molecular-mass fluorescent dye/dextran polymers into different blastomeres at the two, four and eight cell stages. The movement of the dyes revealed that the three axes of the adult zebrafish, dorsal-ventral, left-right and anterior-posterior, are defined by lineage progenitors from the earliest stages. Previously it had been thought that mixing of

the blastomeres of zebrafish prevented them from giving rise to a recognisable fate map (Kimmel and Law 1985a, b, Kimmel and Warga 1986, 1987, Kimmel *et al.* 1990). Kimmel *et al.* (1988) even concluded that final cell position, rather than cell lineage, made a greater contribution to cell fate. Although early cell fates may be difficult to determine (Kimmel and Warga 1987), at the gastrula stage cells regularly generate clones restricted to a single kind of tissue (Kimmel and Warga 1986). Extensive studies of the zebrafish show a segmental pattern of development within the embryo following gastrulation (Hanneman *et al.* 1988, Kimmel *et al.* 1988, Kimmel *et al.* 1991, Ho 1992). The cellular events controlling segmentation in the zebrafish appear to operate rather late during embryonic development. Extensive cell migrations cease prior to any visible signs of segmentation (Kimmel *et al.* 1988). The terminal cell divisions generating early muscle fibres and neurones occur at about the time cell movements cease along the embryonic axis (Kimmel and Warga 1986, Myers *et al.* 1986). Kimmel *et al.* (1988) suggested that only after cells have reached their definitive positions will segmentation begin. Segmented peripheral tissues in zebrafish include the trunk and tail myotomes which are derived from the somitic mesoderm (Van Raamsdonk *et al.* 1977, 1979). The spinal cord and hindbrain are also segmentally organised (Hanneman *et al.* 1988).

Pattern of innervation with development

Development of the hindbrain and spinal cord of the zebrafish embryo follows a segmental pattern (review- Kimmel and Westerfield 1988). In the ventral hindbrain and spinal cord of embryos, the first identifiable neurones appear as single cells or small clusters of cells distributed somite lengths apart along the body axis (Hanneman *et al.* 1988). Present within the hindbrain are a series of neuromeres also equal in length to the somites, the earliest neurones being located within the centre of each neuromere. As embryonic development proceeds and

more neurones differentiate, Hanneman *et al.* (1988) observed the initially similar patterning of the cells in the hindbrain and spinal cord regions diverging. In the spinal cord a continuous longitudinal column of developing neurones appeared, while in the hindbrain there was an alternating series of large and small neurones.

Studies of the organisation of spinal cord motor neurones and the innervation of white muscle fibres in the myotomes of adult zebrafish show that there are two classes of motor neurones, primary and secondary (Westerfield *et al.* 1986). Primary motor neurones have larger cell bodies and larger diameter axons than secondary motor neurones. In addition, the cell bodies of primary motor neurones occupy a more dorsal and medial position within the spinal cord than those of the secondary motor neurones. Westerfield *et al.* (1986) distinguished three primary motor neurones in each segment on each side of the spinal cord. The adult primary motor neurones corresponded both in position and morphology to the primary motor neurones found in embryonic and larval zebrafish (Eisen *et al.* 1986, Myers *et al.* 1986). The secondary motor neurones are positioned more ventrally and laterally in the spinal cord, have smaller axons and characteristically smaller fields. Zebrafish motor neurones innervate cell-specific subsets of contiguous muscle fibres in mutually exclusive regions of their own body segment. Each individual muscle fibre receives inputs from only one primary motor neurone but up to three secondary motor neurones (Westerfield *et al.* 1986).

The development of synaptic connections within the zebrafish embryo necessitates the successful navigation of neuronal growth cones to their correct target cells. Previous studies highlighted a small group of early-developing, individually identifiable primary motor neurones, the growth cones of which pioneer the peripheral nerves (Eisen *et al.* 1986, Myers *et al.* 1986). Studies of both embryonic (Eisen *et al.* 1986) and larval zebrafish (Myers *et al.* 1986) showed that

the growth cones of the primary motor neurones project directly to their cell-specific target muscles along stereotyped pathways. Individual primary motor neurones of the zebrafish embryo pioneer cell-specific peripheral motor nerves, the growth cones of the secondary motor neurones extending along the pathways pioneered by the primary motor axons. Pike *et al.* (1992) found that ablation of the primary motor neurone pioneering the ventral nerve caused a delay in ventral nerve formation, but the nerve was eventually formed. In the absence of the primary motor neurone normally acting as a pioneer for the dorsal nerve however, the secondary motor neurones formed dorsal nerves in an atypical position and not in the normal location. The two different primary motor neurones both appear to have distinct roles in guiding the growth cones of the secondary motor neurones. Certainly it seems that pioneer neurones are able to perform various functions during the developmental process and that numerous cues combine to guide growth cones along the appropriate pathways (Pike *et al.* 1992).

Somitogenesis and the differentiation of muscle fibres

In the teleost *Brachydanio rerio*, the somites divide from the presumptive somitic mesoderm along the rostro-caudal axis of the developing embryo (Waterman 1969, Van Raamsdonk *et al.* 1974, 1977, Hanneman and Westerfield 1989). Somitogenesis in teleosts is similar to that in amphibians, but differs from the process of somitogenesis in birds and mammals. In birds and mammals somite formation is preceded by the regression of Hensen's node through a preformed mass of mesodermal tissue (Flint *et al.* 1978, Veini and Bellairs 1983). The trunk somites in teleosts and amphibians develop from a preformed mass of mesodermal tissue, while the caudal somites are formed by the presegmental extension of a zone of proliferation situated in the central region of the tail bud (Elsdale and Davidson 1983). The proposed model for somite

formation in the zebrafish (Hanneman 1992) is that proliferative cells in the tail bud produce a morphogen. As the cells become committed to the presumptive somitic mesoderm they acquire acetylcholinesterase (AChE) activity, which has been observed occurring approximately five somites caudal to the zone of somite formation (Hanneman 1992). AChE is thought to degrade the morphogen, the degradation products then become or cause the production of an activating signal which initiates somite formation. Certainly when the presumptive somitic mesoderm was unable to initiate AChE activity because of inhibition by di-isopropylfluorophosphate (DFP), abnormal somites developed (Hanneman 1992).

Immediately after their formation all somites consist of loose central cells surrounded by a simple epithelium (Waterman 1969, Van Raamsdonk *et al.* 1974, 1977, 1978). Myogenesis begins at the medial surface of the zebrafish somite, close to the notochord (Waterman 1969). The first myofilaments were found to appear at the ten somite stage, in the cranial somites, within uninuclear cells (Waterman 1969, Van Raamsdonk *et al.* 1974). In the centre of the somites, cells fused to become polynucleate prior to myofilament formation (Van Raamsdonk *et al.* 1974). Waterman (1969) suggested that different fibre types developed from the superficial and deep cells within the somites. However, Van Raamsdonk *et al.* (1978), found no evidence to suggest that the different fibre types were generated from distinct populations of myoblasts.

Locomotion and patterns of swimming behaviour

The swimming behaviour of fish larvae changes as they grow and develop (Hunter 1972, Weihs 1980, Webb and Weihs 1986, Fuiman and Webb 1988). In the first few days after hatching larvae of the northern anchovy, *Engraulis mordax*, possess a different mode of swimming from both older larvae and later stage fish (Weihs 1980). At hatching yolk-sac larvae undergo

bouts of continuous, energetic swimming, interspersed with periods of floating motionlessly (Hunter 1972). A new swimming pattern emerges three to four days after hatching when larvae can be seen combining alternate periods of swimming and gliding. The appearance of intermittent swimming coincides with the absorption of the yolk-sac and the start of exogenous feeding (Hunter 1972). Fuiman and Webb (1988) also quantified ontogenetic changes in spontaneous swimming of a teleost larvae, that of the zebrafish, *Brachydanio rerio*. Swimming speeds and mean acceleration rate increased throughout the larval period, the most rapid changes occurring at 5-15 mm total length. Fish longer than 15 mm showed only a small rate of increase in performance. The changes in swimming performance were tentatively linked to rapidly occurring morphological changes in larval structure (Fuiman and Webb 1988). As the adult structure was attained the changes in swimming performance declined (Fuiman and Webb 1988). The swimming behaviour of yolk-sac larvae has been shown to be a more efficient method of locomotion at this stage of the lifecycle because routine activities of the early life history stages of fishes occur in an intermediate hydrodynamic environment (Webb and Weihs 1986). The hydrodynamic environment depends on two physical properties of water, viscosity and density, and the size and speed of objects moving through the water. The small size of the larval stages of fish therefore places them in a hydrodynamic regime with somewhat different properties than that occupied by adult fish (Vlymen 1974, Weihs 1980, Batty 1984). Reynolds number (Re), a function of both fish length and speed, defines the relative importance of viscous and inertial effects on the hydrodynamic resistance to motion. When $Re < 20$ viscous forces are paramount, the drag of any body is proportional to viscocity and velocity (Wu 1976, Weihs 1980), making continuous high speed swimming energetically efficient. Inertial forces dominate when $Re > 200$; viscous effects are confined to a narrow region adjacent to the body surface, the so called 'boundary layer', where most resistance

to motion arises. When inertial forces are more important, as is the case for longer larvae, juveniles and adults, beat- and glide- swimming is more energetically efficient. Since Re is dependent upon body length and velocity, the hydrodynamic regime changes as larvae grow or alter speed, changing the balance between viscous and inertial forces.

Phenotypic plasticity of muscle

Muscle as a tissue has the ability to respond effectively to a wide range of conditions and constraints and has been found to adapt to different circumstances in a variety of different ways. An increase in work load can produce hypertrophy, an increase in the size of the component muscle fibres (Baldwin *et al.* 1976, Goldspink 1985), or alter the relative proportions of fibre types (Green *et al.* 1979). Muscle fibres may also respond to functional demands by changes in metabolism, increasing or decreasing enzyme levels or activity (Goldspink and Waterson 1971, Green *et al.* 1983). Muscle fibre ultrastructure is also influenced by function. High work loads result in an increase to the number of capillaries per fibre (Hoppeler and Lindstedt 1985) and also affect mitochondrial density (Baldwin *et al.* 1972). However, the essential plasticity of muscle is ensured primarily because of the pronounced polymorphism of the contractile proteins. Each individual muscle fibre is able to respond to functional demands by changes in the phenotypic expression of one or more myofibrillar components. Even within a single muscle fibre it is possible for the contractile filaments to consist of several isoforms of a particular myofibrillar protein (Salviati *et al.* 1982, Bottinelli *et al.* 1991).

Polymorphism of the myofibrillar components

The myofibrils consist of the thick and thin filamentous contractile proteins held together by the Z-discs to form sarcomeres. Each sarcomere is made up of two sets of thin

actin (I) filaments and one set of thick myosin (A) filaments together with various other protein components such as M-line protein, C protein, titin and nebulin, tropomyosin and the troponins. M-line protein and C protein are both involved in filament assembly. Proteins in the M-line are thought to keep the myosin filaments in the correct spatial arrangement for the actin filaments. C protein may be involved in the aggregation of the myosin monomers and regulation of thick filament length. Titin forms elastic filaments which apparently link the myosin filaments to the Z-lines. The titin filaments are also thought to position the myosin filaments at the centre of the sarcomere (Horowits *et al.* 1989). Nebulin is considered to be inextensible, it is found associated with the thin filaments of the sarcomere. Trinick (1992) proposed that nebulin regulates thin filament assembly by acting as a framework for the contractile proteins. A similar role for titin to regulate thick filament assembly was suggested by Whiting *et al.* (1989). Actin and myosin are the major myofibrillar components, comprising 80% of the myofibril, (actin 25%, myosin 55%). Associated with the actin filament are the regulatory proteins troponin and tropomyosin. Force generation in muscle occurs as the thin actin filaments and thick myosin filaments slide past one another as ATP is hydrolysed (Taylor 1979, Trayer 1993). Myosin heads projecting from the thick filaments undergo a cyclic interaction with the actin residues on the thin filaments. The orientation of the myosin heads, with respect to the actin filaments, changes as a consequence of ATP binding, hydrolysis and product release (Trayer 1993).

Thin filament proteins

Actin has always been assumed to play a passive role in muscle contraction aside from the activating the magnesium ATPase activity of myosin. Recent studies (Drummond *et al.* 1990, Prochniewicz and Yanagida 1990, Schwyter *et al.* 1990), have shown that actin cannot be considered solely as the

activator of the myosin heads (S1 subfragment), but is also involved in the contractile mechanism. The modification of a single amino acid within the actin molecule can result in noticeable modifications in the mechanical performance of muscle (Morel and Merah 1992).

Tropomyosin and the troponins are associated with the actin thin filament of the myofibrils. Tropomyosin plays a regulatory role in the contraction of striated muscle, it has been found to be essential for the calcium sensitivity of actomyosin ATPase when troponin is present (Schachat *et al.* 1987). The function of tropomyosin is thought to be related to its ability to interact and form a linear aggregate with actin, possibly stabilising the filament structure (Perry 1985).

Troponin T is also a key protein in the regulation of skeletal muscle contraction by calcium (Greaser and Gergely 1973). The molecule shows extreme diversity based upon alternative splicing of the troponin T primary RNA transcript (Imai *et al.* 1986). The N-terminal region of the troponin T molecule is a hypervariable domain, which is generated by alternative splicing of five short 5'exons (Breibart *et al.* 1985). The N-terminal region of troponin T is functionally important in the contractile response of muscle fibres because it stabilises the interaction between adjacent troponin-tropomyosin regulatory complexes (Breibart *et al.* 1985). In mammalian muscles a different pattern of expression of troponin T molecules has been observed in each muscle and fibre type. The different troponin T molecules do not appear to be muscle or fibre type specific, but the component proportions of fast and slow troponin T species do vary with fibre type (Moore *et al.* 1986, Moore *et al.* 1987).

Troponin I is a specific inhibitor of the magnesium stimulated actomyosin ATPase reaction (Perry 1979). It forms a complex with troponin C that is stable in high urea concentrations and is calcium-dependent. Calcium binding to

the low affinity states of troponin C opens a hydrophobic cavity, the site for interaction with troponin I. When troponin I binds to troponin C it frees the actin-binding site allowing the interaction of actin and myosin (Grabarek *et al.* 1992). Developmental isoforms of troponin I and troponin T have been identified in teleosts (Crockford and Johnston 1993, Yamano *et al.* 1991). Stage specific isoforms of troponin T are also sequentially expressed with development by both birds and mammals (Dhoot and Perry 1980, Saggin *et al.* 1990, Briggs *et al.* 1990).

Thick filament proteins

All vertebrates possess a similar myosin sub-unit composition, consisting of two heavy chains and four light chains (Gazith *et al.* 1970, Weeds and Lowey 1971, Focant and Huriaux 1976, Huriaux and Focant 1977). Myosin polymorphism is common in vertebrate muscle, and is not only observed in relation to different muscle fibre types (Biral *et al.* 1982, Salviati *et al.* 1982, Bottinelli *et al.* 1991), but also in single fibres from the same muscle (d'Albis *et al.* 1979, Sweeney *et al.* 1986, Lannergren 1987, Greaser *et al.* 1988). Myosin isoforms may be distinguished on the basis of their different heavy chain sub-units, different light chain components or a combination of both. Myosin isoforms often have very distinct properties, such as immunological specificities or ATPase activity. As a major component of skeletal muscle, myosin plays a central role in determining its physiological performance (Whalen 1985, Perry 1985). The myosin sub-unit composition of fast muscle fibres from most vertebrates, including teleosts, is distinct from that of slower muscles (Lowey and Risbey 1971, Biral *et al.* 1982, Huriaux and Focant 1985, Focant and Huriaux 1976, Focant *et al.* 1976, Martinez *et al.* 1990, Hoh *et al.* 1976). Bovine extraocular muscles (Sartore *et al.* 1987) are extremely fast contracting but generate very low tensions, so the presence of a distinct form

of myosin in these fibres is probably related to their particular functional characteristics.

Developing muscles have been found to show a sequential expression of stage-specific myosin heavy chain isoforms (Dalla Libera 1981, Bandman *et al.* 1982, Van Horn and Crow 1989, Hoh and Hughes 1989, Whalen *et al.* 1979, Merrifield *et al.* 1989, Rieser *at al.* 1988, Scapolo *et al.* 1988). Foetal myosin heavy chains have also been identified in muscle regenerating after injury (Sartore *et al.* 1982).

Production of contractile protein isoforms

The different isoforms of the myofibrillar proteins are produced in a variety of ways. Isoforms may result from the transcription of different genes (Periasamy *et al.* 1984, Buckingham 1985, Dalla Libera *et al.* 1991), or by alternative transcription of the same gene (Medford *et al.* 1984, Wilkinson *et al.* 1984). Isoforms may also arise from post-translational modifications (Bandman *et al.* 1982). Isoforms of the thin filament proteins tropomyosin and troponin T appear to be mainly derived from a single promotor by differential splicing (Medford *et al.* 1984, Wilkinson *et al.* 1984, Breibart *et al.* 1985, Imai *et al.* 1986, Bucher *et al.* 1988). By contrast the myosin heavy chain component of the thick filament proteins is produced primarily by the transcription of different genes (Rushbrook and Stracher 1979, Periasamy *et al.* 1984, Buckingham 1985). However Bandman *et al.* (1982) obtained results indicating that differences in myosin heavy chain isoforms may also arise from post-translational modifications. Myosin light chain isoforms have been found to be produced both by alternative transcription and splicing, and from different genes (review, Barton and Buckingham 1985).

Regulation and control of developmental transitions in the contractile protein isoforms

How transitions from foetal to adult contractile protein isoforms are regulated and controlled is unclear. Mechanisms suggested are fibre innervation and changes in hormone levels. The developmental transition of myofibrillar isoforms may, however, be an intrinsic property of each fibre, independent of external factors. Control of isoform switching may also be regulated by a combination of two or more processes.

Innervation

Experiments in which the foetal muscles of mice have been denervated by injection with ß-bungarotoxin (Weydert *et al.* 1987), have shown that the sequential expression of developmental myosin heavy chain isoforms is not regulated by innervation. The transition from foetal to neonatal to adult myosin heavy chains occurred even within the primary myotubes of ß-bungarotoxin treated foetuses, suggesting that it is nerve-independent. Myosin light chain expression in ß-bungarotoxin treated mice foetuses was investigated by Barton *et al.* (1989). In the foetuses treated with ß-bungarotoxin, developing in the absence of functional nerves, the isoforms of myosin light chain 1 underwent normal transitions from the foetal to the adult form. The accumulation of myosin light chain 3 mRNA in denervated foetuses was, however, significantly retarded, and may be linked with the innervation of the muscle fibres.

While the earliest stages of muscle formation may occur independently of innervation or contractile activity (Harris 1981), the later development of muscle fibres appears to be under extensive neuronal control. Bonner (1978, 1980) found in chickens that secondary myotube formation was entirely dependent upon innervation. The nerves appear to regulate muscle development by regulating mitosis in populations of

myoblasts with different properties and by changing the patterns of protein synthesis within multinucleate muscle cells (Harris *et al.* 1989). Extensive growth of the muscle fibre pattern established early in the development of the embryo occurs later, during the foetal period. Van Horn and Crow (1989) found that this period of growth was dependent upon the establishment and maintenance of functional neuromuscular contacts. Appearance of the late embryonic myosin heavy chain isoforms could be blocked by chronic treatment of the established fibres with d-tubocurarine, a neuromuscular blocking agent. In addition, cell cultures of embryonic chicken skeletal muscle, which differentiated in the absence of motor neurones, expressed little of the late embryonic myosin heavy chain isoform, a further indication that the expression of developmental myosin heavy chain isoforms was dependent upon functional nerve-muscle interactions. Studies by Condon *et al.* (1990) also suggest that innervation is required for normal myogenesis and fibre maintainance during the development of muscle fibre types in the prenatal rat hindlimb. However the initial differentiation of fibre types occurs even in the complete absence of innervation.

Hormonal

The sequential expression of developmental isoforms of the contractile proteins have also been found to be affected by hormones. In the guinea pig, *Cavia coboya*, there is a difference between males and females in the temporalis muscle of the jaw; in females the muscle contains a fast-red myosin heavy chain, in males a fast-white myosin heavy chain. This dimorphism is both initiated and maintained by testosterone (Rubenstein *et al.* 1988). Developmental modulation of myosin expression has also been found to be hormonally influenced in chicken skeletal muscle (Gardahaut *et al.* 1992). The thyroid hormones apparently modulate the appearance of neonatal fast myosin heavy chain isoforms and

the disappearance of isomyosins transiently expressed during embryogenesis. However it is also possible that there is an interaction between the thyroid hormones and other factors leading to the observed transitions. In rats a correlation has been found between maturation of the motor neurones, neuromuscular junctions and muscle fibres, with increasing serum thyroid hormone concentrations (Rubenstein *et al.* 1988), coordinated development leading to coordinating changes in properties. Thyroid hormones can also induce changes in the expression of myosin heavy chain isoforms independently of muscle fibre innervation by acting directly on muscle tissue (Russell *et al.* 1988). Certainly all members of the rat myosin heavy chain multigene family respond to thyroid hormone in a highly tissue-specific manner (Izumo *et al.* 1986). Each of the myosin heavy chain genes analysed responded to thyroid hormone, even within the different muscles. In addition, hypothyroidism in certain of the adult muscles reinduced myosin heavy chain genes transiently expressed during the embryonic and neonatal period (Izumo *et al.* 1986).

Myogenic

Muscle fibres have been shown to be still capable of switching from embryonic to neonatal to adult fast myosin heavy chain in the absence of both hormones and motor neurones (Rubenstein *et al.* 1988). Hoh *et al.* (1988) proposed that the emergence of primary fibres and secondary fibres in cat limb fast and slow muscles during postnatal development was determined myogenically. Each fibre type becoming functionally specialised because of the differential expression of the genes coding for myosin and other muscle proteins. This would mean that the particular range of a given fibre was an intrinsic property of that fibre, depending upon muscle type and the specific tag imprinted on it during development. Hoh (1991) suggested that the cellular basis of myogenic regulation of muscle properties stemmed from the pattern of

commitment of myoblasts during myogenesis and that the role of the nerve was restricted to modifying the fibre phenotype within a myogenically determined range.

Fish Muscle - an extremely plastic tissue

Temperature is able to directly affect the performance of fish muscle through a variety of extrinsic and intrinsic mechanisms (Johnston 1985a). In temperate fish, cold water temperatures may either cause dormancy or initiate a range of homeostatic responses serving to offset the passive effects of reduced temperature (Johnston and Dunn 1987). These responses include changes in muscle structure, contractile protein composition, muscle fibre ultrastructure and muscle contractile properties.

Muscle structure

Differences in temperature have been shown to alter the relative proportions of the different fibre types in goldfish myotomes, *Carassius auratus* (Johnston and Lucking 1978). Cold acclimation results in a shift to a more aerobic type of metabolism, significantly increasing the cross-sectional area of red and pink muscle fibres present in the myotomes. This increase in red and pink fibre area with cold acclimation was because of a substantial increase in total fibre number; fibre diameters were also larger. The fibre composition of goldfish fin muscles following acclimation was examined by Heap *et al.* (1987). They found that cold acclimation was associated with an increase in the proportion of oxidative fibre types composing the pectoral fin adductor muscles. The muscles from cold-acclimated fish had an average of 23% more red and pink fibres than muscle from warm-acclimated fish.

Contractile protein composition

The thermostability of fish skeletal muscle actomyosin and myofibril preparations have been clearly related to the temperature at which the fish lives (Johnston *et al.* 1973, Connell 1961). The activities of Antarctic fish ATPases are significantly higher at low temperatures than those of temperate and tropical species (Johnston *et al.* 1975, Johnston and Walesby 1977). Fish species regularly exposed to large seasonal fluctuations often have the ability to adapt their contractile proteins. Common carp, *Cyprinus carpio,* do not alter their fibre composition at different acclimation temperatures. Instead, the acclimation mechanism apparently involves a change in the expression of certain of the contractile proteins. Carp acclimated to low temperatures show an increase in myofibrillar ATPase activity (Crockford and Johnston 1990). The ATPase activity of myofibrils at 8°C was found to be higher in carp acclimated to 8°C than in those acclimated to 20°C (Crockford and Johnston 1990). In goldfish, *Carassius auratus,* biochemical studies have provided evidence for adaptive changes in the thermodynamic properties of Mg^{2+} Ca^{2+} myofibrillar ATPase at different environmental temperatures (Johnston and Lucking 1978). The ATPase activity of goldfish myotomal muscles acclimated to 31°C was significantly higher compared to the ATPase activity of myotomal muscle from fish acclimated to 3°C (Johnston and Lucking 1978). Goldfish fin muscles responded in the opposite way to temperature acclimation (Heap *et al.* 1987). Fin muscles from goldfish acclimated to 10°C possessed a significantly higher myofibrillar ATPase activity than fin muscles from fish acclimated to 28°C (Heap *et al.* 1987). The difference between the two ATPases was most apparent at the lower measurement temperatures. Watabe *et al.* (1992) demonstrated further differences in the myosin structure of fast muscle fibres from carp acclimated to different temperatures. Myosin subfragment-1 (S1) prepared from carp acclimated to 10°C has a higher acto-S1 Mg^{2+}-ATPase activity

and a lower thermostability than myosin subfragment-1 isolated from fish acclimated to 30°C. Temperature acclimation also caused changes in the rod region of myosin (Watabe *et al.* 1992). The troponin I and myosin light chain isoforms expressed by carp skeletal muscle fibres have also been found to vary at different temperatures (Crockford and Johnston 1990, Langfeld *et al.* 1991). Cloning and partial characterisation studies in carp indicate the presence of at least 28 different genes encoding myosin heavy chain (Gerlach *et al.* 1990).

Muscle fibre ultrastructure

Certain teleost species respond to changes in temperature by alterations to muscle fibre ultrastructure. Acclimation to cold temperatures (5°C) for several weeks results both in hypertrophy of the goldfish slow muscle fibres and extensive intracellular reorganisation (Tyler and Sidell 1984). The volume density of mitochondria increases, as do intracellular lipid deposits (Tyler and Sidell 1984). The mitochondrial diffusion path in the slow muscle fibres of the goldfish is actually 23% shorter in cold- than in warm-acclimated fibres (Tyler and Sidell 1984). A similar response was observed in the slow muscle fibres of crucian carp, *Carassius carassius*, acclimated to temperatures of 2°C and 28°C (Johnston and Maitland 1980). Mitochondria occupied 25% of slow fibre volume in carp acclimated to 2°C but only 14% in fish maintained at 28°C (Johnston and Maitland 1980). Striped bass, *Morone saxatilis*, respond differently to temperature. The muscle fibres of striped bass acclimated to cold temperatures (5°C) have a significantly lower mean mitochondrial spacing than fibres from warm-acclimated (25°C) individuals (Egginton *et al.* 1987). The lower diffusion distances between mitochondria are not, however, achieved by an increase in the volume density of muscle mitochondria located in the subsarcolemmal zone (Egginton and Sidell 1989). Within the red muscle fibres of fish acclimated to 5°C,

mitochondria occur in clustered arrays, the arrangement is such that the surface area of mitochondria exposed to the muscle cytoplasm is independent of acclimation temperature (Egginton and Sidell 1989).

Crucian carp, after two months acclimation to either 2°C or 28°C demonstrate significant differences both in the mitochondrial content and capillarisation of the myotomal muscles (Johnston 1982). Increases in the mitochondrial compartment were apparent in both the red and white muscle fibres of the cold-acclimated fish, and there was an increase in the capillary supply to both fast and slow muscles of cold-acclimated fish (Johnston 1982). Temperature acclimation is also associated with changes in the amount of sarcoplasmic reticulum present within muscle fibres. Following temperature acclimation red muscle fibres from warm-acclimated carp have a higher surface density of sarcoplasmic reticulum and terminal cisternae than cold-acclimated fish (Fleming *et al.* 1990). In goldfish skeletal muscle there is an increase in the surface area of the sarcoplasmic reticulum following cold acclimation (Penney and Goldspink 1980). Carp are also capable of modifying the fluidity of the membrane forming the sarcoplasmic reticulum and the molecular structure of the Ca^{2+}-ATPase to compensate for fluctuating ambient temperature (Ushio and Watabe 1992). The Ca^{2+} ATPase activity of sarcoplasmic reticulum-enriched microsomes prepared from the white muscle fibres was 60% higher at 8°C in cold- than warm-acclimated carp (Fleming *et al.* 1990). Changes in muscle fibre ultrastructure induced by temperature acclimation may help to counteract the effect of temperature change on the diffusion rate of molecules through the cytoplasm, thus maintaining flux rates between the different cellular components (Sidell and Hazel 1987).

Muscle contraction

The common carp, *Cyprinus carpio,* demonstrates a range of muscle capacity adaptations which enable the species to remain active throughout the year. When temperatures are reduced from 20°C to 10°C the speed at which white muscle fibres are first recruited in 16-20 cm carp decreases from 2.6 to 1.4 bodylengths per second (Rome *et al.* 1984). This suggests that at lower temperatures slow muscle fibres are no longer capable of providing all the power necessary for cruising speeds and so faster contracting fibre types are recruited at slower swimming speeds (Rome *et al.* 1984). However, following several weeks acclimation to 8°C, the recruitment threshold for fast muscle fibres has been shown to have increased (Rome *et al.* 1985). Carp acclimated to cold temperatures are able to swim faster with their aerobic muscle and have have higher sustained swimming speeds than acutely cooled fish (Rome *et al.* 1985). Cold acclimation actually results in modest improvements to the contractile performance of red muscle fibres at low temperatures (Langfeld *et al.* 1991). Bundles of red fibres had similar maximum tetanic tensions when measured at the acclimation temperature of the fish and the relationship between force and velocity (P-V) was independent of acclimation temperature. However at 8°C the maximum tetanic tension was 32% higher in fibres from 8°C- than in those from 20°C-acclimated fish and unloaded contraction velocity (Vmax) of fibres was 17% higher in 8°C- than in 20°C-acclimated fish (Langfeld *et al.* 1991). The maximum power output from the red muscle fibres of carp was therefore calculated to be 26.5 W/kg in cold-acclimated fish and 18.0 W/kg in warm-acclimated carp (Langfeld *et al.* 1991). Johnson and Johnston (1991) examined the effects of acute and seasonal temperature change on the power output of sculpin, *Myoxocephalus scorpius,* fast muscle fibres performing oscillatory work. The maximum power output of the muscle fibres showed little seasonal variation at 4°C, ranging from 20-25 W/kg. In contrast, at 15°C, maximum

muscle power output increased from 9 W/kg in the winter- to 30 W/kg in the summer-acclimatised fish (Johnson and Johnston 1991). The higher power output of muscles in the summer-acclimatised fish is largely the result of an increase in average force during the shortening part of the contraction cycle (Johnson and Johnston 1991).

The influence of temperature on muscle structure in fish larvae

Previous experiments investigating the effects of temperature on teleost embryos and larvae have tended to concentrate on rate of development and the efficiency of yolk utilisation (Kinne and Kinne 1962, Ryland 1966, Ryland and Nichols 1967, Ryland *et al.* 1975, Solberg and Tilseth 1987, Miranda *et al.* 1990, Polo *et al.* 1991). Those experiments that have examined the influence temperature has on muscle development have shown that different teleost species react in different ways (Stickland *et al.* 1988, Vieira and Johnston 1992), and that responses within the same species can vary from year to year (Johnston 1993). Incubation temperature influences the number of muscle fibres and the diameter of those fibres via differential effects on hypertrophy and hyperplasia (Stickland *et al.* 1988, Vieira and Johnston 1992, Johnston 1993). The number of myosatellite cells present within newly hatched herring larvae is affected by temperature as is the ultrastructure of the muscle fibres (Johnston 1993). Both mitochondrial and myofibrillar volume density are significantly influenced by temperature during the embryonic and larval stages of the herring (Vieira and Johnston 1992, Johnston 1993). In turbot, *Scophthalmus maximus,* relatively small differences in rearing temperature are sufficient to affect the distribution of muscle fibre types and the energy storage levels of larval and juvenile fish (Calvo and Johnston 1992).

Developmental transitions in myofibrillar protein isoforms have been observed in the sea bass, *Dicentrarchus labrax*, (Scapolo *et al.* 1988), the herring, *Clupea harengus*, (Crockford and Johnston 1993, Johnston and Horne 1993) and the barbel, *Barbus barbus*, (Focant *et al.* 1992). Calvo and Johnston (1992) also observed a continuous change in histochemical staining properties of myosin ATPase activity over several months in the white muscle of larval and juvenile turbot, *Scophthalmus maximus*. The only study of how temperature affects the myofibrillar protein isoforms present in fish larvae was made by Crockford and Johnston (1993). In adult fish, temperature has been shown to influence the expression of the individual myofibrillar components to varying extents, resulting in potentially unique combinations of isoforms (Johnston *et al.* 1990, Gerlach *et al.* 1990, Langfeld *et al.* 1991). In larvae of the herring the combination of myofibrillar isoforms present at any developmental stage was found to be dependent on the rearing temperature (Crockford and Johnston 1993).

The primary aim of this study was to examine the development of skeletal muscle in the teleost *Pleuronectes platessa*. In addition it was proposed to investigate the influence of ambient water temperature upon the differentiation, structure and development of the muscle fibres.

Chapter 2

Materials and Methods

Collection, rearing and maintenance of animals

Eggs and Larvae

Mature *Pleuronectes platessa* L. were trawled from the Firth of Clyde in February and March 1990, 1991 and 1992, just prior to their naturally spawning in the wild. Fish were maintained in the aquarium at the Dunstaffnage Marine Laboratory, Oban, in tanks supplied with seawater of ambient temperature. When ripe, eggs were stripped from adult females by gentle massage of the ovaries and collected in a sterilised beaker. The eggs were fertilised with milt produced by ripe adult males and collected in a sterile syringe after gentle pressure on the gonads. Once fertilised the eggs were left to stand in 10 volumes of seawater at 8°C for an hour, they were then rinsed several times with more seawater. Any unfertilised eggs were removed by increasing the salinity of the water to 40% by the addition of solid NaCl, causing fertilised eggs to float, unfertilised eggs were siphoned off the bottom of the beaker (Holliday and Pattie Jones 1967).

The fertilised eggs were transferred to tanks in constant temperature rooms. In 1990 the rooms had seawater circulating at 5°C, 10°C and 15°C. Before eggs were placed in the 15°C room they were first warmed to 10°C and then 12°C so that there was no sudden temperature shock. Very few larvae successfully hatched at rearing temperatures of 15°C, so in the subsequent two years eggs were incubated at 5°C, 8°C and 12°C, with varying degrees of success.

In 1992, in addition to the larvae reared at Dunstaffnage Marine Laboratory, fertilised eggs were transported to the Gatty Marine Laboratory, St. Andrews, and raised in a constant

temperature room kept at 8°C. The fertilised eggs were placed in a large sterilised glass jar together with at least 3 litres of seawater, initially at 8°C. The water was kept circulating by means of an airstone connected to a battery operated air-pump. The eggs were transported on ice, in an insulated cool box. On arrival eggs were transferred to tanks in an 8°C constant temperature room. Seawater entering the room had first passed through a 100 µm filter, a 3 µm filter and an ultra-violet filter. Each tank was provided with an air-supply to keep the eggs moving. Illumination in the room was constant, 24 hours light. Dead eggs were removed from the tanks every other day by siphoning them off the bottom.

Once hatched, larvae were fed on the nauplii of *Artemia salina*. Food was given 3 or 4 days after hatching, before the yolk-sac was completely absorbed, to accustom larvae to the presence of the food organism. Artemia nauplii were a nutritionally adequate food source for the larvae, until 20 weeks after hatching when the growth of the juveniles began to decline. Unfortunately an adequate substitute could not be found. Juveniles (total length = 19.1 ± 3.8 mm, n = 10, mean ± SD) refused to eat pelleted foods or other alternatives such as chopped mussels.

All larval samples were identified by their age in days/weeks from hatching, until metamorphosis. The completion of metamorphosis was taken to be the point at which the migrating eye reached the edge of the head (Ryland 1966).

In the first larval season both eggs and larvae were sampled daily but in the following two seasons, once larvae had hatched, weekly sampling was found to be sufficient, but the embryos were still sampled daily.

Adults

Plaice, *Pleuronectes platessa* L., standard length, 25.75 cm (S.D. ± 2.28, n = 10) and body mass, 268 g (S.D. ± 97, n = 10), were trawled from St Andrews Bay or the Firth of Clyde throughout the year. Fish were maintained in recirculating seawater aquaria at ambient temperature (5-15°C) and photoperiodic regime (12 hours light: 12 hours dark). Fish were fed a mixture of chopped mussels and raw minced fish muscle. Before experimentation fish were stunned by a blow to the head, pithed and decapitated.

Fixation and Embedding

Plaice larvae and eggs were found to be extremely difficult to fix to obtain good tissue preservation. During the first two larval seasons a variety of fixatives were tried. Before fixing hatched larvae were anæthetised in a 1:5000(v/v) benzocaine solution in seawater and their heads and yolk-sacs or alimentary tract removed. Eggs were placed directly into fixative, after puncturing the chorion with a fine needle. Developing embryos were too small to dissect out of the egg without damage to the tissues.

Wax histology

Eggs and larvae were fixed in Formol saline, Bouin's Fluid, Zenker's Fluid and Heidenhain's Susa (see Appendix). Specimens were dehydrated through a graded series of alcohols, cleared in chloroform and embedded in paraffin wax (melting point 56°C). Serial sections were cut at 8 µm intervals along the entire length of each fish. The sections were dewaxed and stained with Ehrlich's Hæmatoxylin and eosin.

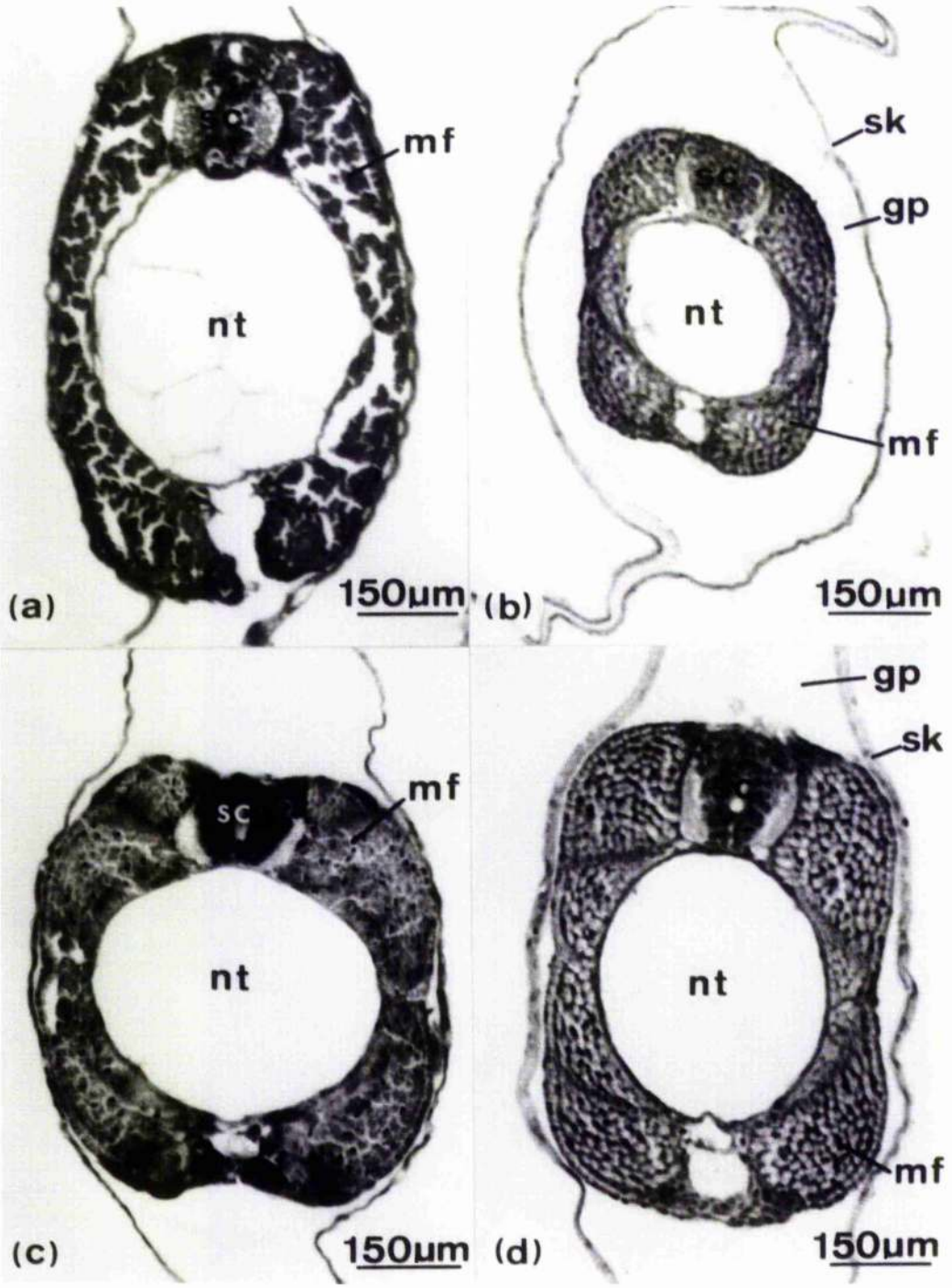
The different fixatives varied in their effectiveness at preserving the structure of the muscle fibres within the

larvae. Formol saline is a commonly used histological fixative consisting of formaldehyde in NaCl solution. The formaldehyde hardens the tissues and is a good general fixative. Unfortunately it does not provide any protection for the cells when they are dehydrated and embedded in paraffin wax. When used on newly hatched larvae (Fig. 1a) it did not preserve the structure of the muscle fibres and there was a large degree of shrinkage.

Bouin's Fluid is a combination of picric acid, formaldehyde and acetic acid. The picric acid precipitates out proteins in the tissue but causes greater shrinkage than any other fixative and only slowly penetrates tissue. The acetic acid present in the Bouins penetrates tissue rapidly and swells the tissue helping to counteract the action of the picric acid; it precipitates nucleoproteins but does not fix the cytoplasmic proteins eg. myofibrils. Although the Bouins fixed tissue did not shrink to the same extent as that fixed in formol saline, cytological detail within the specimens is not clear and staining is weak (Fig. 1b), probably as a result of the fixative penetrating the skin too slowly. Plaice larvae have a very thick skin and also a sub-dermal glycoprotein layer which would impede the passage of the fixative through to the muscle fibres (Fig. 1b). The slow penetration of picric acid could be the reason why muscle fibres towards the centre of the fish are more often damaged than the peripheral fibres. The acetic acid in the fixative penetrates more rapidly, causing the fibres to swell before the arrival of the picric acid, which counteracts the swelling.

The combination of the skin and the sub-dermal glycoprotein layer acting as a barrier to fixation meant that a very rapid fixative needed to be employed. Mercuric chloride, which is present both in Zenker's Fluid and Heidenhain's Susa, is a powerful protein precipitant, which penetrates and hardens tissues rapidly. It also shrinks tissues less than any other protein precipitant and may actually swell them. Considerable

Figure 1. The effects of different histological fixatives on newly hatched plaice larvae. (a) Formol saline. (b) Bouin's Fluid. (c) Zenker's Fluid. (d) Heidenhain's Susa.
gp: glycoprotein layer; mf: muscle fibres; nt: notochord; sc: spinal cord; sk: skin.


mf
nt
(a)
150µm
sk
gp
nt
mf
(b)
150µm
mf
sc
nt
(c)
150µm
gp
sk
nt
mf
(d)
150µm

shrinkage still occurs in the alcohol and paraffin wax, but less than is seen with the other fixatives.

Zenker's Fluid also contains potassium dichromate which has a strong fixative action on certain lipids. Larvae fixed in Zenker's Fluid (Fig. 1c), showed a certain amount of shrinkage in the muscle fibres but staining was more intense and more detail could be seen than in Bouins fixed tissue.

Heidenhain's Susa in addition to mercuric chloride also contains trichloroacetic acid, acetic acid and formaldehyde. As a fixative it gives excellent cytological detail and penetration is both rapid and even. Specimens fixed in Susa (Fig. 1d) showed less distortion amongst the muscle fibres than was observed with other fixatives, and staining was fairly intense. Overfixation did not result in any damage to the tissue itself but staining became progressively less intense.

Transmission Electron Microscopy

Samples to be examined under the transmission electron microscope (T.E.M.) were fixed in one of four fixatives:-

(a) 2% glutaraldehyde in phosphate buffer (100 mM NaH_2PO_4, 100 mM Na_2HPO_4), pH 7.2 overnight,

(b) 4% paraformaldehyde in phosphate buffered saline (160 mM NaCl, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4), pH 7.2,for 4 hours,

(c) in 2% osmium tetraoxide for 1 hour

(d) in 4% paraformaldehyde, 4% gluteraldehyde, 40 mM cacodylate buffer, 16 mM NaCl, 1.2% sucrose, pH 7.2 with 1N NaOH, for 24 hours.

After fixation samples were rinsed with their respective buffers:- (a) phosphate buffer pH 7.2,

(b) phosphate buffered saline pH 7.2,

(c) distilled water,

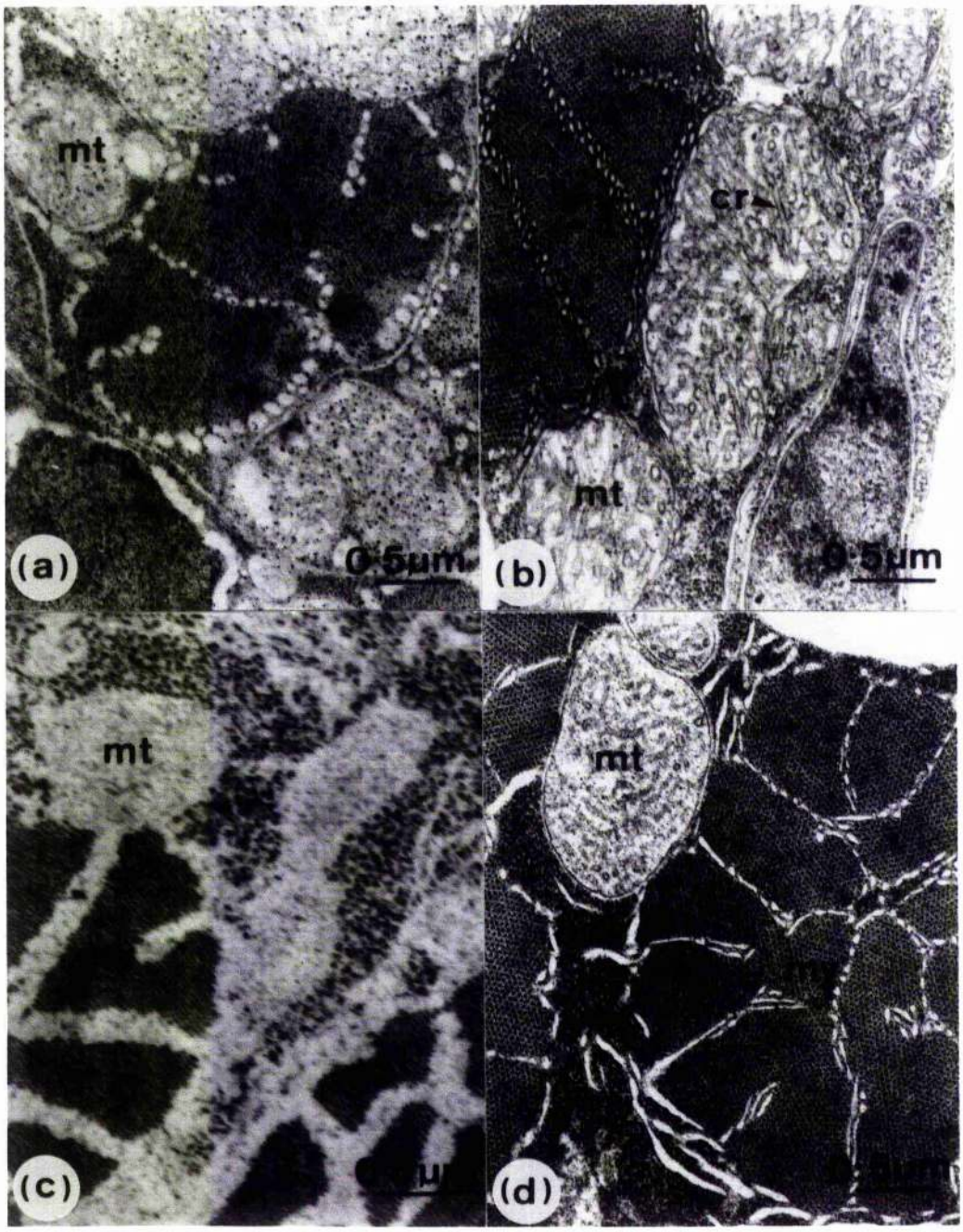
(d) sodium cacodylate buffer pH 7.2.

All specimens, except those originally fixed in osmium, were post-fixed with 2% osmium tetraoxide for 1 hour. After further buffer or distilled water rinses larvae were stained *en bloc* with uranyl acetate, then dehydrated with ethanol and epoxypropane and embedded in Araldite resin. Once embedded both semi-thin (0.5-1 μm) and ultra-thin (50-60 nm) sections were cut from specimens. These were cut using a Reichart OMU2 ultramicrotome fitted with glass knives. Semi-thin sections were stained with toluidine blue and examined under a light microscope. Ultra-thin sections were mounted on 200 mesh copper grids and stained with aqueous saturated uranyl acetate and Reynolds lead citrate, before examining them on a Phillips 301 transmission electron microscope at 60 kV.

The presence of a glycoprotein layer just beneath the skin also affected fixation of specimens for transmission electron microscopy. Glutaraldehyde in phosphate buffer was a particularly poor fixative (Fig. 2a). It did not preserve any membranes present within the tissue and because of the poor fixation osmium and uranyl acetate were precipitated out within the tissue further obscuring the ultrastructural details.

Paraformaldehyde fixed tissue was well preserved, osmication was even and membranes were very clearly visible (Fig. 2b). However this fixative could be erratic, fixation tended to vary from year to year as did the penetration of araldite into this tissue.

Figure 2. Fixation of newly hatched plaice larvae for examination under the transmission electron microscope. (a) 2% glutaraldehyde. (b) 4% paraformaldehyde. (c) osmium tetraoxide. (d) 4% paraformaldehyde, 4% glutaraldehyde.
cr: cristae; mt: mitochondria; my: myofibrils.


mt
(a)
0·5μm
cr
mt
(b)
0·5μm
mt
(c)
mt
(d)

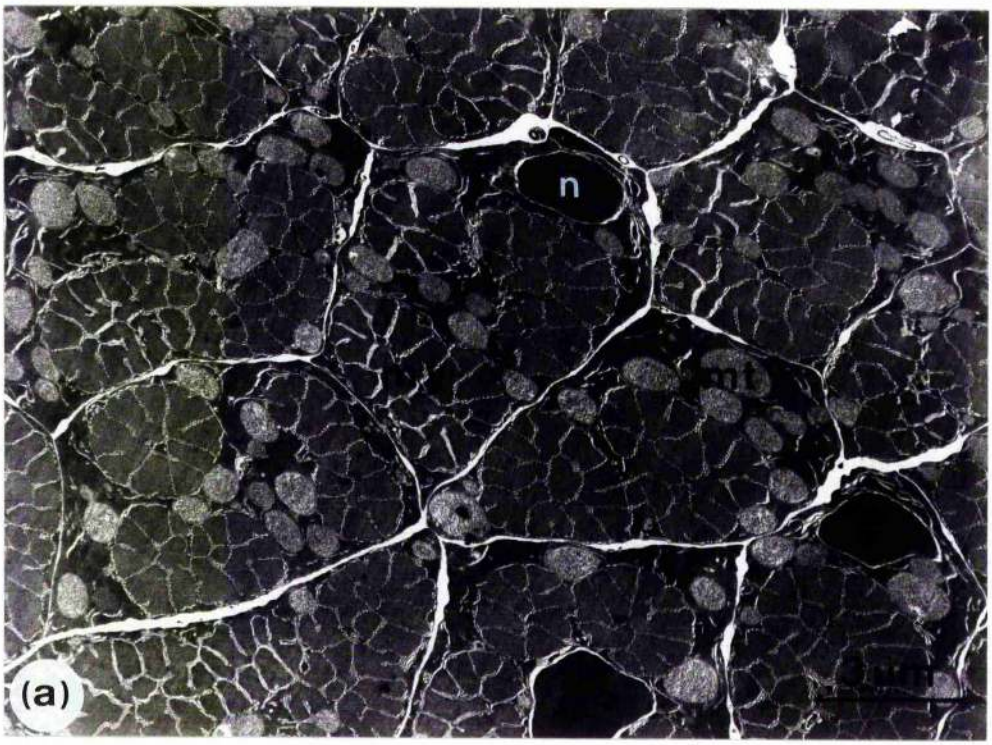
Osmium has been successfully used as a fixative on tissue from adult plaice (Cobb personal communication). The myofibrils were very densely stained, but no membranes or cristae were preserved (Fig. 2c).

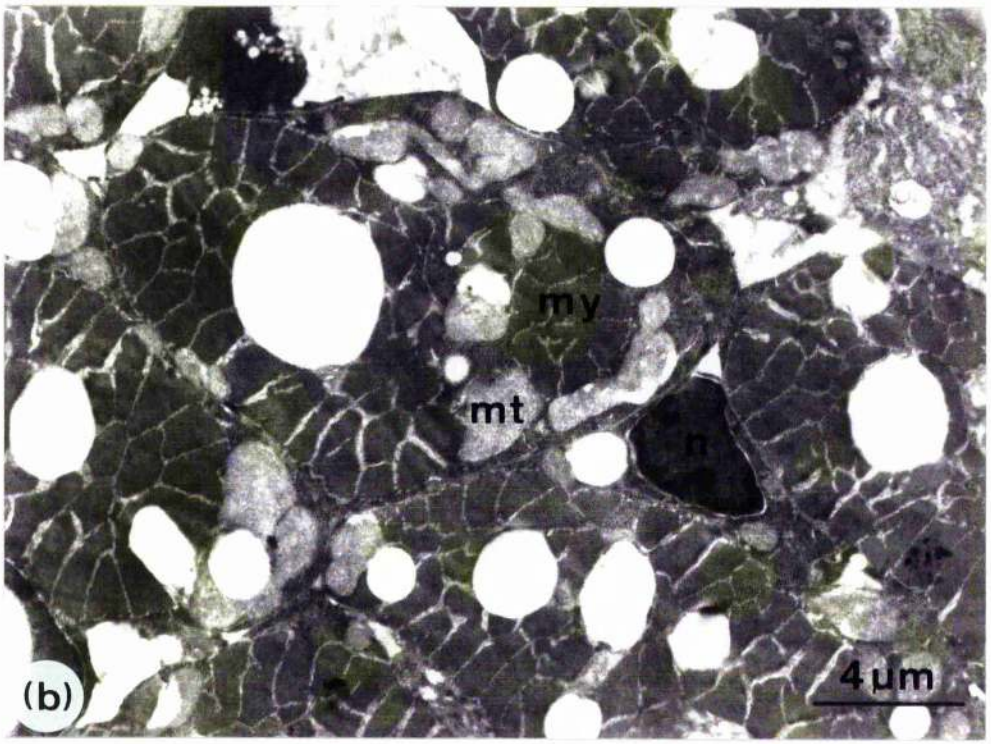
The problem most fixatives appear to have is penetrating the skin and subsequent layer of glycoprotein. The paraformaldehyde and glutaraldehyde mixture was developed to penetrate the surface cuticle of small marine crustaceans (Laverack personal communication), another very resistant layer, so it was decided to test this fixative on plaice larvae (Fig. 2d). In newly hatched larvae this was found to be a very good fixative. Membranes are clearly visible as are the myofibrils and mitochondria, and it was more consistant than paraformaldehyde on its own (Fig. 2d and 3a). Unfortunately after deciding to use this fixative for all tissue at all developmental stages it was discovered that as the muscle fibres developed, the fixative began to damage the fibres (Fig. 3b). Smaller fibres were still adequately fixed but the larger ones were full of holes where the tissue had been ruptured by osmotic shock.

Specimen Fixation

Finding a fixative which gave good tissue preservation was complicated by the presence of a subdermal glycoprotein layer in newly hatched plaice larvae. This space between the integument and the underlying mesoderm was described by Shelbourne (1956a and b), who related it to the pelagic lifestyle. Pelagic eggs do not develop a complex system of circulatory vessels around the yolk, instead the heart opens directly into the yolk-sac sinus, a subdermal space formed when the extra-embryonal mesoderm around the yolk breaks down into mesenchyme cells during gastrulation. The yolk-sac sinus communicates laterally with the subdermal space of the dorsal marginal fin and so there is free access of fluid yolk derivatives to all the growing tissues. Throughout embryonic development soluble nutrients are withdrawn from the dilute

Figure 3. 4% paraformaldehyde, 4% glutaraldehyde fixation of plaice larvae at different developmental stages.
(a) Newly hatched larvae. (b) 6 week old larvae.
mt: mitochondria; my: myofibrils; n: nucleus.


n
mt
(a)


my
mt
n
(b)
4 µm

yolk fluid to be used in the synthesis of the compact embryonic axis and respiration. If the egg is to remain buoyant there must be a limit to embryonic water loss, determined by buoyancy reserves at fertilisation and weight loss because of the excretion of respiratory metabolites (Shelbourne 1956a). Although the yolk-sac fluid derivatives are utilised throughout embryonic development the low density yolk diluent remains, filling the voluminous subdermal spaces and maintaining an overall embryonic volume relevant to the buoyancy requirements of the egg. Direct yolk appropriation, independent of a blood vascular system continues well into the larval phase and could indirectly explain the decline in the effectiveness of the T.E.M. fixatives with the development of the larvae. As the yolk-sac diminishes, so does the subdermal space (Shelbourne 1956b) and so a less penetrative fixative was needed. To some extent the decline of the subdermal space might be balanced by the development of the epidermis (Holliday and Pattie Jones 1967), which seems to be the case for specimens fixed in Susa but not in the paraformaldehyde, gluteraldehyde mixture.

Histochemical Identification of Fibre Types

Muscle blocks were dissected from the lateral line region of adult fish and metamorphosed juveniles. The tissue was mounted in Tissue Tek OTC compound on metal chucks and rapidly frozen in isopentane cooled to near its melting point in liquid nitrogen (-130°C). After equilibrating the blocks to -20°C frozen sections were cut using a Bright cryostat. Transverse sections 13 μm thick were collected on coverslips and left to air dry for 45 minutes before staining.

Myofibrillar ATPase

The method of Guth and Samaha (1970) was used to demonstrate myosin ATPase activity. Sections were rinsed in 18 mM calcium chloride ($CaCl_2$), 100 mM Tris buffer pH 7.8, and

preincubated in an alkaline solution containing (in mM/litre), 18 mM $CaCl_2$, 100 mM Sigma 221 buffer pH 10.4 for 2 minutes. After rinsing in 18 mM $CaCl_2$, 100 mM Tris pH 7.8, sections were incubated for 10 minutes in 2.7 mM ATP, 50 mM KCl, 18 mM $CaCl_2$ in 100 mM 221 buffer pH 9.4. Sections were subsequently treated as follows:-

(a) 2 washes in 1% $CaCl_2$
(b) 2 minutes incubation in 2% $CoCl_2$
(c) 3 washes 100 mM 221 buffer pH 9.4
(d) 1 minute incubation in 1% ammonium polysulphide

Succinic dehydrogenase (SDHase)

Tissue sections were stained for SDHase using the method of Nachlas *et al.* (1957). Sections were incubated in 1 mg/ml nitroblue tetrazolium, 80 mM sodium succinate, 50 mM phosphate buffer pH 7.5, at room temperature for 30 minutes, rinsed with distilled water and mounted on slides with glycerol gelatin.

Glycogen

The periodic acid Schiff technique (Pearse 1972) was used to stain sections for glycogen. Sections were incubated in 1% (w/v) periodic acid for 30 minutes, rinsed in distilled water, left to stain in Schiffs reagent (Sigma) for 30 minutes, washed three times with 0.5% bismetasulphate and mounted in glycerol gelatin. All solutions were used at room temperature.

Preparation of Myofibrils

Samples of adult red and white muscle fibres were dissected, on ice, from the dorsal side of the body. Care was taken to avoid cross-contamination of fibre types. Muscle samples were also frozen and stored in liquid nitrogen.

Myofibrils were prepared essentially as described by Focant *et al.* (1976). All steps were carried out at 0-4°C.

Muscle was finely minced with scissors and homogenised in 10 volumes of 100 mM NaCl, 10 mM Tris-HCl pH 7.2 and a cocktail of the following proteolytic enzyme inhibitors, 50 µg/ml n-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 50 µg/ml phenyl-methylsulphonyl fluoride (PMSF), 0.5 µg/ml leupeptin and 1 µg/ml pepstatin A. The homogenate was centrifuged at 2000 g, 4°C, for 5 minutes and the supernatant discarded. The pellet was washed 3 times in the same buffer. After the third wash the pelleted myofibrils were resuspended in 100 mM NaCl, 10 mM Tris-HCl pH 7.2.

Purification of Myosin

Method 1

Initially the method of Huriaux and Focant (1990) was used. Approximately 50 g of white muscle was dissected, cut with scissors, and minced in a cooled Universal No.1 grinder. All procedures were performed on ice and solutions were kept at 4°C. Muscle was extracted with 0.5 M KCl, 30 mM $NaHCO_3$, 10 mM sodium pyrophosphate ($Na_4P_2O_7$), 1 mM $MgCl_2$, 1 mM PMSF, pH 8.4. and centrifuged at 3000 g for 15 minutes. The pellet was discarded and actomyosin precipitated from the supernatant by dilution with 6 volumes of ice cold deionised water and acidified to pH 6.3 with HCl. The precipitated actomyosin was removed by centrifuging at 17 000 g for 15 minutes and washed once with a solution of 30 mM KCl. Washed actomyosin was dissolved in 120 mM Tris-HCl pH 7.5, 250 mM NaCl and myosin prepared by neutral ammonium sulphate fractionation between 40% and 60% saturation, in the presence of 0.4% w/v ATP and 20 mM magnesium sulphate ($MgSO_4$). In order to remove tropomyosin and troponin myosin was dialysed for 24 hours against 2 mM $NaHCO_3$ pH 8.7, isolated by centrifugation at 39 000 g for 30 minutes and

redissolved in 120 mM Tris-HCl, 250 mM NaCl pH 7.5. At each stage of the preparation samples of supernatant and pellet were taken to run on 13% SDS polyacrylamide gels to determine which proteins were present.

Method 2

Method 2 was based on Giambalvo and Dreizen (1978) for purifying frog skeletal myosin with modifications (Margossian and Lowey 1982). Approximately 50 g of white muscle or 35 g of red muscle were carefully dissected from fish ranging from 220-375 g in size. Muscle was minced and washed three times in 5 volumes of 15 mM potassium phosphate buffer, 10 mM $MgCl_2$, 2 mM ATP, 1 mM DL-dithiothreitol (DTT), 3 mM sodium azide (Na_3N), protease inhibitors - 0.0035% pentamidine isethionate, 0.525% *E*-amino-*n*-caproic acid and 0.017% *p*-aminobenzamidine HCl, final pH 7.5, retaining the mince in a muslin bag. The mince was suspended in wash solution and centrifuged at 5000 g for 10 minutes. The precipitate was suspended in 3 volumes of 0.15 M potassium phosphate buffer, 300 mM KCl, 10 mM $MgCl_2$, 10 mM ATP, 1 mM DTT, 3 mM Na_3N and protease inhibitors, pH 6.6, and left on ice for 30 minutes after which was added an equal volume of ice-cold deionised water. After centrifuging at 13 000 g for 10 minutes the muscle mince was discarded and the supernatant was diluted with 6.5 volumes of ice-cold deionised water, ATP was added to a final concentration of 1 mM and the pH of the solution was adjusted to pH 6.1 using 1 M KH_2PO_4. This solution was left overnight in the fridge and centrifuged next morning at 17 000 g for 10 minutes. The resulting precipitate was dissolved in 60 mM potassium phosphate buffer, 1 M KCl, 10 mM ATP, 1 mM DTT, 3 mM Na_3N and inhibitors pH 6.4 and left stirring in the fridge. Once all the precipitate had dissolved the solution was dialysed against 6.5 volumes of ice cold deionised water overnight. After centrifuging at 10 000 g for 20 minutes the precipitate was dissolved in 50 mM potassium phosphate buffer pH 7, 0.5 M KCl, 10 mM $MgCl_2$, 10 mM ATP, 1 mM DTT, 3

mM Na_3N and protease inhibitors, and centrifuged at 150 000 g for 3 hours. The top third of the supernatant was removed and dialysed against 400 mM KCl, 5 mM Tris, 0.1 mM EDTA, 2 mM pyrophosphate, 3 mM Na_3N pH 7.7, over 3 days. The resulting solution of myosin was diluted with an equal volume of glycerol and stored frozen.

Protein Assay

The protein concentration of each solution of myofibrils was ascertained using the Sigma Diagnostics Protein Assay kit (Procedure No. P5656). This kit is based upon Peterson's modification of the micro-Lowry technique. Proteins were first precipitated from solution with aqueous sodium deoxycholate (DOC) 1.5 mg/ml and trichloroacetic acid (TCA) 72% (w/v), because Tris buffer affects the direct Lowry procedure (Kuno and Kihara 1967). The dissolution of proteins is facilitated by the presence of sodium dodecylsulphate (SDS) in the Lowry reagent. Optical density was read at 750 nm. Protein standards prepared from bovine serum albumin (BSA) were used to construct a calibration curve over the range 50-400 µg/ml.

Identified Muscle Fibre Types

Bundles of single fibre types identified by their different light scattering properties under dark field illumination, were dissected from various regions of the myotome.

Fibre bundles were crushed gently in 50 µl of 100 mM NaCl, 10 mM Tris-HCl pH 7.2, 50 µg/ml TPCK, 50 µg/ml PMSF, 1 µg/ml pepstatin and 0.5 µg/ml leupeptin at the bottom of a 2ml Eppendorf tube with a teflon homogeniser and centrifuged at 13 000 g for 5 minutes. The supernatant was carefully blotted away and the remains of the fibres dissolved in 50 µl of 8 M urea, 1% 3-10 Ampholyte, 5% glycerol, 5% ß-

mercaptoethanol, 2% Noniodet P-40 (NP40), (IEF sample buffer).

Samples were warmed to 30°C in a water bath and left for 60 minutes before use.

Electrophoretic Techniques

SDS polyacrylamide gel electrophoresis (PAGE) was performed according to the methods described by Laemmli (1970) and SDS PAGE in the presence of 8 M urea as described by Sender (1971).

Alkali-urea gel electrophoresis techniques were based upon the method of Focant and Huriaux (1976) with modifications by Crockford (1989). Isoelectric focusing (IEF) and two dimensional methods were essentially taken from Crockford (1989) and O'Farrell (1975) with various modifications

Preparation of Samples for Electrophoresis

SDS PAGE: Aliquots of known protein concentration were centrifuged at 13 000 g for 5 minutes at 5°C. The precipitated myofibrils were dissolved in 60 mM Tris-HCl pH 6.75, 2% SDS, 10% glycerol, 1% ß-mercaptoethanol and 0.001% BPB to give a final protein concentration of 2 mg/ml. The samples were heated to 90°C for 5 minutes and either used immediately after cooling to room temperature or stored frozen at 0°C.

Myosin samples were first dialysed against 5 mM Tris, 50 mM NaCl and then against 5 mM Tris, 0.1% SDS (w/v) to remove KCl, which precipitates SDS. Once the SDS was no longer precipitated samples could be diaylsed against sample buffer and used as myofibrillar samples.

Urea SDS PAGE: Crystalline urea was added to samples prepared as for SDS PAGE with vigorous mixing after each addition until a saturated solution was obtained.

Alkali-urea gels: Myofibrils were dissolved in 3 volumes of 20 mM glycine pH 8.9 with 1 M Tris base, 12 M urea, 5% ß-mercaptoethanol, 0.001% BPB and 10 mM EGTA, final protein concentration 2 mg/ml. Samples were kept in a water bath at 30°C for 60 minutes before use; thereafter they were stored at 0°C.

Myosin samples were prepared for alkali-urea gels by dialysing samples against 3 changes of deionised water and then dissolved in sample buffer as above.

IEF gels: Samples were prepared by centrifuging aliquots of myofibrils of known protein concentration (2 mg/ml) and homogenising the precipitate in 8 M urea, 1% 3-10 Ampholyte, 5% glycerol, 5% ß-mercaptoethanol, 2% Noniodet P-40 (NP40). Samples were warmed to 30°C in a water bath and left for 60 minutes before use.

All samples were loaded onto gels using a clean Hamilton syringe. Between 10 µg and 150 µg of protein was loaded onto each well.

Gel Composition and Running Conditions

SDS PAGE:-

STACKING GELS:- Each contained 0.125 M Tris-HCl pH 6.8 at 20°C, total acrylamide 4%, BIS cross-linker 2.67%, and 0.1% SDS. 1 µl of TEMED per 1 ml of gel and 0.5 mg ammonium polysulphate per 1 ml of gel were used as polymerising agents.

RESOLVING GELS:- Total concentration of acrylamide depended on the percentage gel required, this ranged from 12.5

to 14%. BIS cross-linker made up 2.67% of the total acrylamide in all cases. Gels also contained 0.375 M Tris-HCl pH 8.8 at 20°C, 0.1% SDS, 1 µl/ml TEMED and 0.5 mg/ml ammonium polysulphate were the polymerising agents.

UREA SDS PAGE:- These gels contained 8 M urea in both the stacking and resolving parts in addition to the reagents listed above.

ELECTRODE BUFFER:- 25 mM Tris, 0.192 M glycine, 0.1% SDS pH 8.3.

RUNNING CONDITIONS:- Samples were run into the gel at 50 volts, once they had entered the gel the voltage was increased to 200 V and this was maintained until the bromophenol blue front reached the end of the gel.

Alkali-urea PAGE:- Alkali urea gels consisted of 8 M urea, 100 mM glycine pH 8.9 using 1 M Tris base, 10% total acrylamide, 2.67% BIS cross-linker and polymerising agents; 1 µl/ml TEMED, 0.5 mg/ml ammonium polysulphate.

ELECTRODE BUFFER:- 100 mM glycine pH 8.9 with 1 M Tris base.

RUNNING CONDITIONS:- Gels were pre-ran at 400 V for 30 minutes. The samples were loaded and the gel ran at 50 V until the samples had entered, when the running voltage was raised to 400 V, until the bromophenol blue front reached the bottom of the gel.

Isoelectric focusing gels:- IEF gels consisted of 5% Ampholyte mixture, 8 M urea, 2% NP40, 9% total acylamide, 15% DATD cross-linker. Polymerisers as previous gels.

The Ampholyte mixture used depended upon the proteins under study. For basic proteins gels contained 1.67%

Pharmalyte 3-10 (Pharmacia. Uppsala, Sweden.) and 3.33% Ampholyte 9-11 (Sigma). Gels used to separate acidic proteins contained equal volumes of Pharmalyte 3-10, Pharmalyte 4-6.5 and Pharmalyte 2.5-5.

ELECTRODE BUFFERS:-

Cathodic Buffer- 20 mM NaOH, throughly degassed,

Anodic Buffer- 10 mM Phosphoric acid (H_3PO_4).

RUNNING CONDITIONS:- Gels used to separate basic proteins were run from anode to cathode and those separating acidic proteins were run from cathode to anode with the samples being loaded into NaOH solution. All IEF gels were pre-run before samples were loaded. Gels were overlaid with 5 µl sample buffer containing 8 M urea, 1% 3-10 Pharmalyte, 5% glycerol, 5% mercaptoethanol and 2% NP40. Gels were run at 200 V for 0.2 hours, 300 V for a further 0.2 hours and 400 V upto a total of 0.6 hours. This helped to establish the pH gradient through the gels. Samples were then loaded into the reservoirs and overlaid with 4 M urea, 2% NP40, 1% 3-10 Pharmalyte, 2% glycerol and 0.001% bromophenol blue. Gels were focused initially at 200 V, this was increased after 0.2 hours to 300 V, 450 V after another 0.2 hours, raised to 600 V and finally to 750 V for a total of 3.5 kVhours.

2-Dimensional Electrophoresis and Related Techniques

IEF gels were exuded from tubes and loaded onto SDS PAGE mini-gels with a 14% resolving gel, cast with 1 mm spacers. Instead of using a well former, the stacking gel was overlaid with isobutanol before polmerisation. This gave a smooth even surface on which to position the tube gel. Once in position the tube gel was covered with 0.25 M Tris-HCl pH 6.75, 8% SDS, 40% glycerol, 4% mercaptoetanol and 0.004% bromophenol blue.

the depth of the stacking gel, which was at least 1.5 cm deep, double that normally used. The gel strip was carefully positioned between the glass plates so that no air bubbles were trapped between it and the surface of the stacking gel. It was wedged securely in place with a folded strip of filter paper; this stopped it floating off when the upper chamber was filled with buffer. The filter paper was overlaid with a small quantity (less than 50 µl) of 4x concentration sample buffer to give a level surface and 100 µl of sample buffer containing 10 units/ml protease was gradually loaded over the denser 4x sample buffer. Gels were run at 20 V until the bromophenol blue front was seen to have entered the stacking gel and at 30 V whilst the front passed through the stacking gel. Once the bromophenol blue front had entered the resolving gel the voltage was raised to 200 V and this was maintained until the marker dye had run off the end of the gels. Proteolysis actually occurred whilst the samples were passing through the stacking gel. Two proteases were initially used, V8 protease from *Staphylococcus aureus* (Sigma) and Type 1-S Chymotrypsin from bovine pancreas (Sigma). However the V8 gave clearer peptide maps and the results were more reproducible and so this was the protease finally used. Peptide maps were silver stained and scanned on a Shimadzu CS-9000 densitometer.

Staining Methods

Coomassie blue G250 Colloidal Stain. (Neuhoff *et al.* 1988).

Gels were fixed for at least 1 hour in a solution of 12% trichloroacetic acid (TCA) and 3% sulphosalcyclic acid, before transferring them to 2% phosphoric acid for at least 15 minutes to wash out urea or SDS from the gel. After washing gels were placed in stain, 2% phosphoric acid, 15% (w/v) ammonium sulphate, 0.01% (w/v) Coomassie blue G250 and 20% methanol. Gels were destained by washing in Milli Q water.

Silver Staining. (Bloom *et al.* 1988).

Gels were fixed overnight in 12% TCA, 3% sulphosalicyclic acid and 0.5 µl/l formaldehyde. After fixing gels were washed in at least two changes of Milli Q water for at least 5 minutes each change. After washing they were placed in 0.2 g/l sodium thiosulphate for 1 minute and then back into Milli Q water, two changes, each of 20 seconds. Then gels were left to equilibrate with 0.1 g/l silver nitrate solution for 10 minutes before washing once again with Milli Q water and placing them in the developer solution. The gels were left in developer, 0.3 g/l sodium carbonate, 0.4 mg/100 mls sodium thiosulphate and 50 µl/100 ml formaldehyde, until the bands were clearly visible. Further staining was prevented by placing gels in 3% citric acid solution and washing until the pH was neutral.

"Stains All" (1-ethyl-2-[-3-(ethylnaptho[1,2-d]-thiazolin-2-ylidene)-2-methylpropenyl]naphtho[1,2-d]-thiazolium bromide.)

Gels were stained with "Stains All" essentially using the method of Campell *et al.* (1983), but with modifications. Instead of fixing gels in 25% isopropanol, a solution of 12% TCA, 3% sulphosalicyclic acid was used. SDS gels were washed exhaustively with Milli Q water to remove SDS which interferes with the stain, before placing them in 0.0025% "Stains All", 25% isopropanol, 7.5% formamide and 30 mM Tris pH 8.8. Alkali urea gels could be placed directly in the staining solution after fixation. Staining and subsequent storage of gels had to be done in the dark.

Chapter 3

The influence of temperature on somitogenesis and organogenesis in embryos of the plaice, *Pleuronectes platessa* L.

Introduction

The influence of temperature on the growth and development of plaice embryos (*Pleuronectes platessa* L.) has previously been studied by Johansen and Krogh (1914), Ryland and Nichols (1967) and Ryland *et al.* (1975). The relationship between temperature and embryonic development was observed to be curvilinear, best expressed by the equation $D = k/(t-t_0)+D_0$, where t = temperature, D = embryonic development time and k, D_0 and t_0 are constants (Ryland *et al.* 1975). Embryos maintained at temperatures greater than 12°C were more likely to display structural abnormalities and less likely to survive to hatching (Johansen and Krogh 1914). Survival of the embryos to hatching was greatest at 8°C (Ryland *et al.* 1975) the temperature at which larval yolk-sac utilisation was most efficient (Ryland and Nichols 1967). The relative timing of organogenesis may also vary in embryos incubated at different temperatures (Hayes *et al.* 1953, Fukuhara 1990). Hayes *et al.* (1953) suggested that the rates of differentiation of different embryonic tissues may show a range of temperature dependencies. Temperature has also been found to affect meristic characteristics such as vertebrae and fin ray number (Tåning 1952). Dannevig (1950) found that vertebrae number is higher with decreasing temperature in the plaice. The meristic characters of a species are apparently influenced most by the temperature experienced by the embryo during gastrulation and before eye formation (Tåning 1952). Variation in meristic characters is thought to have both an environmental and a genetic component (Barlow 1961, Beacham and Murray 1986).

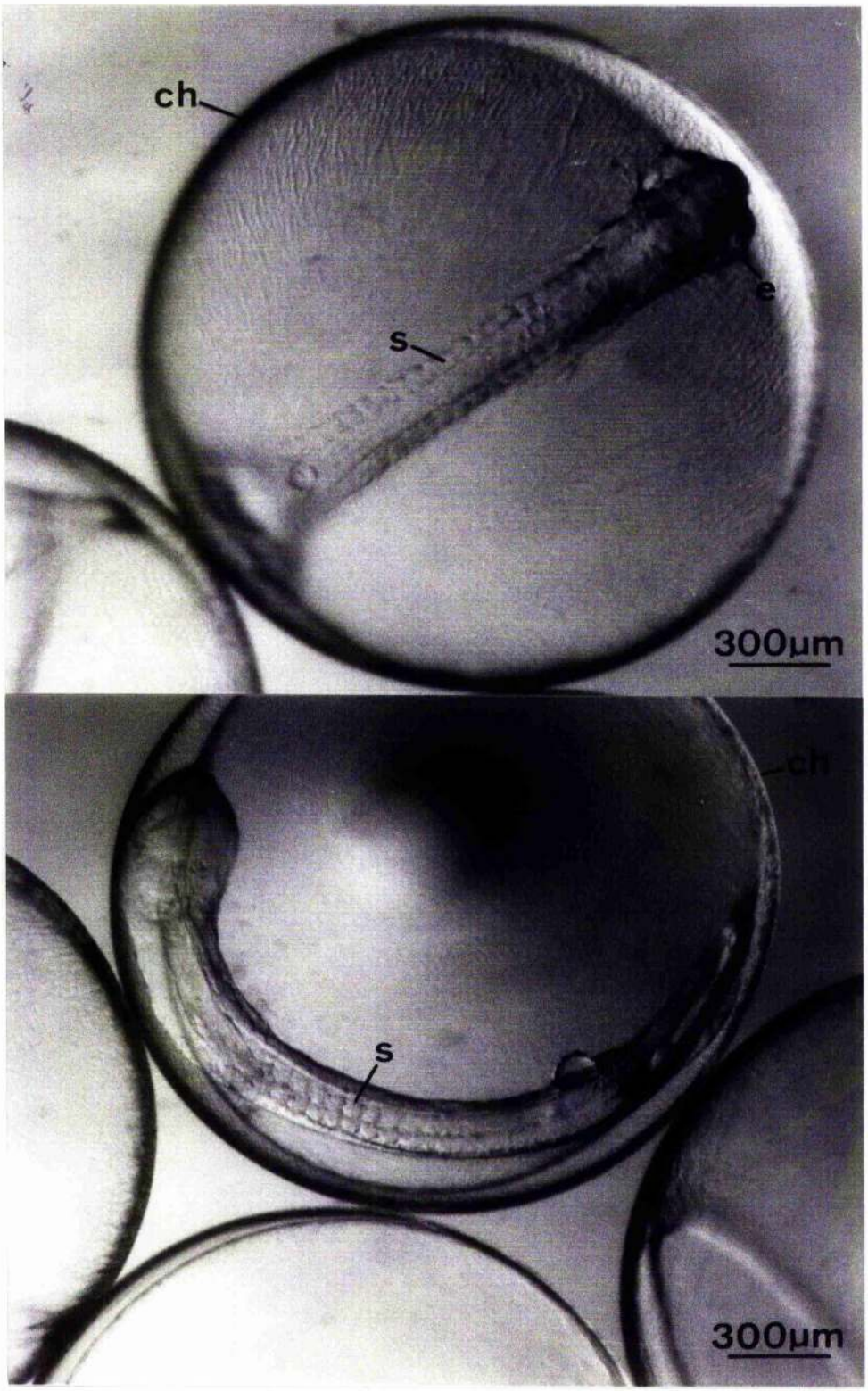
The objectives of this study were to examine the influence of temperature on somitogenesis and organogenesis in plaice embryos.

Materials and Methods

Plaice eggs incubated at two temperatures, 8°C and 12°C, were sampled daily from fertilisation. Eggs were fixed in Heidenhain's Susa (see Appendix) and embedded in paraffin wax (melting point 56°C). Serial sections (8 μm) of the eggs were prepared. Sections were dewaxed and stained with Haematoxylin and eosin and examined under the light microscope.

In March 1993 eggs were stripped from 6 mature adult females and fertilised with sperm from 5 ripe males. The fertilised eggs were maintained at 8°C for 24 hours in the aquarium at Dunstaffnage Marine Laboratory before being transported to the Gatty Marine Laboratory where the eggs were gradually transferred to temperatures of 5°C and 12°C over the next 24 hours. Somite numbers were counted in living embryos, photographed using dark field illumination (Fig. 1) and from embryos fixed in neutral-buffered formalin (see Appendix). Embryos fixed in neutral-buffered formalin had to be dissected out of the chorion before somite numbers could be counted. Results are presented as means ± S.D., n = number of embryos/temperature. Somite numbers and larval lengths at the two different temperatures were compared using the Student's t test (Minitab Software, Minitab Inc. USA.). Data were normally distributed and showed no significant difference in variance.

Figure 1. Plaice embryos reared at 12°C, 65.5 hours after fertilisation, photographed under a dissection microscope. ch: chorion; e: eye capsule; s: somite.


ch
e
s
300µm
ch
s
300µm

Results

Development of the fertilised egg and the effects of temperature

At 18 hours after fertilisation there were already visible differences in the rate of meroblastic cleavage (Fig. 2a and b), cleavage having proceeded more rapidly at the higher temperature. The blastula stage was not clearly delineated at 18 hours post-fertilisation, even at 12°C. The blastula consists of a multicellular blastoderm composed of three cell types, an outer layer of enveloping cells firmly attached to one another; a yolk syncytial layer covering the yolk globule and between these two layers the cells which subsequently develop into the embryo. Gastrulation was estimated to have occurred approximately 2 days post-fertilisation at 12°C and about 2.5 days after fertilisation in 8°C embryos. However the embryos were not sampled at frequent enough intervals to allow gastrulation to be precisely defined. Gastrulation is characterised by epiboly, when the blastoderm spreads over the yolk globule behind the leading edge of the yolk syncytial layer. The movement of the cells leads to a rearrangement of the deep cells to form the germ ring, from which the embryonic axis and then the embryo arise following aggregation of the deep cells at the future dorso-caudal side of the egg.

The gut, notochord and spinal cord started forming 3 days post-fertilisation in the 12°C embryos (Fig. 2c). At 8°C, only the embryonic axis was clearly visible 3 days after fertilisation (Fig 2d). Four days after fertilisation a well-defined spinal cord and gut were present in the 12°C embryos and the eyes were almost fully formed (Fig. 3a). At 12°C, five days after fertilisation myoblasts could be seen arranged in rows prior to their fusion to form myotubes (Fig. 4). The pronephros was also visible, as were the pectoral fins and the dorsal ventral finfold, five days post-fertilisation. Six days

Figure 2. Transverse wax sections through plaice embryos reared at 8°C and 12°C, at different stages of development. Sections were stained with Haematoxylin and eosin.
(a) Embryo 18 hours after fertilisation, rearing temperature 12°C.
(b) Embryo reared at 8°C, 18 hours after fertilisation.
(c) Embryo 3 days after fertilisation, rearing temperature 12°C.
(d) Embryo maintained at 8°C, 3 days after fertilisation.
ch: chorion; g: gut; n: notochord; sc: spinal cord; y: yolk.

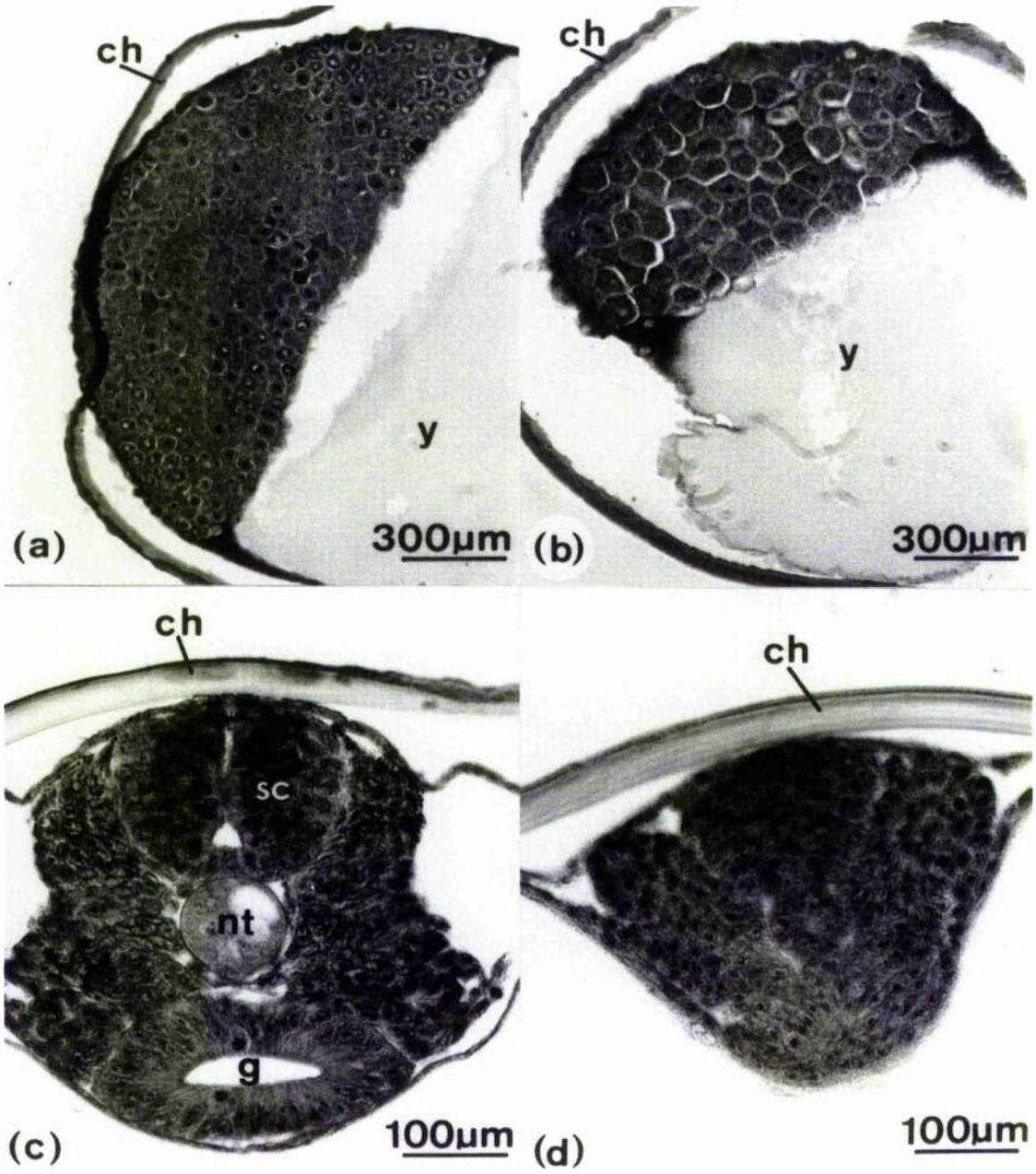
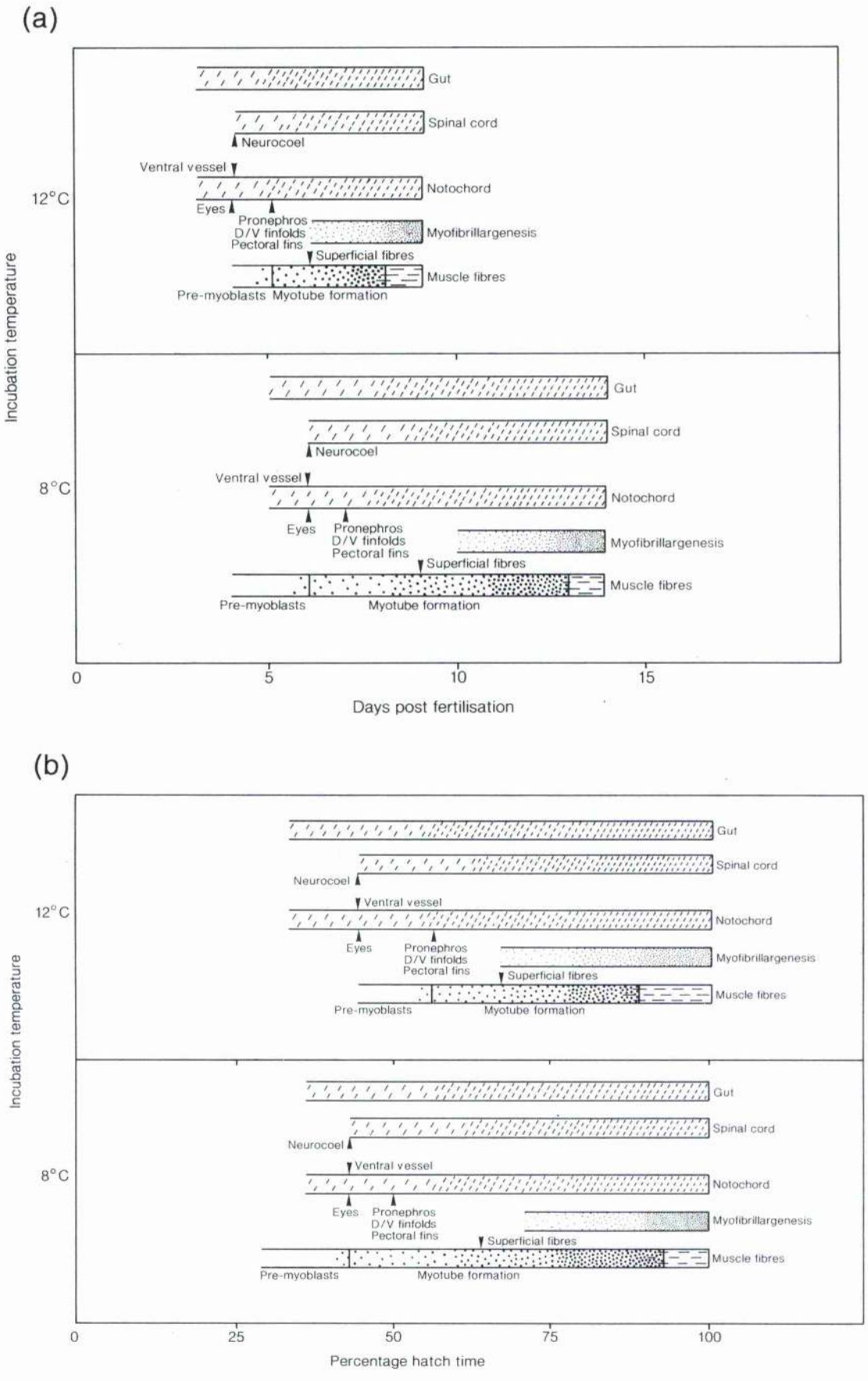

ch
y
(a)
300µm
ch
y
(b)
300µm
ch
sc
nt
g
(c)
100µm
ch
(d)
100µm

Figure 3. Diagrammatic representation of organogenesis during the embryonic development of the plaice.
(a) Formation of organs within the 8°C and 12°C embryos expressed in days post fertilisation.
(b) Organ formation at 8°C and 12°C shown as a proportion of total development time.
Development was assessed by examining transverse and longitudinal wax sections of embryos sampled daily between fertilisation and hatching.
Horizontal bars illustrate myogenesis, myofibrillargenesis and formation of the notochord, spinal cord and gut in the anterior myotomes. Formation of the eyes, neurocoel, ventral vessel (presumptive dorsal aorta), dorsal (D) and ventral (V) finfolds, pronephros and pectoral fins are indicated by arrows. Muscle fibre formation was divided into three stages: (1) formation and division of pre-myoblast cells; (2) alignment and fusion of myoblasts to form myotubes; (3) appearance of muscle fibres containing discrete myofibrils. Progressive changes in the appearance (maturity) of the notochord, spinal cord, gut and muscle fibres are illustrated by the density of pattern within the horizontal bars.


(a)
Incubation temperature
12°C
8°C
Gut
Spinal cord
Neurocoel
Ventral vessel
Notochord
Eyes
Pronephros
D/V finfolds
Pectoral fins
Myofibrillargenesis
Superficial fibres
Muscle fibres
Pre-myoblasts
Myotube formation
0
5
10
15
Days post fertilisation
(b)
Incubation temperature
12°C
8°C
Gut
Spinal cord
Neurocoel
Ventral vessel
Notochord
Eyes
Pronephros
D/V finfolds
Pectoral fins
Myofibrillargenesis
Superficial fibres
Muscle fibres
Pre-myoblasts
Myotube formation
0
25
50
75
100
Percentage hatch time

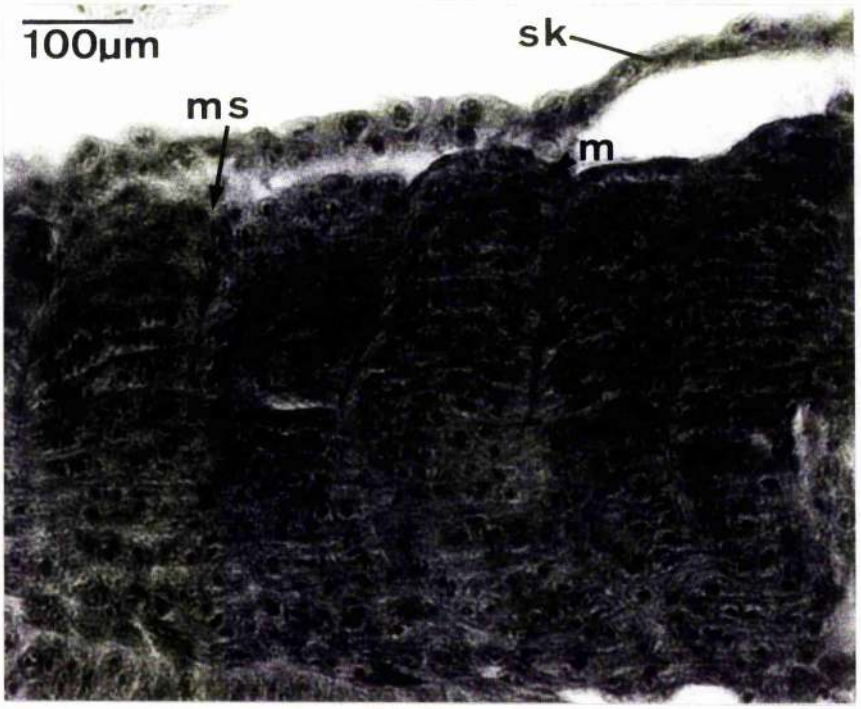


Figure 4. Plaice embryo reared at 12°C, five days after fertilisation. Myoblasts are arranged in rows, parallel to the notochord. Longitudinal wax section stained with Haematoxylin and eosin.
m: myoblast; ms: myosept; sk: skin.

after fertilisation it was possible to distinguish the superficial muscle fibres from the inner fibres in the embryos maintained at 12°C (Fig. 3a).

Organogenesis was slower at 8°C; at 6 days post-fertilisation the 8°C embryos reached the same developmental stage as the 12°C embryos had reached at 4 days post-fertilisation (Fig. 3a). The order in which the various organs and tissues formed in the embryos was the same at both incubation temperatures. At both 8°C and 12°C the different organs differentiated from the embryonic mesoderm at equivalent points between fertilisation and hatching and were present in the developing embryo for a very similar proportion of the period between fertilisation and hatching (Fig. 3b).

The information obtained from the wax sections can only be used to give an estimated time for the events comprising muscle fibre differentiation in plaice embryos. The magnifications provided by the light microscope (x20-1000) are too low to distinguish the initial stages of myofibrillargenesis and myotube formation. T.E.M data is required for a more accurate timetable. Plaice eggs were fixed and embedded for examination with the T.E.M, but the chorion, even when punctured was found to completely block the passage of osmium and unfortunately the developing embryos were too small to successfully survive the embedding process when dissected free from the egg capsule.

Effect of incubation temperature on embryonic somite development

Development time, defined as the period between fertilisation and 75% of the embryos hatching, was 456 hours at 5°C and 210 hours at 12°C. The head and eye capsules had formed when the embryos were examined after 22% (104 hours) development time at 5°C and 23% (46 hours) development time at 12°C but distinct somite boundaries were

not present. Somites could first be counted after 25% (114 hours) development time in embryos reared at 5°C, when 17 ± 1.1 (n = 8) somites were present (Fig. 5). At 12°C, 14 ± 1.5 (n = 8) somites were visible after 31% (65 hours) development time (Fig. 5). This suggests that formation of the initial somites occurs relatively later in development at high than at low temperatures. At any given stage of development embryos reared at 5°C had significantly more somites than the embryos maintained at 12°C ($p < 0.05$). For example, after 35% development time 5°C embryos possessed 33 ± 0.9 somites while 12°C embryos, after 36% development time, had 26 ± 1.2 somites (n = 8). After 70% and 69% development time respectively, 5°C embryos had 52 ± 1.1 somites and 12°C embryos 46 ± 1.0 somites (n = 8). At both stages the differences in somite numbers were statistically significant ($p < 0.05$). Spontaneous contractions of the anterior somites and a regular heart beat were observed after 47% of development time at both temperatures, corresponding to the 50 somite stage at 5°C and the 44 somite stage at 12°C (Fig. 5). Somitogenesis proceeded more rapidly at both temperatures before the embryos began moving (Fig. 5a and b). At 5°C, somites were added to the tail at a rate of 0.35 somites/hour. Prior to spontaneous movement in the 12°C embryos the rate of somite addition was 0.8 somites/hour (Fig. 5a).

When the embryos hatched the total length of the larvae reared at 5°C was 6.61 ± 0.36 mm (n = 10) while the 12°C larvae measured 6.07 ± 0.22 mm (n = 10), a 9% difference ($p < 0.05$). Newly hatched 5°C larvae possessed 61 ± 1.5 body somites (n = 8), 9% more than the 56 ± 1.3 (n = 8) somites of 12°C larvae at hatching ($p < 0.05$).

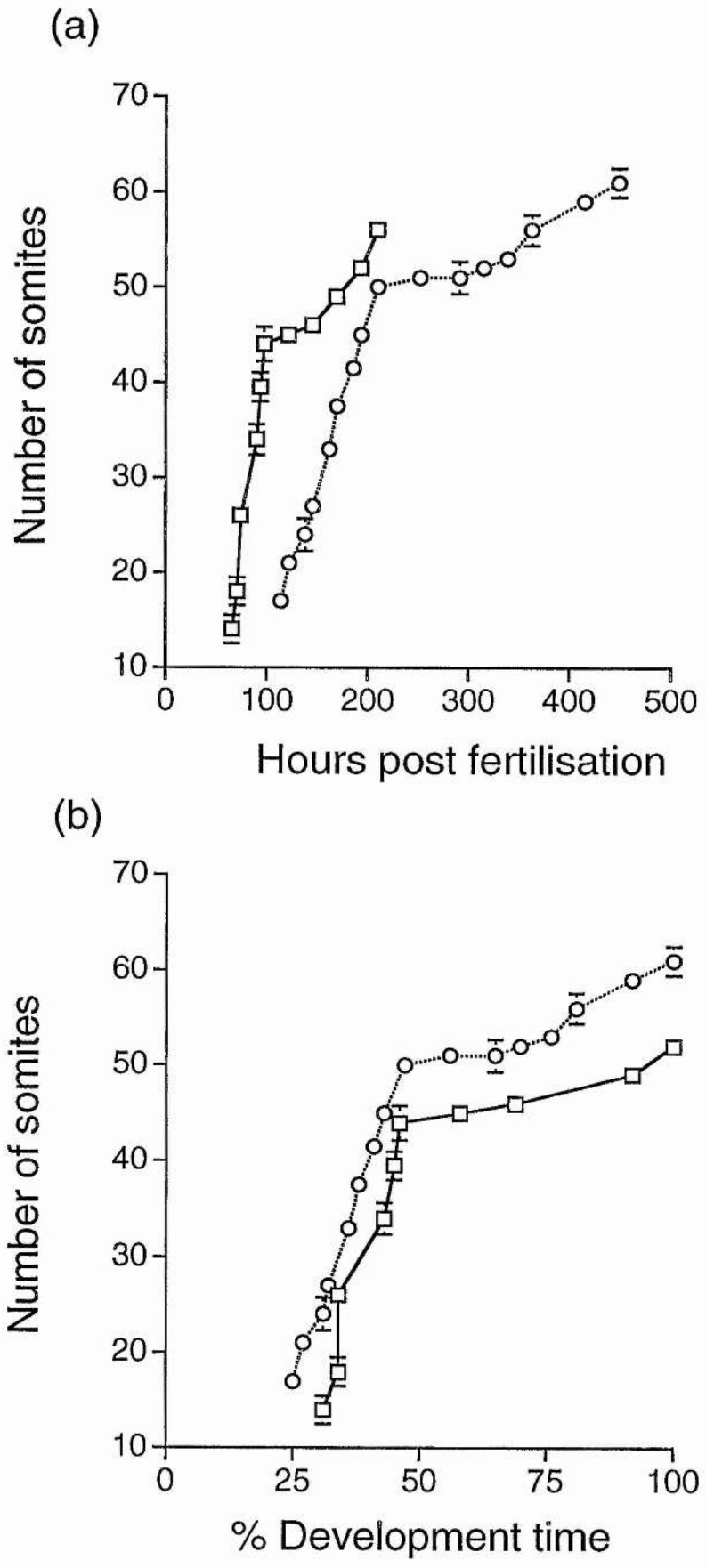
Figure 5.

(a) The rate of somite development in embryos maintained at 5°C and 12°C.

(b) Somite development in embryos reared at 5°C and 12°C expressed as a percentage of the total embryonic period (defined as the period between fertilisation and 75% of the embryos hatching).

Values represent mean ± standard deviation (n = 8 embryos/temperature). Note some of the error bars fall within the symbols. Arrows indicate the start of spontaneous movement by the embryos.

□-12°C, ○-5°C.


(a)
Number of somites
70
60
50
40
30
20
10
0
100
200
300
400
500
Hours post fertilisation
(b)
Number of somites
70
60
50
40
30
20
10
0
25
50
75
100
% Development time

Discussion

Predictably plaice embryos reared at 8°C took longer to develop than those maintained at 12°C, organogenesis proceeding more rapidly in the embryos at the higher temperature. The order of appearance of anatomical features was unaltered by temperature (Fig. 3). Muscle fibre differentiation did, however, begin slightly earlier in plaice embryos maintained at 8°C, compared with the embryos reared at 12°C (Fig. 3). By contrast, embryos of the herring, *Clupea harengus,* showed a degree of asynchronous development, when incubated at widely different temperatures (Johnston 1993). The gut, notochord, eyes and ventral vessel all appeared at the same relative point between fertilisation and hatching whatever the rearing temperature, but at 5°C the formation of the pronephros, pectoral fin buds and muscle fibres occurred earlier than at 8°C and 12°C (Johnston 1993). Garside (1959) found evidence indicating that the differentiation of successive structures in salmonid embryos was confined to fairly rigid limits. If a metabolic block prevented the formation of a structure at its normal position during morphogenesis the structure would completely fail to develop. Osse (1990) suggested that in fish the ontogenetic sequence of development of functional systems was linked to the constantly changing demands of both environment and function. This could ensure that embryonic development followed essentially the same pathways of organogenesis and tissue differentiation, unless environmental stress was very severe, completely disrupting the development of whole organs and body regions (Garside 1959). Temperature is unlikely to dramatically alter the order in which the various organs and tissues appear in plaice embryos, although small variations may be possible.

Somite formation began earlier at 5°C than at 12°C in plaice embryos, but how temperature affects the mechanism of somitogenesis is unclear. In embryos of the zebrafish,

Brachydanio rerio, somite formation appears to be regulated by acetylcholinesterase (AChE) activity (Hanneman 1992). Prior to the onset of somitogenesis the nuclei of presumptive somitic mesodermal cells show AChE activity. The appearance of AChE activity follows a rostro-caudal sequence, as cells become committed to the presumptive somitic mesoderm they acquire AChE activity in a region extending approximately 5 somite lengths caudal to the last formed somite. Inhibition of AChE activity by diisopropylfluorophosphate disrupts somitogenesis in zebrafish embryos (Hanneman 1992). Myogenesis begins at the 20- to 25-somite stage in embryos of the zebrafish, *Brachydanio rerio,* and progresses laterally along the embryo (Waterman 1969). As the somites form, myofilaments differentiate medially outwards from the notochord (Waterman 1969). It is possible that myogenesis begins earlier in the plaice embryos reared at 5°C where somite pairs appeared after 25% development time compared to 31% development time at 12°C.

Van Raamsdonk *et al.* (1977, 1979) proposed that the shape of the somites was regulated by a combination of muscle contractions and embryonic movements. Plaice embryos started exhibiting movement at the 51-somite stage in 5°C embryos and at the 44-somite stage in 12°C embryos, after which there was a marked reduction in the rate of somitogenesis at both rearing temperatures. Zebrafish embryos demonstrated movements much earlier than plaice embryos. From the sixteen somites stage onwards the zebrafish embryo performs lateral swinging movements produced by contractions of the differentiated anterior somites (Van Raamsdonk *et al.* 1974). When first formed the somites of the zebrafish are 'block shaped' (Waterman 1969). The midbody somites become V-shaped soon after the formation of myofibrils in the medial somitic cells, the myosepts forming an angle of 35° with the body axis (Van Raamsdonk *et al.* 1977, 1979). The anal and post-anal somites differentiate after the start of embryonic movement and are

V-shaped before containing myofilaments, possibly because these somites are added onto somites which are already V-shaped (Van Raamsdonk *et al.* 1974, 1977). Certainly the immobilisation experiments carried out by Van Raamsdonk *et al.* (1977, 1979) indicated that body movements did determine somite shape and help to stabilise that shape. In plaice embryos the relationship between somite shape and embryonic movement was less marked, V-shaped somites could be distinguished in many of the embryos before the onset of spontaneous movement. Why somite formation should decline so markedly after plaice embryos began moving is unclear; a similar decline has not so far been observed in other species.

Disturbances in the rate of differentiation by temperature also causes minor morphological variations such as influencing the number of meristic characters (Tåning 1952, Beacham and Murray 1986, Murray and Beacham 1989). Temperature appears to influence the number of somite pairs forming in plaice embryos in a similar way to vertebrae numbers, low temperatures resulting in higher numbers (Dannevig 1950, Molander and Molander-Swedmark 1957). Hempel and Blaxter (1961) had previously established that both rearing temperature and salinity have a modifying effect on myotome numbers in newly hatched herring larvae. However any relationship between myotome numbers and temperature is obscured by the formation of the caudal fin supports and musculature later in development (Hempel and Blaxter 1961). Temperature, while it has been found to influence the number of vertebrae and vertebral myotomes has not been found to influence the number of caudal fin supports (Fowler 1970).

Chapter 4

Muscle development in plaice, *Pleuronectes platessa* L.

Introduction

Various patterns of muscle development have been documented in teleosts apparently related to the size, and locomotory capacities of the larvae at hatching (Waterman 1969, Van Raamsdonk *et al.* 1978, Veggetti *et al.* 1990, Johnston 1993). Newly hatched plaice larvae, *Pleuronectes platessa,* have a functional mouth and jaws and heavily pigmented eyes. The heart has started beating and a circulatory system is present, although there are no erythrocytes or haemoglobin (Shelbourne 1956a, de Silva 1974). The alimentary canal is not fully formed, larvae have an internal food source, the yolk-sac, and as this diminishes they begin feeding on diatoms and small zooplankton. Shortage or absence of food affects the behaviour of the larvae; when food is scarce larvae become more active, swimming for longer periods, which should increase the chance of finding food (Wyatt 1972).

Plaice larvae show optimal growth and yolk-sac utilisation at temperatures between 6.5 and 8°C (Ryland and Nichols 1967), corresponding to the mean sea temperatures of the spawning areas in the southern North Sea during February to April. As plaice larvae grow the optimum temperature for growth increases (Ryland and Nichols 1967). Field studies of growth, otolith growth and RNA/DNA ratios of larval plaice in the North Sea by Hovenkamp and Witte (1991) showed that somatic growth rates were strongly correlated with seawater temperature. Age at metamorphosis was found to decline with increasing temperature, but there was no relationship between size at metamorphosis and temperature.

The myotomes of newly hatched herring, *Clupea harengus,* sea bream, *Pagrus major,* and roach, *Rutilus rutilus,* larvae all

contain a single superficial layer of small diameter muscle fibres that surround an inner mass of larger diameter fibres (Batty 1984, Vieira and Johnston 1992, Matsuoka and Iwai 1984, El Fiky *et al.* 1987). Incubation temperature has been shown to affect the number and average diameters of the superficial and inner muscle fibres at hatching in the Atlantic salmon (Stickland *et al.* 1988, Johnston *et al.* personal communication) and the herring (Vieira and Johnston 1992), although the results reported are somewhat variable even for the same species (Johnston 1993). Temperature has also been observed to affect the spatial organisation and volume densities of muscle fibre organelles (Vieira and Johnston 1992).

Muscle growth during the larval stages of teleost species such as sea bass (Veggetti *et al.* 1990), zebrafish (Waterman 1969, Van Raamsdonk *et al.* 1978) and rainbow trout (Nag and Nursall 1972), occurs not only by hypertrophy but also by the recruitment and development of undifferentiated myoblasts. The continuation of hyperplasia in conjunction with hypertrophic growth throughout the post-larval period is thought to cause the mosaic appearance of teleost white muscle (Carpene and Veggetti 1981). Veggetti *et al.* (1990) suggested that new fibres were recruited from germinal areas specific for each fibre type. In juvenile fish, myotomal growth continued to occur by both hypertrophy and hyperplasia. However new fibres appear to be generated from myosatellite cells deep within the myotome and not from the specific germinal zones observed in larvae (Veggetti *et al.* 1990, Stickland 1983).

The aim of this study was to investigate the influence of rearing temperature on the fine structure of larval swimming muscles and to examine changes in the distribution and ultrastructure of myotomal muscle fibre types throughout development.

Materials and Methods

The effects of temperature on muscle fibre ultrastructure were examined using larvae reared at five temperatures: 5°C, 8°C, 10°C, 12°C and 15°C. Two fixatives were used. Newly hatched larvae reared at 5°C, 10°C and 15°C were fixed for 4 hours in 4% paraformaldehyde in phosphate buffered saline (160 mM NaCl, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4), pH 7.2, at 4°C and embedded in Araldite resin. Larvae reared at 8°C and 12°C were fixed in 4% paraformaldehyde, 4% gluteraldehyde in 40 mM cacodylate buffer pH 7.2, overnight, at 4°C (see Chapter 2).

Semi-thin (0.5-1 μm) and ultra-thin (50-60 nm) transverse sections, were cut from the region immediately posterior to the yolk-sac, where the gut just terminated. Five individuals from each temperature regime were sectioned.

Photographs were taken of semi-thin sections using a light microscope and enlarged to a final magnification of X550. The number of inner muscle fibres in each cross-section was counted directly from the photographs. The cross-sectional areas of the individual inner muscle fibres were also determined from the photographs using a Videoplan Image Analysis System (Kontron Electronics, Basel). Measurements were made of 50-70% of all inner fibres in the cross-section. The resulting data was analysed using a one-way analysis of variance (Minitab software, Minitab Inc., USA).

Ultrastructural analysis

Four superficial and four inner muscle fibres were photographed per larvae using 1/4 plate film at magnifications of 7100-19000X, making a total of twenty per fibre type at 5, 10 and 15°C. Negatives of whole muscle fibre cross-sections were enlarged 3.5 times and volume densities (Vv, Σ organelle cross-sectional areas/muscle fibre cross-sectional area) of the organelles were determined using stereological analysis

software (Kontron Electronic, Basel). The surface density of mitochondrial cristae (Sv, cr,mit) was determined from randomly selected micrographs taken at x34000-91000 and enlarged 2.92-4.14 times, using a line intercept system with a grid spacing of 0.06-1.12 μm and 80-300 intersections per mitochondrion (Weibel 1989). A total of 20 fibres was analysed for each temperature and fibre type. No corrections were made for the effects of section thickness or compression.

The data from the different rearing temperatures was compared using a one-way analysis of variance (Minitab Software, Minitab Inc. U.S.A.). Where one-way analysis of variance showed that results did not all belong to one population adjacent data sets were compared by Student's t test (Minitab Software, Minitab Inc. USA.). Fibre cross-sectional areas were also analysed using the Mann-Whitney test (Kontron Electronics, Basel.).

Muscle fibre development

The development of the muscle fibres during the period from hatching to metamorphosis was observed from larvae reared at 8°C and fixed in Heidenhain's Susa for wax histology and 4% paraformaldehyde, 4% gluteraldehyde, 40 mM sodium cacodylate buffer pH 7.2, for T.E.M. The histochemical characteristics of myotomal muscle fibres were determined from larvae that had passed through metamorphosis (total length = 12.2 ± 1.4 mm, mean ± S.D.), 0-group wild caught juveniles (total length = 29.1 ± 1.8 mm, mean ± S.D.) and adult fish, as described in Chapter 2.

Results

Plaice eggs hatched after a mean duration of 20, 13, 11, 9 and 8 days at 5, 8, 10, 12 and 15°C respectively. The percentage of larvae hatching from eggs kept at 15°C was very low and none of these survived more than a few days. Larvae hatching at the lowest temperature were significantly longer (from 19-28%), than those hatching at any of the four higher temperatures (Table 1, $p < 0.05$). Two types of muscle fibres could be distinguished in the newly hatched larvae, beneath the skin was a single layer of smaller diameter muscle fibres (Fig. 1, superficial fibres) which completely surrounded the larger diameter inner muscle fibres (Fig. 2).

Muscle fibre cross-sectional area and fibre number both varied with temperature. Larvae hatching at 5°C had significantly fewer ($p < 0.05$) but larger inner muscle fibres than 10°C larvae. (Fig. 3a and b). Larvae reared at both 12°C and 15°C possessed inner fibres of larger cross-sectional area than those larvae reared at 5, 8 and 10°C, there were also more of them (Fig. 3a and b). The size distribution of the inner muscle fibres was also affected over the range of rearing temperatures studied (Fig. 4). At 15°C larvae had a significantly broader distribution of fibre sizes compared to larvae reared at 5 and 10°C ($p < 0.05$).

Superficial and inner muscle fibres had different ultrastructural characteristics (Table 2). The superficial muscle fibres had a higher volume density of mitochondria (24-33%) than the inner fibres ($p < 0.05$), while the inner muscle fibres had a significantly higher volume density of myofibrils ($p < 0.05$, Table 2). Superficial muscle fibres had almost equal proportions of mitochondria (24-33%) and myofibrils (22-29%), the mitochondria being clustered to the side of the fibre (Fig. 1) next to the subcutaneous glycoprotein layer and the skin. The distribution of mitochondria within the superficial fibres did not alter with rearing temperature.

Table 1

The body lengths (unfixed material) and cross-sectional areas (fixed material) of newly hatched plaice larvae reared at different temperatures. Values are mean ± standard deviation. Number of larvae measured = 6

Rearing temperature	Total body length (mm)	Cross-sectional area of trunk (mm^2)
5°C	7.81 ± 0.02	0.10 ± 0.009
8°C	6.60 ± 0.76	0.10 ± 0.004
10°C	6.06 ± 0.16	0.09 ± 0.011
12°C	6.48 ± 0.66	0.14 ± 0.012
15°C	6.10 ± 0.22	0.13 ± 0.005

Figure 1. Electron micrographs of superficial muscle fibres (transverse sections) from newly hatched plaice larvae reared at three different temperatures.
(a) 5°C, (b) 10°C, (c) 15°C.
ec: external cells; gp: glycoprotein matrix; if: inner muscle fibres; mt: mitochondria; my: myofibrils; n: nucleus; sf: superficial muscle fibres.

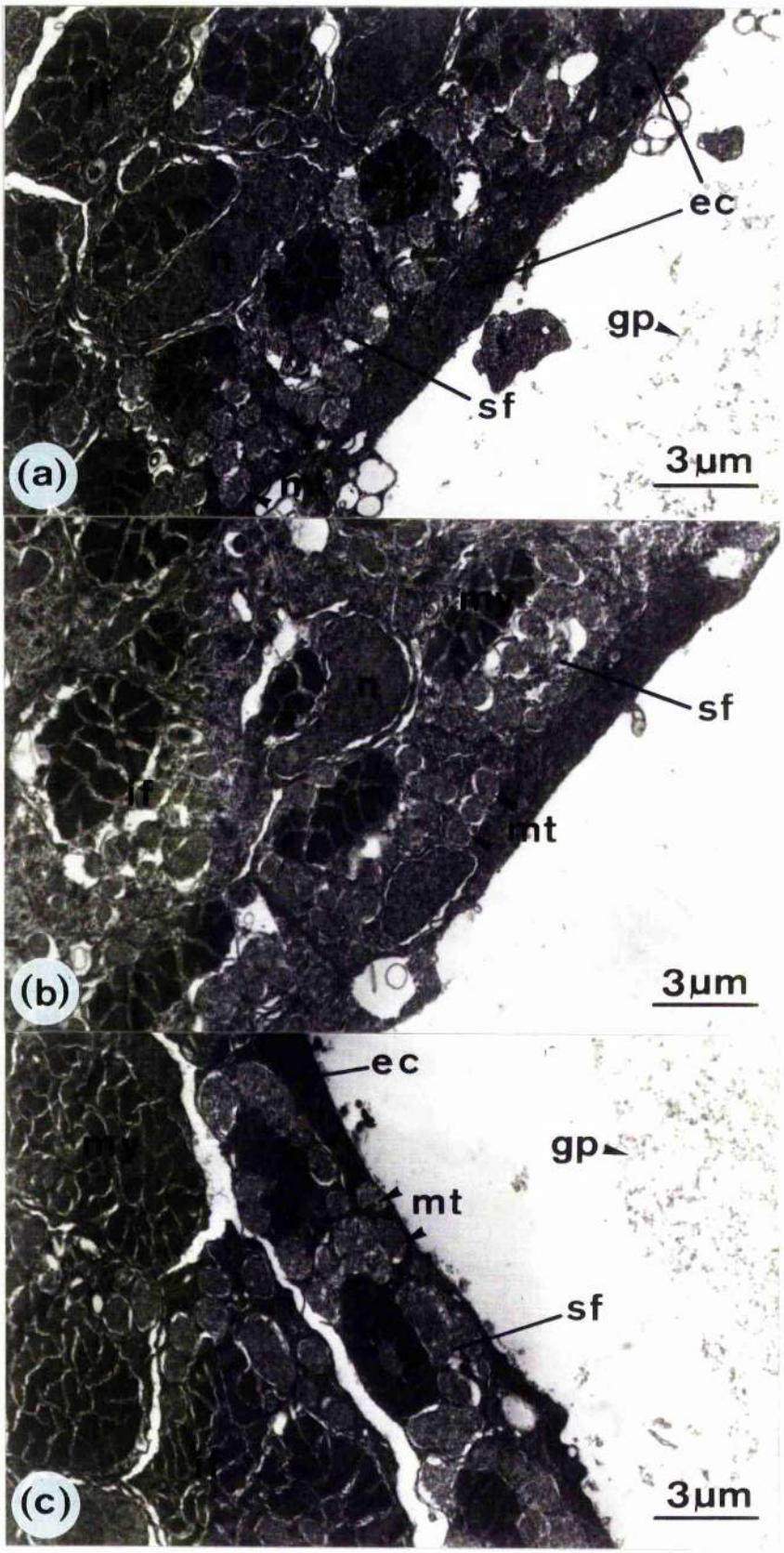

ec
gp
sf
3µm
(a)
mv
n
sf
lf
mt
3µm
(b)
ec
gp
mt
sf
3µm
(c)

Figure 2. Electron micrographs of inner muscle fibres (transverse sections) from newly hatched plaice larvae reared at three different temperatures. (a) 5°C, (b) 10°C, (c) 15°C.
mt: mitochondria; my: myofibrils; n: nucleus.

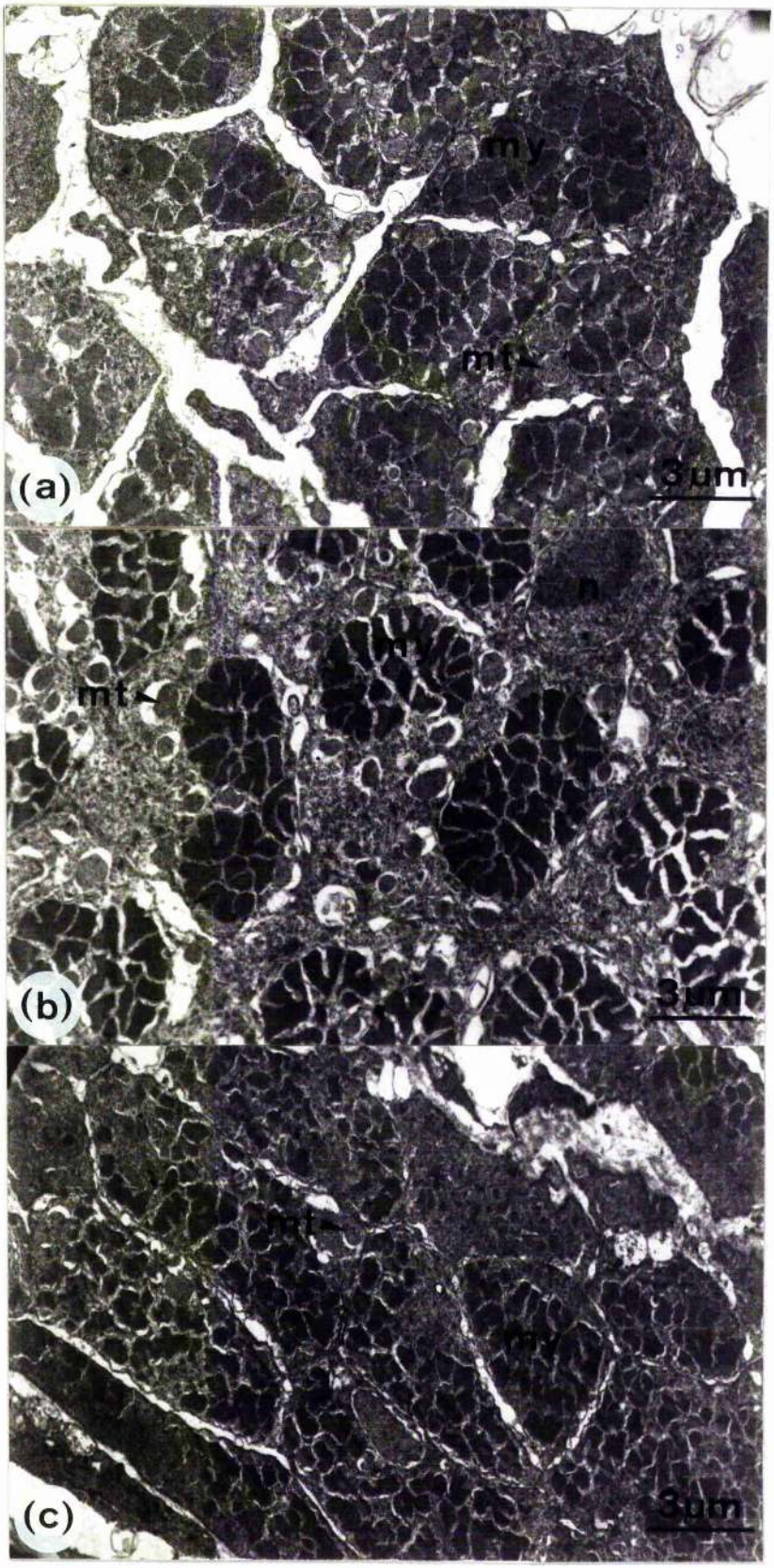

my
mt
(a)
3um
n
my
mt
(b)
3um
mt
my
(c)
3um

Figure 3. (a) Total number of inner muscle fibres in the myotomes immediately posterior to the yolk-sac of newly hatched larvae reared at five different temperatures. Values plotted are means ± standard deviation (n = 5 larvae/temperature).
(b) Average cross-sectional area of inner muscle fibres from newly hatched larvae reared at five different temperatures. Values plotted are means ± standard deviation. (n = 5 larvae/temperature).

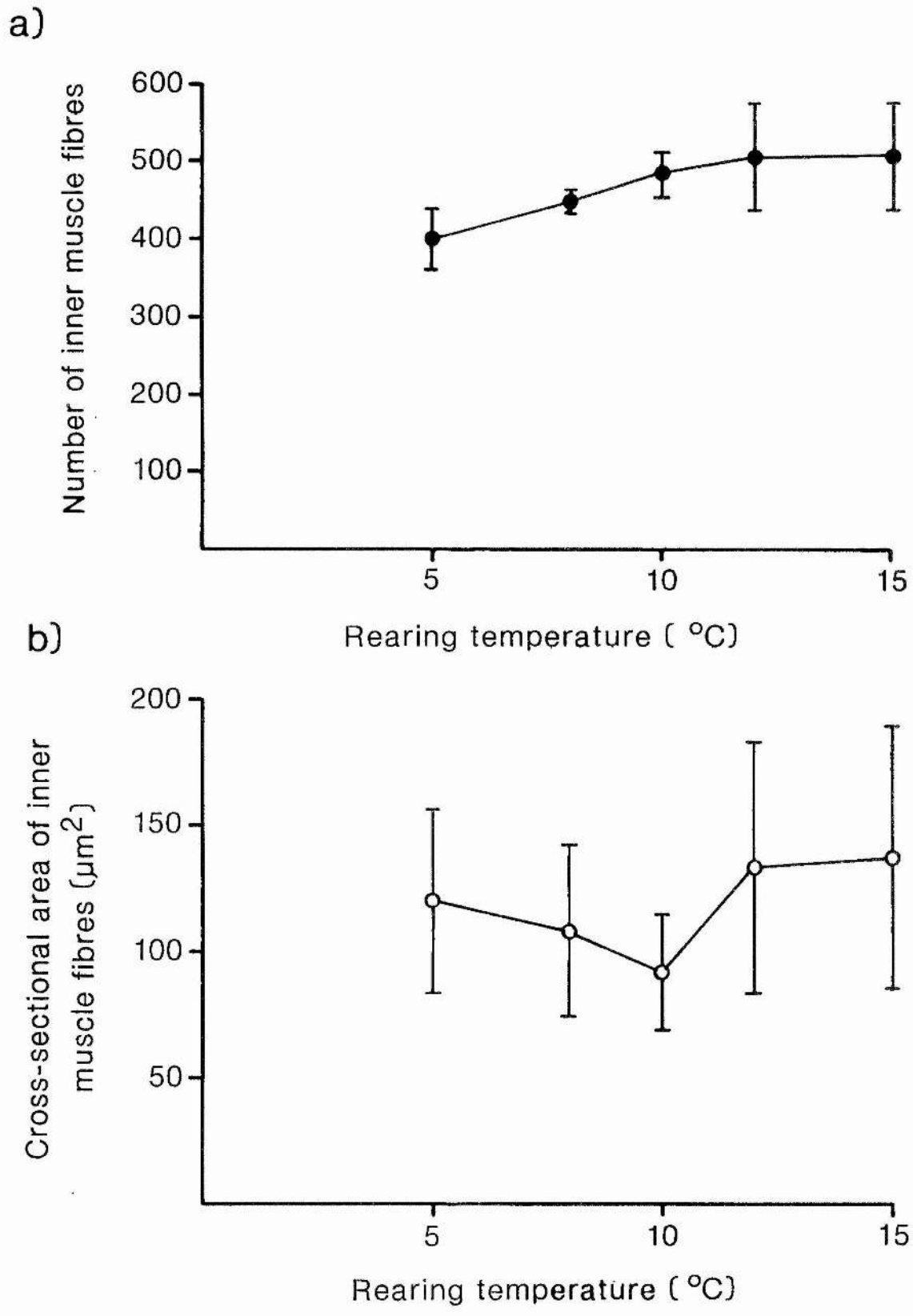

a)
Number of inner muscle fibres
600
500
400
300
200
100
5
10
15
Rearing temperature (°C)
b)
Cross-sectional area of inner muscle fibres (µm²)
200
150
100
50
5
10
15
Rearing temperature (°C)

Figure 4. Distribution of fibre sizes within the inner muscles of newly hatched plaice larvae reared at three different temperatures. (a) 5°C, (b) 10°C, (c) 15°C.

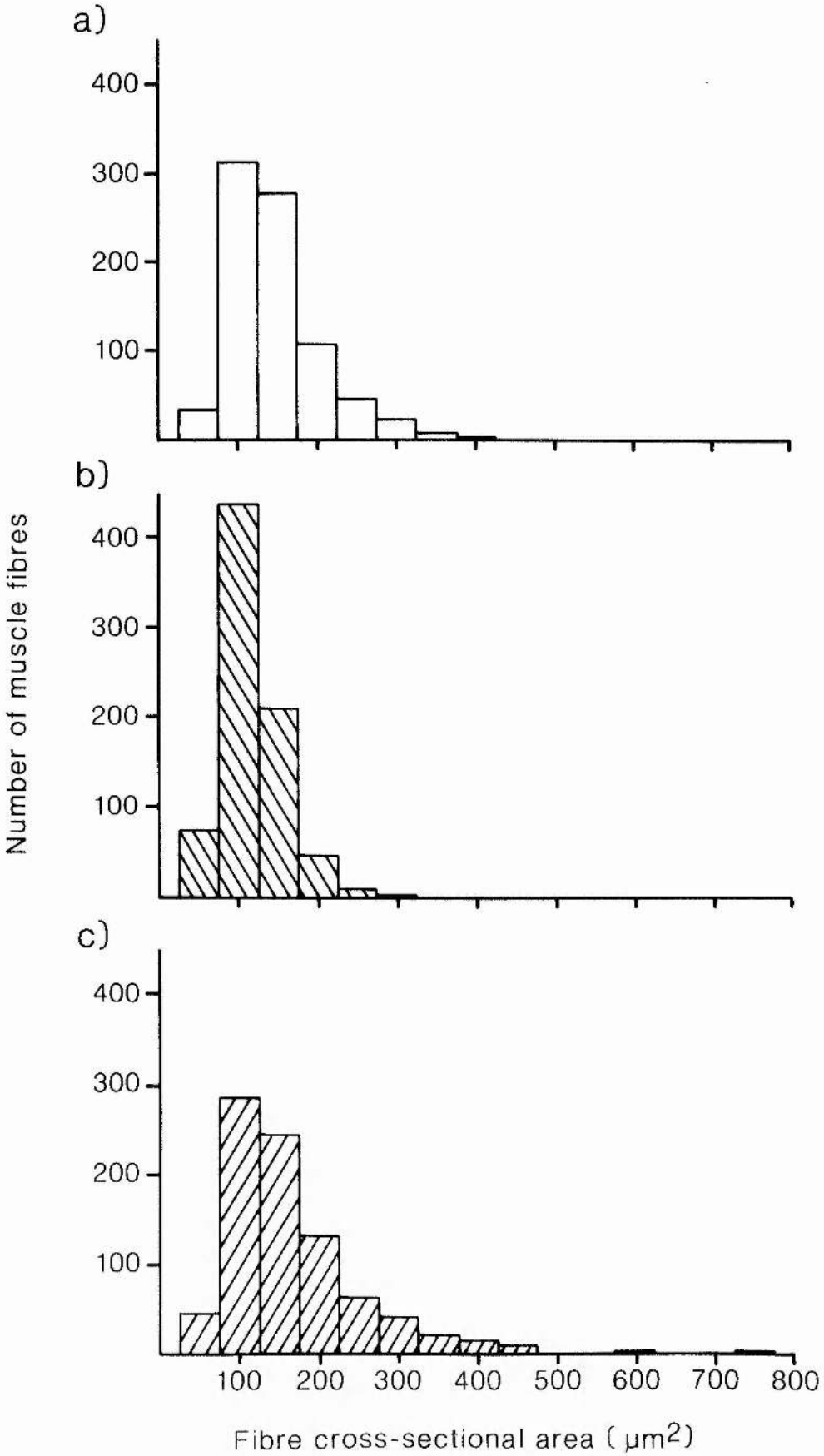

a)
400
300
200
100
b)
400
300
200
100
Number of muscle fibres
c)
400
300
200
100
100
200
300
400
500
600
700
800
Fibre cross-sectional area (μm2)

Table 2

Ultrastructural characteristics of superficial and inner muscle fibres from plaice larvae hatched at three temperatures.

Superficial fibres	5°C	10°C	15°C
Vv (mit,f)	0.24 ± 0.06	0.27 ± 0.04	0.33 ± 0.05*
Sv (cr,mit)	9.47 ± 1.62	10.02 ± 1.10	9.23 ± 0.91
Vv (myo,f)	0.26 ± 0.02	0.22 ± 0.01	0.30 ± 0.05
Inner fibres	**5°C**	**10°C**	**15°C**
Vv (mit,f)	0.081 ± 0.01	0.069 ± 0.01	0.072 ± 0.01
Sv (cr,mit)	8.95 ± 2.56	9.24 ± 1.52	8.33 ± 1.99
Vv (myo,f)	0.41 ± 0.08	0.39 ± 0.04	0.61 ± 0.05*

Vv (mit,f)- Σ Total cross-sectional area of mitochondria/total cross-sectional area of fibre

Sv (cr,mit)- Cristae density

Vv (myo,f)- Σ Total cross-sectional area of myofibrils/total cross-sectional area of fibre

Values represent mean ± standard deviation

Number of observations = 20 fibres/fibre type/temperature

At 15°C the superficial fibres were more elongated (Fig. 1c) and more irregular in shape. Inner muscle fibres had a similar ultrastructural appearance at all three temperatures (Fig. 2). The volume density of muscle fibre organelles was influenced by the temperature at which the embryos were incubated (Table 2). The volume density of mitochondria in the superficial muscle fibres was significantly higher at 15°C ($p < 0.05$, Vv,mit = 33%). At rearing temperatures of 5°C and 10°C the proportion of the superficial muscle fibre volume occupied by mitochondria was 24% and 27% respectively. In contrast, mitochondria occupied a similar fraction of fibre volume in the inner muscle fibres at all three rearing temperatures. The surface density of mitochondrial cristae, however, was not significantly influenced by rearing temperature in either the superficial or the inner muscle fibres.

The volume density of myofibrils in the superficial fibres was unaffected by rearing temperature. However the myofibrillar density (Vv(myo,f)) of the inner muscle fibres was significantly higher in larvae raised at 15°C (61%), than at 5° (35%) and 10°C (36%), respectively (Table 2, $p < 0.05$).

Muscle growth and development in larvae and juveniles

As plaice larvae grow and develop (Fig. 5) their external appearance changes dramatically. The yolk-sac is absorbed and has completely disappeared by the time the larvae are two weeks old (Fig. 5b). Their bodies increase in size both lengthwise and dorso-ventrally (Fig. 5c and d), but there is very little growth laterally across the body. During metamorphosis (Fig. 5e) the left eye migrates over the dorsal ridge onto the the right hand side of the body and fish settle onto the substratum.

The development of the muscle fibres within the myotomes corresponds very closely to the changes in body shape (Fig. 6), the majority of growth occurs along the the dorso-ventral axis

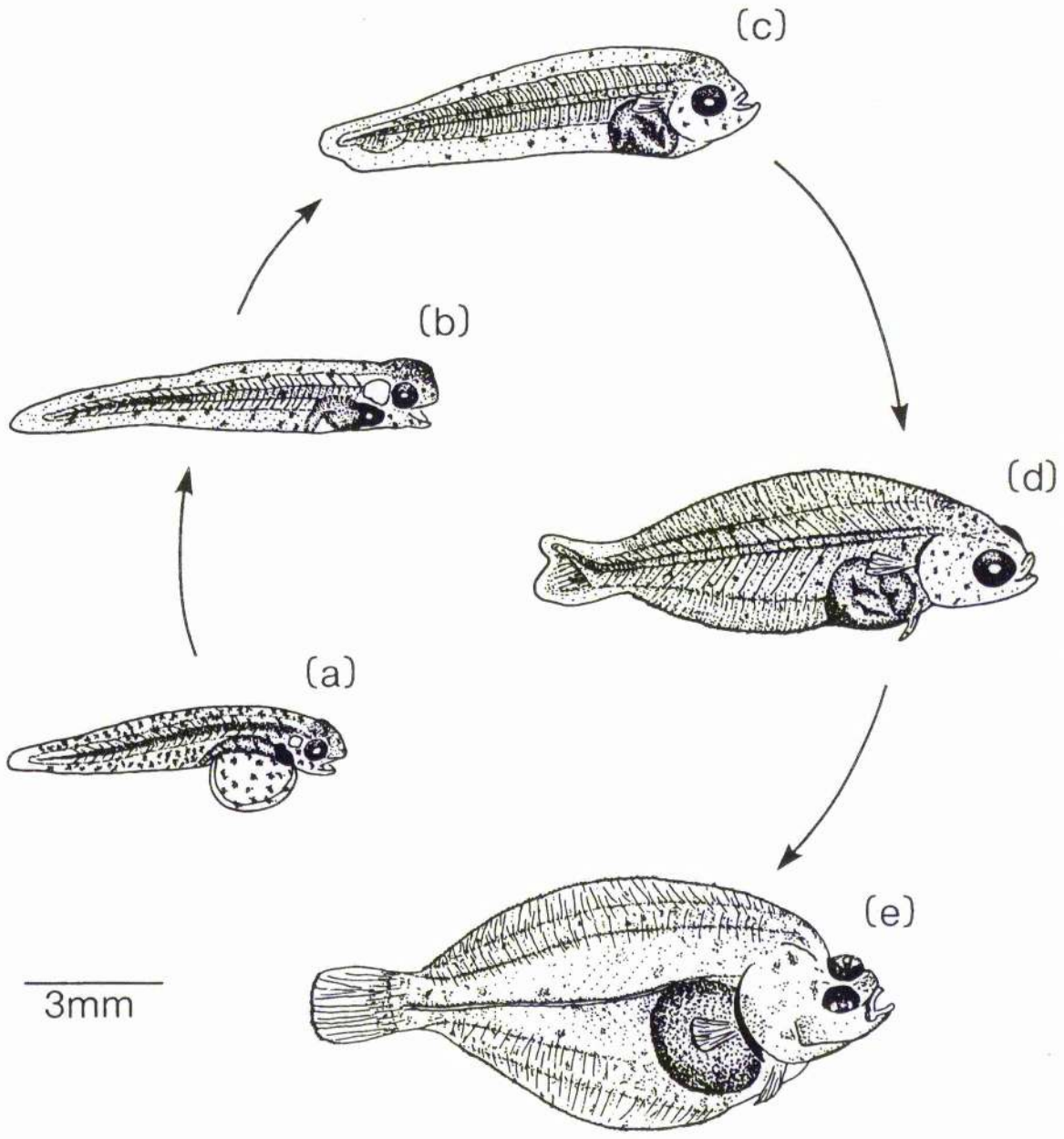


Figure 5. Diagrammatic representation of the different larval stages of the plaice.
(a) Newly hatched yolksac larva. (b) Larva at the end of the yolksac period (approximately 1-2 weeks post hatching). (c) Larva aged between 4-5 weeks, caudal fin starting to develop. (d) Larva at onset of metamorphosis (approximately 7 weeks post hatching). (e) Metamorphosis completed (approximately 10 weeks post hatching).

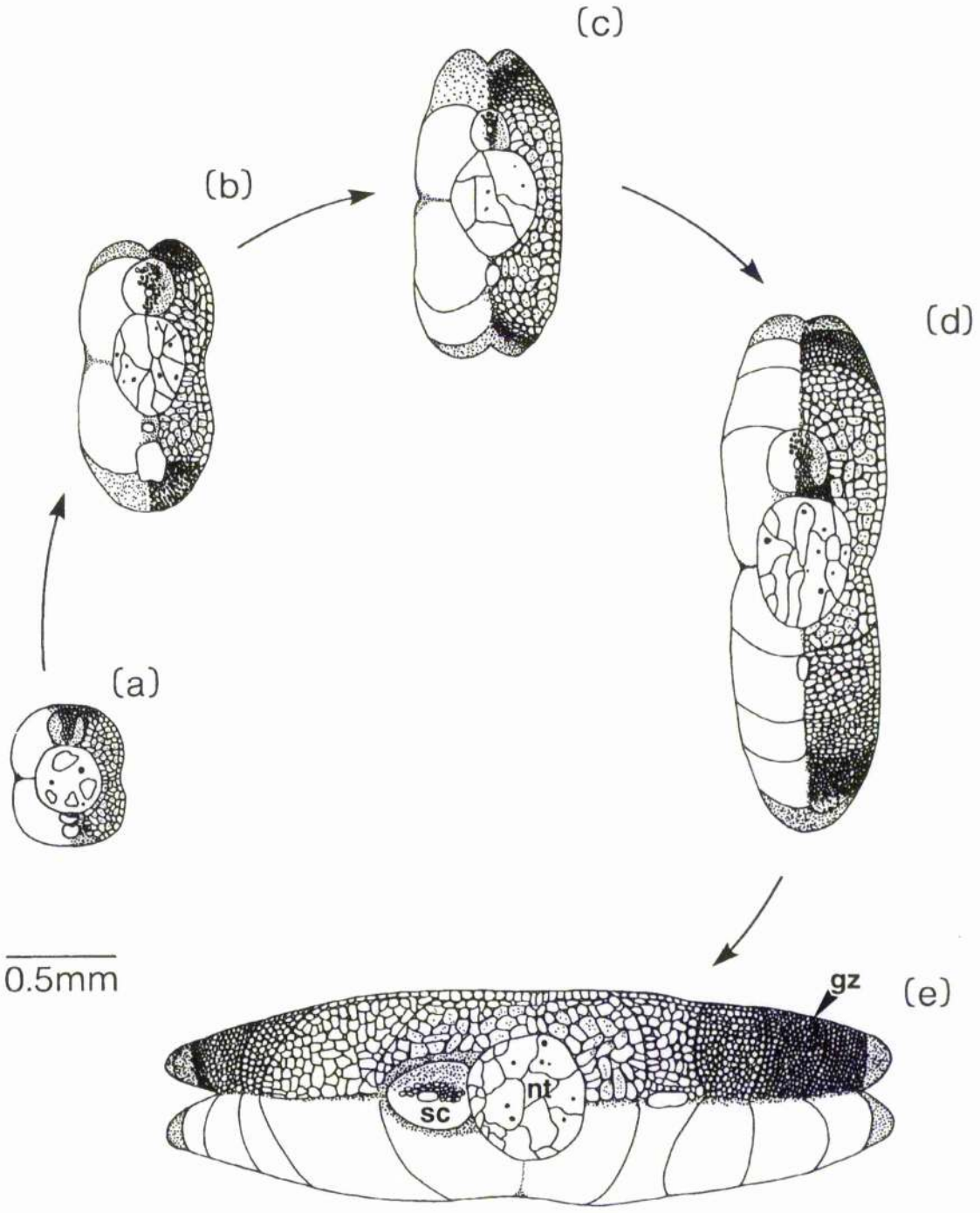


Figure 6. Diagram showing the development of the trunk musculature of plaice larvae between hatching and metamorphosis. (Drawn from semi-thin transverse sections stained with toluine blue).

(a) Section through larva at hatching. (b) Larva aged two weeks. (c) Larva at 5 weeks post hatching. (d) Larva at 7 weeks post hatching. (e) 10 week old juvenile after the completion of metamorphosis.

nt: notochord; sc: spinal cord; gz: germinal zone.

of the body. At 5 weeks, muscle fibres extend dorsal to the spinal cord but there has been little or no change to the central region of the fish since hatching (Fig. 7 and 8b). Smaller fibres were concentrated in zones adjacent to the dorsal and ventral fin muscle insertions (Fig. 8b), at the body apexes. These germinal zones consisted of numerous undifferentiated myoblasts (Fig. 8a), identifiable by a large, heterochromatic nucleus, abundant free ribosomes in the cytoplasm and scanty rough-surfaced endoplasmic reticulum, as described by Nag and Nursall (1972). Scattered amongst the myoblasts were very small diameter (< 5μm) muscle fibres which showed free contractile filaments in addition to the early stages of myofibril synthesis (Fig. 8a). Newly hatched larvae had a few undifferentiated myoblasts within the myotome, present at the interface of the superficial and inner fibre layers (Fig. 9b) and throughout the inner fibres (Fig. 9c). None of the cells described here as myoblasts were located between the basal lamina and the sarcolemma of the muscle fibres, which characterises a myosatellite cell.

The hypo- and epi-axial regions of densely packed myoblasts and differentiating muscle fibres first became visible at one week after hatching (Fig. 6), and were still present in fish that had undergone metamorphosis (Fig. 10), although the zones were much smaller than those of the younger fish (Fig. 8b). Between the skin and the superficial fibres there was a discontinuous layer of external cells (Fig. 9a) similar to those present in sea bass larvae (Veggetti *et al.* 1990). The external cells were very similar in appearance to the myoblasts but contained no contractile filaments, remaining unchanged in appearance throughout the larval period. Transverse wax sections from fish at metamorphosis (Fig. 10) showed a gradient of fibre sizes, the largest muscle fibres surrounding the notochord and the smallest at the body apices.

Figure 7. Plaice larva five weeks post hatching. Transverse wax section stained with Haematoxylin and eosin.
gz: germinal zone; if: inner muscle fibres; sc: spinal cord; sf: superficial muscle fibres; sk: skin; nt: notochord.

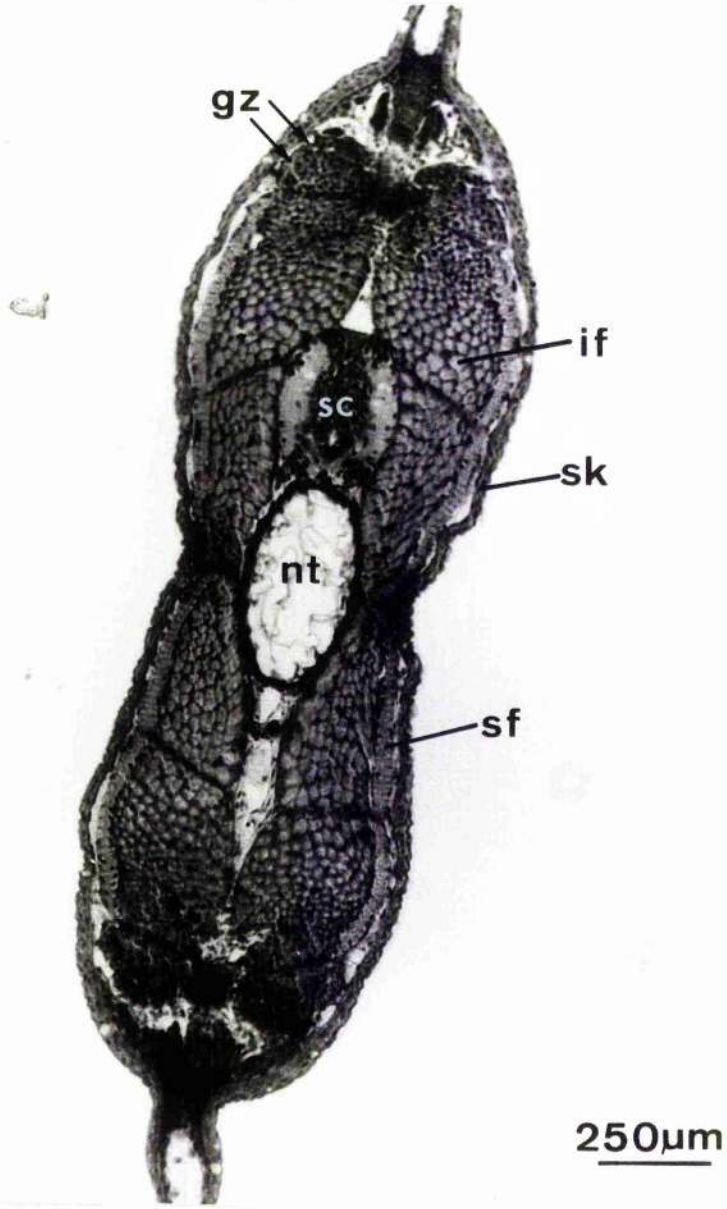
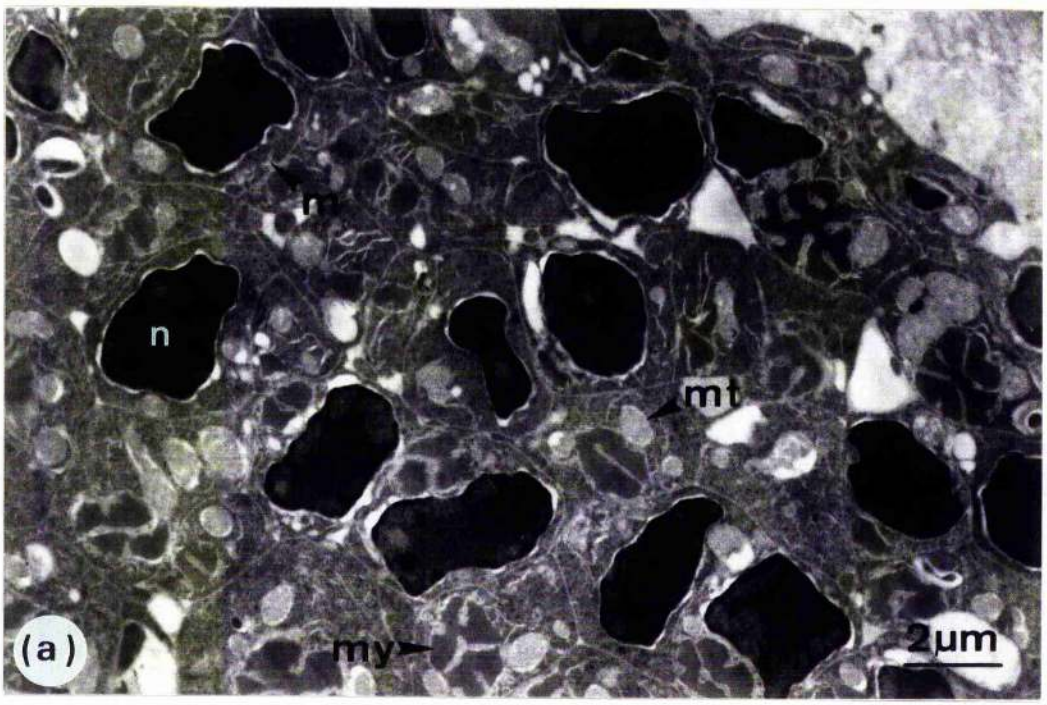

gz
if
sc
sk
nt
sf
250µm

Figure 8. (a) Electron micrograph of the germinal zone from a plaice larva at five weeks post hatching. The region contains both small muscle fibres and undifferentiated myoblasts.
(b) Dorsal myotomes of a five week old larva, showing the germinal zone adjacent to the dorsal fin.
gz: germinal zone; if: inner muscle fibres; m: myoblast; mt: mitochondria; my: myofibrils; n: nucleus; sc: spinal cord; sf: superficial muscle fibres; sk: skin.


n
n
mt
my
(a)
2µm

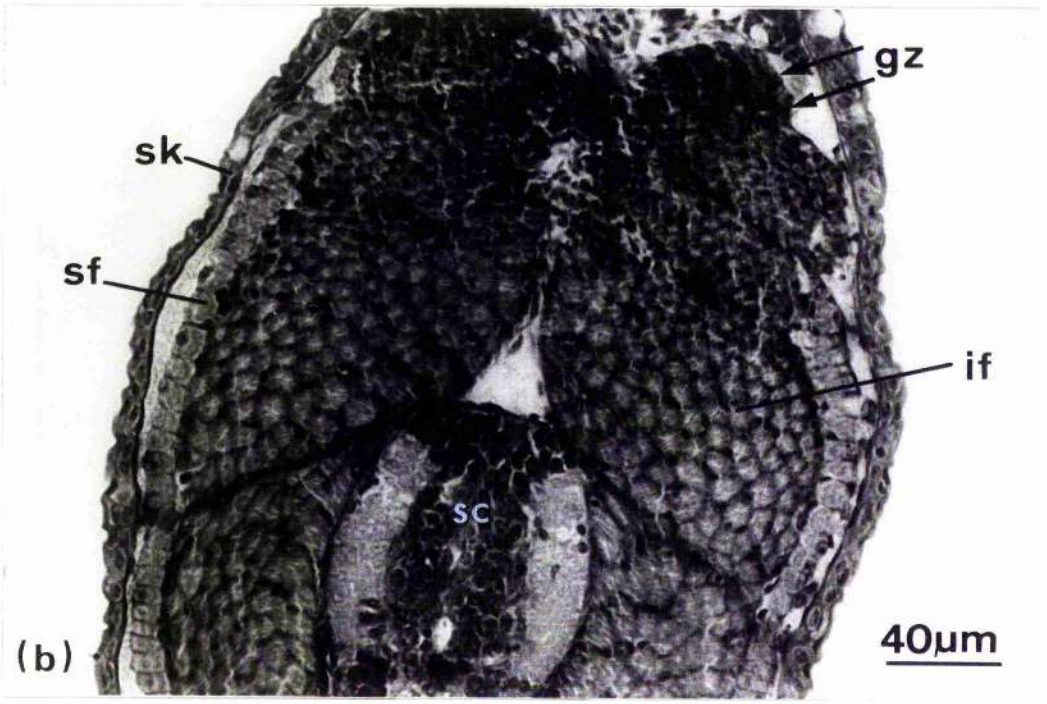

gz
sk
sf
if
sc
(b)
40µm

Figure 9. Transverse ultra-thin sections through newly hatched plaice larvae. (a) External cells forming an interrupted layer adjacent to the superficial muscle fibres, beneath the skin and the glycoprotein layer.
(b) Undifferentiated myoblast lying between the superficial and the inner muscle fibres.
(c) Undifferentiated myoblast completely surrounded by inner muscle fibres.
ec: external cells; gp: glycoprotein layer; m: myoblast; mt: mitochondria; my: myofibrils; n: nucleus; sf: superficial muscle fibre; sk: skin.

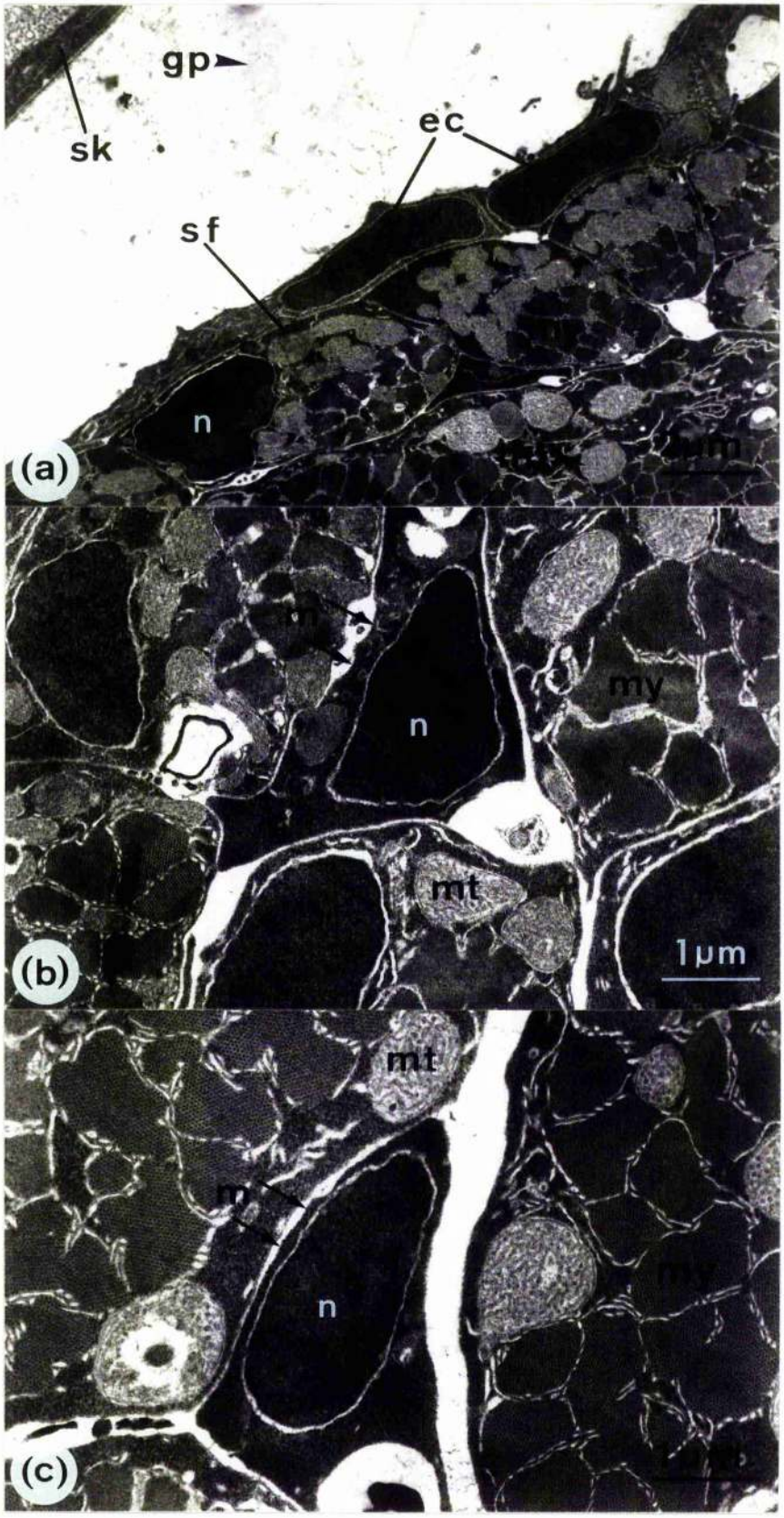
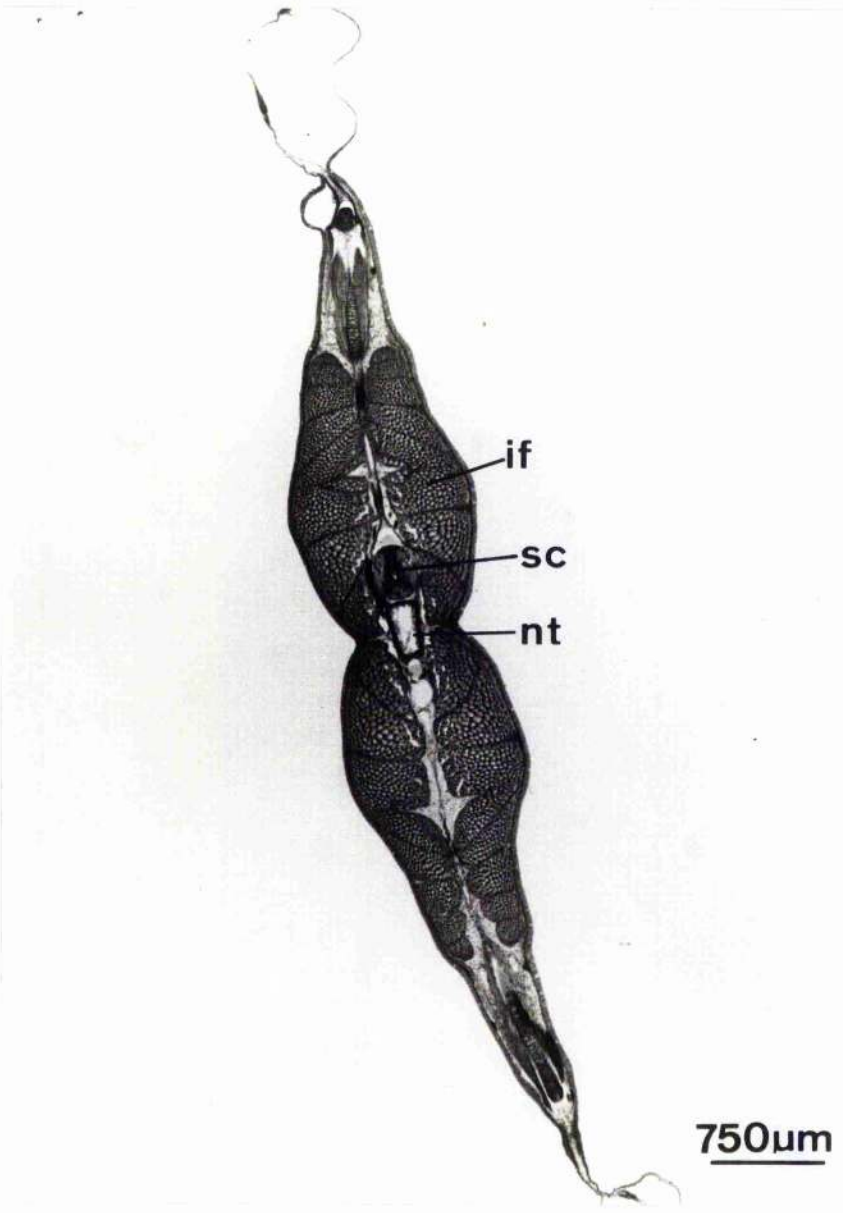

gp
sk
ec
sf
n
(a)
n
n
my
mt
(b)
1μm
mt
m
n
my
(c)

Figure 10. Transverse section through a ten week old juvenile after the completion of metamorphosis. Wax section stained with Haematoxylin and eosin.
if: inner muscle fibres; nt: notochord; sc: spinal cord.


if
sc
nt
750µm

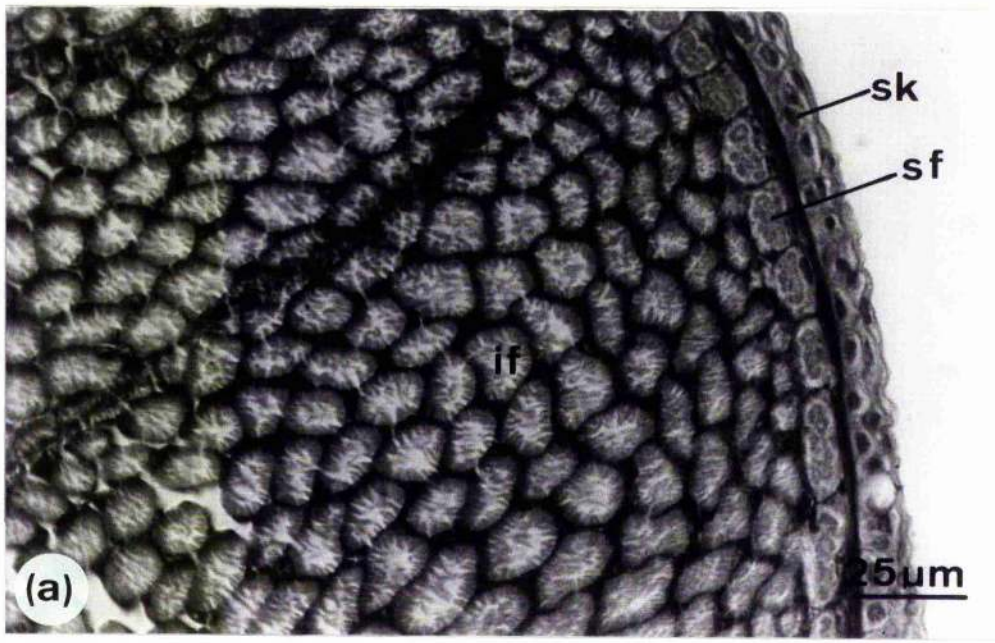
After metamorphosis, although the fish had the physical appearance of adults they did not as yet have the different, histochemically distinct adult fibre types (Fig. 11). Cryosections of metamorphosed fish stained with nitroblue tetrazolium to show succinic dehydrogenase activity revealed the presence of two muscle fibre types;- a single layer of densely stained fibres directly under the skin, surrounding a core of larger, unstained inner fibres (Fig. 11b).

The adult distribution of red muscle fibres did not begin to develop until fish reached a length of 25 mm. 0-group wild juveniles no longer possessed a single layer of densely stained superficial muscle fibres. In the lateral line region (Fig. 12a) and at various places beneath the skin (Fig. 12b), the superficial muscle layer was several fibres deep.

Histochemical Analysis of Adult Fibre Types

At least five distinct fibre typres could be identified in the adult fish following staining for myofibrillar ATPase (Fig. 13a.). Directly beneath the skin there were fibres which stained weakly for all three of the stains used; SDHase, myofibrillar ATPase and PAS. These weakly staining fibres were smaller in diameter than any of the others present in the myotome and are analogous to the tonic fibres described in a range of teleosts (Johnston 1981a, Kilarski and Kozlowska 1983). The tonic fibres formed an interrupted layer up to four fibres deep between the skin and the adjacent layer of muscle fibres (Fig. 13b and f). Red muscle fibres stained heavily for myosin ATPase, without pre-incubation (not shown) and following 2 minutes alkaline preincubation pH 10.4, (Fig. 13b). Red fibres were also heavily stained with nitroblue tetrazolium (Fig. 13e), a marker for mitochondria. Red muscle fibres also have greater glycogen reserves than other fibre types present in the myotome, staining more intensely with Schiff's reagent (Fig. 13f).

Figure 11. Transverse sections through the myotomes of juvenile plaice after the completion of metamorphosis.
(a) Wax section stained with Haematoxylin and eosin.
(b) Cryosection stained with nitroblue tetrazolium.
if: inner muscle fibres; sf: superficial muscle fibres; sk: skin.


sk
sf
if
(a)
25um

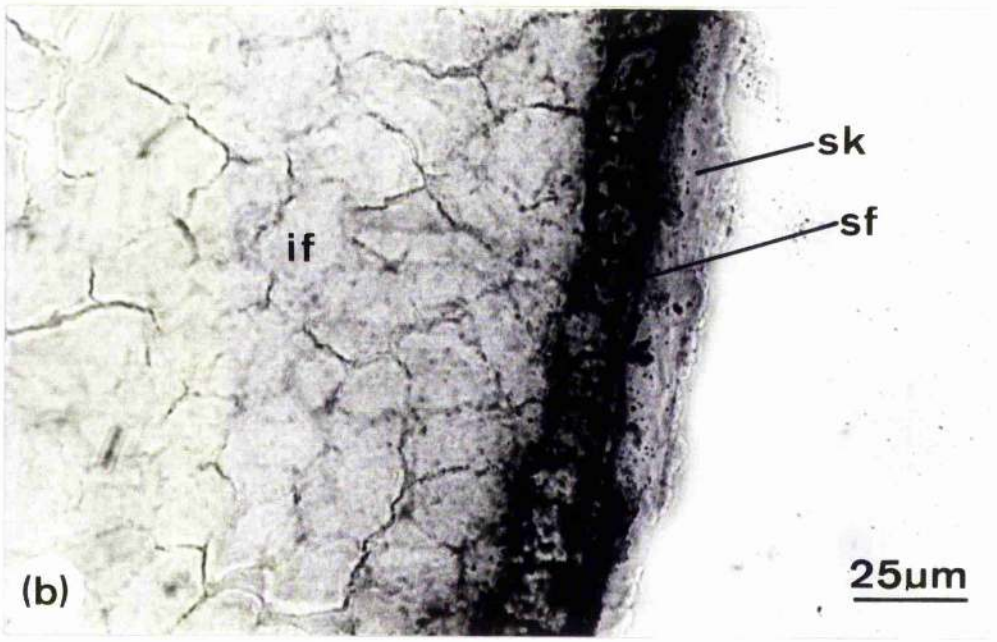
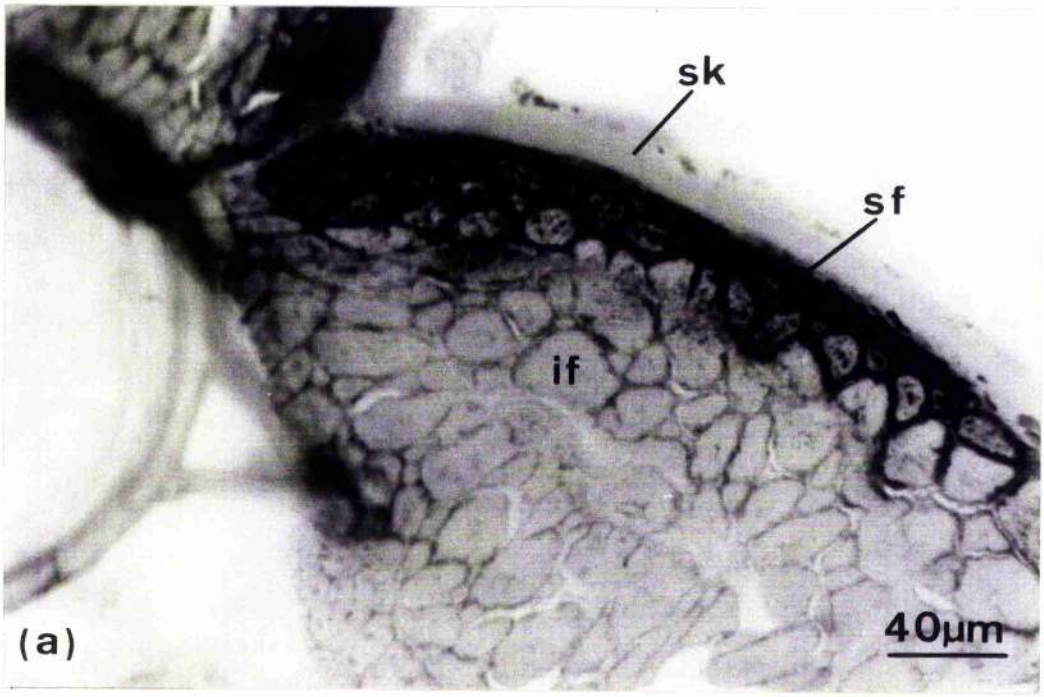

sk
sf
if
(b)
25µm

Figure 12. Transverse frozen sections through wild 0-group plaice showing the thickening of the superficial fibre layer. Sections were stained with nitroblue tetrazolium.
(a) Lateral line region.
(b) Dorsal surface, between the lateral line and the dorsal fin.
if: inner muscle fibres; sf: superficial muscle fibres; sk: skin.


sk
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40µm

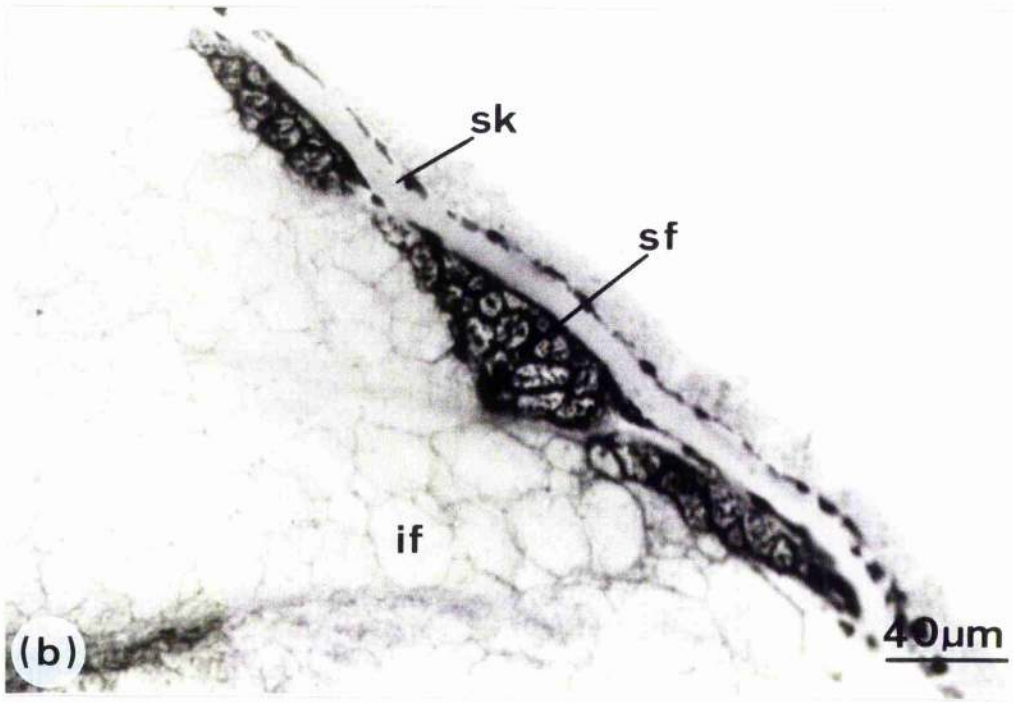
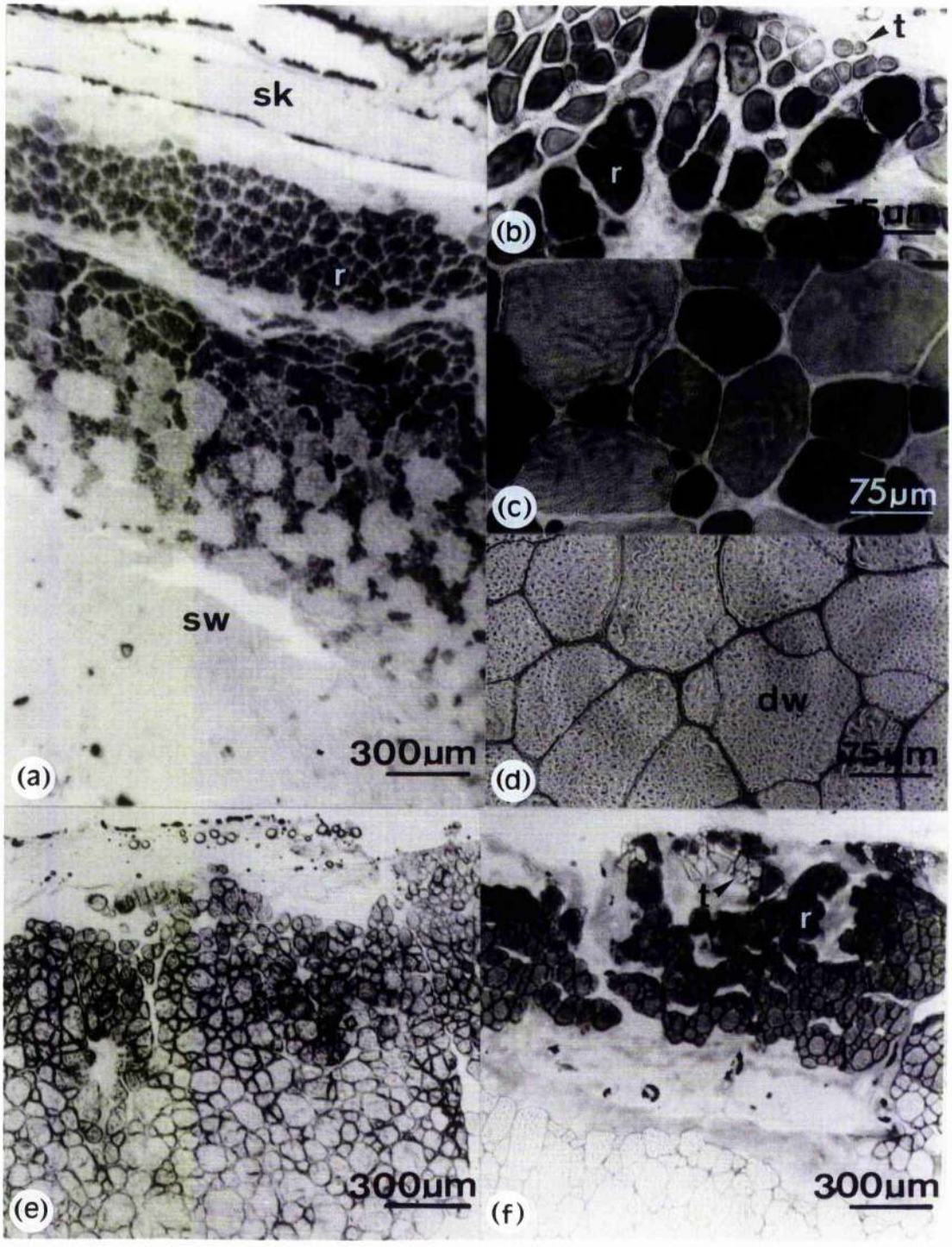

sk
sf
if
(b)
40µm

Figure 13. Transverse cryosections through adult myotomal muscle fibres.
(a) Muscle fibres in the lateral line region stained to show mATPase activity.
(b) Tonic and red muscle fibres stained for mATPase.
(c) Superficial white fibres stained for mATPase.
(d) Deep white muscle fibres.
(e) Adult muscle fibres stained with nitroblue tetrazolium to show SDHase activity.
(f) Adult muscle fibres stained with PAS.
dw: deep white fibres; r: red muscle fibres; sk: skin; sw: superficial white fibres; t: tonic fibres.


sk
r
sw
300µm
(a)
t
r
(b)
(c)
75µm
dw
(d)
(e)
300µm
t
r
300µm
(f)

Beneath the densely stained red fibres was an intermediate zone of large and small diameter fibres. The small diameter fibres stained more intensely for myosin ATPase and succinic dehydrogenase than the larger fibres surrounding them, giving the intermediate muscle zone a mosaic appearance.

Deep white fibres formed the bulk of the myotomal muscle (Fig. 13a), staining heavily for myosin ATPase without preincubation (not shown). Preincubation at alkaline pH (10.4 for 2 minutes) resulted in the inactivation of the white muscle fibres (Fig. 13d). The white fibres stained relatively weakly for SDHase and PAS and so contained fewer mitochondria and less stored glycogen than the red fibres.

Discussion

Newly hatched plaice larvae are not only very different in appearance from the adult fish, they have a different arrangement of muscle fibre types. More than four fibre types have been identified in the myotomes of adult plaice (Johnston 1981a), whereas only two fibre types can be distinguished in the larvae. The superficial fibres are thought to represent a presumptive red fibre type and the inner fibres a presumptive white fibre type (Veggetti *et al.* 1990). The superficial fibres have a higher volume density of mitochondria (24-33%) than the adult red fibres (24.5%) but a lower volume density of myofibrils, 22-29% compared to 58.6%. Larval inner fibres contain higher volume densities of mitochondria (7-8%) than adult white muscle fibres (2%)(Johnston 1981a). White fibres from adult plaice, however, have a higher volume density of myofibrils (85.7%) than inner muscle fibres from larvae (35-61%), (Johnston 1981a).

When plaice larvae hatch out of the egg they have only rudimentary gill arches (Holliday and Pattie Jones 1967). Respiratory gas exchange takes place across the whole body surface, via the skin and superficial muscle fibre layer (Batty 1984, El Fiky and Wieser 1988). At hatching Vv(mit,f) was significantly higher in the superficial muscle fibres of plaice larvae reared at 15°C than 5°C or 10°C (Table 2). This may reflect a relatively high routine metabolic rate and level of spontaneous activity at 15°C which would reduce the energy available for growth and may provide at least part of the explanation for the poor survival of larvae at this temperature. The Vv(mit,f) of both the superficial muscle fibres (24-33%) and the inner muscle fibres (7-8%) of newly hatched plaice larvae is considerably lower than that of other teleost larvae. The volume density of mitochondria in the superficial muscle fibres of newly hatched herring larvae is within the range 38-46% compared with 16-26% for the inner muscle fibres (Vieira and Johnston 1992). The high volume densities of muscle

mitochondria found in herring larvae may be a reflection of their high level of spontaneous swimming activity. Plaice larvae are far less active during the yolk-sac stage, spending long periods floating motionless in the water column.

Rearing temperature has previously been found to affect myogenesis during the embryonic stage of both the herring, *Clupea harengus,* (Vieira and Johnston 1992), and the Atlantic salmon, *Salmo salar*, (Stickland *et al.* 1988). In plaice, the number of inner muscle fibres in newly hatched larvae tended to increase with temperature and was significantly higher at 15°C than 5°C and 8°C ($p < 0.05$; Fig. 3a), which is similar to the response obtained for herring (Vieira and Johnston 1992). By contrast Stickland *et al.* (1988) found that larvae of Atlantic salmon (*Salmo salar*) reared at 10°C had fewer inner muscle fibres of larger mean cross-sectional area, than larvae reared at 2°C. However, in another study on the same species both muscle fibre number and cross-sectional area were found to be positively correlated with incubation temperature (Johnston *et al.* personal communication). The effects of temperature on muscle cellularity have also been shown to vary between spawning groups in Atlantic herring (Johnston 1993). Variations in muscle fibre number and diameter with temperature could arise if cell division and protein deposition have different temperature dependencies. Factors other than temperature might also influence these parameters, accounting for at least some of the variation observed between spawning groups and species. One such factor which is known to affect growth and differentiation is egg size which varies both between and within herring spawning groups (Blaxter and Hempel 1963). In chum salmon, *Oncorhynchus keta*, early spawning stocks have larger eggs, later fry emergence times and poorer low temperature survival of embryos than later spawning stocks (Beacham and Murray 1987). The efficiency with which yolk was deposited into body tissue also varied between chum salmon stocks, indicating the importance of

genetic as well as environmental factors in controlling the pace and nature of tissue differentiation.

The way in which the ultrastructural differences are related to the performance of the muscle fibres is unclear. Batty *et al.* (1993) found that the test temperature but not the rearing temperatures affected the maximum escape swimming speed of one day old plaice and herring larvae. This might be expected as at each rearing temperature, except for plaice larvae reared at 15°C, the total cross-sectional area of muscle fibres in each myotome was similar (Vieira and Johnston 1992, Table 1). It was not possible to determine the escape velocity of plaice larvae reared at 15°C as too few hatched. The maximum escape swimming speeds of plaice larvae were tested at temperatures of 5°, 8° and 12°C (all larvae had been reared at 8°C). Tail beat frequency was found to increase at higher trial temperatures while C-start contraction time was found to decrease with increasing temperature (Batty and Blaxter 1992). The contraction of larval muscle fibres appears to be affected by temperature in the same way as the adult white muscle fibres. Wardle (1980) found that the contraction time of plaice muscle depended upon the ambient temperature and not upon the adaptation temperature.

Although ambient seawater temperature does apparently influence the differentiation of muscle tissues in embryos of the plaice, *Pleuronectes platessa*, how these changes affect larval survival or influence behaviour are unclear. Changes in muscle fibre number and ultrastructure could possibly influence growth rate (Weatherly and Gill 1985) or indirectly affect the energy budget (Wieser and Medgyesy 1990).

Muscle growth and development in larvae and juveniles

In plaice, during the period from hatching to metamorphosis, muscle growth occurs not only by hypertrophy of the existing fibres but also by the recruitment of new fibres from specific

germinal regions, similar to those observed in the larvae of the sea bass, *Dicentrarchus labrax,* (Veggetti *et al.* 1990). Myotomal growth throughout the larval period occurs mainly in the dorso-ventral direction. At the hypo- and epi-axial extremities of the myotome there appear to be germinal zones of undifferentiated muscle fibres which are small in newly hatched larvae, gradually becoming visible even with the light microscope at approximately 2 weeks of age and then apparently becoming less active once larvae have metamorphosed. The differentiation of the cells in the germinal zones of plaice larvae appeared similar to descriptions of muscle fibre formation in the embryos of other teleosts (Yamamoto 1965, Waterman 1969). Initially the cells are fairly undifferentiated, containing a large nucleus, with mitochondria and numerous ribosomes in the scanty cytoplasm. Myofilaments begin to appear in organised clumps in the cytopasm, gradually coming together and forming myofibrils. Veggetti *et al.* (1990) suggested that fibre generation occurred in two ways. During the first half of larval life the majority of new fibres are recruited from the hypo- and epi-axial germinal zones. Throughout the second period of the larval stage, new fibre generation continued by the differentiation of morphologically undifferentiated cells lying between the large inner fibres and by activation of satellite cells. New inner fibres were also derived from presumptive myoblasts, present in early stages, lying between the inner and superficial fibres. Apart from the extreme dorsal and ventral zones of the myotome, plaice larvae do not appear to have such clearly defined germinal zones. Morphologically undifferentiated cells similar in appearance to myoblasts and myosatellite cells, but without a basal lamina, were observed lying amongst the inner fibres and between the superficial and inner muscle fibre layers. Koumans *et al.* (1991), found that in carp smaller than 2.5 cm in length myosatellite cells lacked a continuous basal lamina. Perhaps as observed in carp, the myosatellite cells of plaice larvae do not become surrounded by a continuous muscle basal lamina until reaching a certain length. From hatching to

metamorphosis the undifferentiated cells within the central zone of the trunk appeared to remain unchanged. Any increase in myotomal thickness was achieved by growth of the existing muscle fibres and not by the recruitment of undifferentiated myoblasts.

Plaice larvae, like other teleost larvae, also possess an external cell layer in close contact with the superficial fibres, beneath the epidermis. Waterman (1969) suggested that the external cells were probably involved with the formation of the epidermal basement membrane, while Veggetti *et al.* (1990) suggested that new superficial fibres are derived from the undifferentiated external cells. In the plaice, the arrangement of the superficial fibres remained unaltered throughout the larval period; no changes were observed in the external cells at metamorphosis, though the skin thickened and developed considerably. If the external cells were involved in dermatome formation it is likely that some evidence of this would have been observed at metamorphosis when there is considerable thickening of the epidermis and an increase in the amount of connective tissue and the number of pigment cells present (Holliday and Pattie Jones 1967).

At metamorphosis, plaice still retained the distribution of superficial and inner muscle fibres characteristic of larvae. Although the superficial muscle fibres increased in size throughout the larval period, at metamorphosis they were still present as a single layer surrounding the inner muscle fibres. A close relationship has previously been observed between the differentiation of gill structures and the rate at which the larval superficial muscle layer disappears (El-Fiky and Wieser 1988, El-Fiky *et al.* 1987, Batty 1984). During the larval period of various cyprinid species the metabolism of the swimming muscles was found to be entirely aerobic whilst respiratory gas exchange was still cutaneous. Together with the development of the gills there was a corresponding increase in the glycolytic capacity of the inner muscles (El-

Fiky and Wieser 1988). Delaying the start of their free swimming existence suppressed gill development and prevented the disappearance of the superficial muscle fibre layer. In herring larvae the superficial muscle fibres develop the adult distribution of the red fibres only after the gills and circulation become fully functional (Batty 1984). De Silva (1974) found that plaice larvae at metamorphosis have well developed gills and a functional circulatory system, in which both erythrocytes and leucocytes could be distinguished. The rate of haemoglobin synthesis was reduced following metamorphosis; however filament number and filament length continued to increase (de Silva 1973). In plaice larvae, any relationship between the differentiation of the gills and the rate at which larval superficial fibres differentiate into the adult red muscle layer is not as clearly defined as in the other teleost larvae studied. The fibre types distinguished in the myotomes of adult plaice differentiate after metamorphosis, during the juvenile phase of the lifecycle. Thickening of the superficial muscle fibre layer was first evident in both laboratory reared and wild caught plaice (total length = 26-30 mm), 4-5 months after hatching, but it was not yet possible to distinguish any tonic or intermediate/superficial white muscle fibres in fish this size. Exactly how the differentiation of the larval muscle fibre types into those of the adult plaice affects swimming performance is unclear. Williams and Brown (1992) reported in another flatfish species, the winter flounder, *Pleuronectes americanus,* that metamorphosis does not result in any decrease in the average speed or duration of escape responses to attacks by predatory amphipods.

Histochemical Identification of Adult Fibre Types

At least five fibre types were identified in the myotomes of adult plaice on the basis of their histochemistry. Tonic fibres have been previously identified in plaice (Johnston 1981a), occurring as an interrupted layer beneath the skin

approximately two to five cells deep. Plaice tonic fibres stained only weakly for all three of the stains used, resembling those of amphibians and reptiles which may play a role in stabilising joints (Johnston 1985b). Kilarski and Kozlowska (1987), suggested that in teleost species which frequently remain suspended motionless in water or resting on the bottom tonic fibres may play a significant part in maintaining body posture. Flume experiments (Arnold and Weihs 1978) have shown that adult plaice usually lie flat, with the whole ventral surface touching the bottom when in still water. Fish exposed to currents of 20-30 cm/s gradually developed an 'arched back' posture. A possible role for the tonic fibres could therefore be to assist in the production of this posture.

The red muscle fibres were arranged in a thin lateral strip constituting only 5-7% of the total musculature (Johnston 1981a). Red fibres stained intensely for SDHase and PAS; in addition, biochemical measurements of Mg^{2+}, Ca^{2+} myofibrillar ATPase in plaice (Johnston 1973) have shown red fibres to have only 32% of the activity of the white fibres. In this study the red fibres of plaice appeared to have an alkali-stable myosin ATPase activity silmilar to that reported for juvenile turbot (Calvo and Johnston 1992). The red muscle fibres of most teleost species have been found to be alkali-labile (Kilarski 1990, Mosse and Hudson 1977, Gill *et al.* 1982, Gill *et al.* 1989, Johnston *et al.* 1974). Only in the stickleback (Kilarski and Kozlowska 1983) are the red fibres apparently absent, with an alkali-stable 'intermediate' fibre type occupying that position in the myotome. Fibres characterised by an alkali-stable (pH 10.4) myofibrillar ATPase activity have been found in some species forming a narrow band between the red and white muscle zones (Johnston *et al.* 1974), these have been termed fast red or pink muscle fibres (Johnston 1983a). Fast red fibres contain large amounts of glycolytic enzymes and their aerobic capacity and myofibrillar ATPase activity is

intermediate between slow red and white muscle fibres (Johnston *et al.* 1977, Kryvi *et al.* 1981).

The transitional zone of muscle fibres between the red and white muscle areas consists of a range of fibre sizes, smaller fibres within this area tend to stain more intensely than the larger fibres, giving a characteristic mosaic appearance. The fibres in this so-called transitional zone have been described as intermediate (Zawadowska and Kilarski 1983, Johnston 1981b, Bone 1978) or pink fibres (Rowlerson *et al.* 1985, Scapolo and Rowlerson 1987, Carpene *et al.* 1982). In carp, electromyographical studies have shown that the order of recruitment is red > pink > white muscle fibres with increasing swimming speed (Johnston *et al.* 1977). The appearance of the zone of intermediate fibres tends to vary with species. It has been suggested that the intermediate fibres constitute an undifferentiated stock of precursor fibres, able to develop into either red or white fibre types (Bone 1966, Mosse and Hudson 1977). The intermediate fibres have also been associated with sustained swimming ability (Patterson *et al.* 1975). Kilarski (1990) considered that intermediate muscle fibres were recruited for swimming speeds slightly faster than slow cruising in the roach. In some species, the pink muscle is clearly a mosaic of red and white fibres (Rowlerson *et al.* 1985).

The bulk of plaice myotomes consists of white muscle fibres, these have a higher myofibrillar ATPase activity (Johnston, 1973) but contain fewer mitochondria (2.2%) than the red muscle fibres (Johnston 1981a). Plaice white muscle fibres utilise anaerobic metabolic pathways with glycogen as the principal fuel (Wardle 1978). White muscle is generally considered to provide the main propulsive force at high burst swimming speeds in most teleost species (Hudson 1973, Mosse and Hudson 1977, Bone 1978, Johnston 1981b). However, the white muscle fibres of plaice are multi-terminally innervated and electromyographical studies have shown that in such

species white fibres are also recruited for sustained activity, particularly when cruising at higher speeds (Hudson 1973, Johnston *et al.* 1977, Bone *et al.* 1978, Johnston and Moon 1980).

Chapter 5

Electrophoretic analysis of red and white muscle myofibrillar proteins from adult plaice, *Pleuronectes platessa* L.

Introduction

Each of the muscle fibre types present in the myotomes of teleosts have been found to differ both in myosin heavy chain sub-unit composition and in the isoforms of the myosin light chains present (Rowlerson *et al.* 1985). Scapolo and Rowlerson (1987), Karasinski and Kilarski (1989) and Johnston *et al.* (1990), all demonstrated that myosin heavy chain isoform may vary with fibre type in fish skeletal muscle. Cloning studies indicate that common carp possess at least twenty eight myosin heavy chain genes (Gerlach *et al.* 1990). The light chain compositions of vertebrate myosins are also characteristic of fibre phenotype (Lowey and Risbey 1971) and so it is possible to further distinguish fibre types by their myosin light chain sub-unit composition (Rowlerson *et al.* 1985, Scapolo and Rowlerson 1987). Studies on various teleost species have shown a general pattern of two myosin light chains specific to red muscle fibres and three myosin light chains specific to white muscle fibres (Langfeld *et al.* 1991, Rowlerson *et al.* 1985, Focant *et al.* 1976).

A minimum of five muscle fibre types have been identified in the myotomes of adult plaice (Johnston 1981a, Chapter 4). Each fibre type may contain a different combination of contractile protein isoforms. The purpose of this study was to analyse the myofibrillar components of the two main muscle fibre types present in the myotomes of adult plaice, *Pleuronectes platessa*, using electrophoretic techniques. This information was essential to provide a basis of comparison when searching for possible developmental isoforms in larval and juvenile fibre types.

Materials and Methods

Plaice, *Pleuronectes platessa* L., mean standard length, 25.75 cm (S.D. ± 2.28, n = 10) and mean body mass, 268 g (S.D. ± 97, n = 10), were trawled from St Andrews Bay or the Firth of Clyde throughout the year. Fish were maintained in recirculating seawater aquaria at ambient temperature (5-15°C) and photoperiodic regime (12 hours light: 12 hours dark). Fish were fed a mixture of chopped mussels and raw minced fish muscle. Before experimentation fish were stunned by a blow to the head, pithed and decapitated. Red and white muscle fibres were dissected, on ice, from the dorsal side of the body. All samples and gels were prepared and run as described in Chapter 2.

Electrophoretic Analysis

White and red myofibrils were run on 13% SDS polyacrylamide gels (Laemmli 1970) together with molecular weight standards, Sigma MW-SDS-70L 14000-70000 kit. (Fig. 1). By plotting the log. molecular weight of the standard proteins against mobility relative to the Bromophenol Blue front it was possible to calculate the apparent relative molecular masses of the various myofibrillar components. The bands were subsequently identified by their characteristic migration on alkali-urea gels (Huriaux and Focant 1974), on SDS gels in the presence of urea (Sender 1971) and on two-dimensional PAGE (Rowlerson *et al.* 1985, Crockford 1989). The apparent molecular masses of the myofibrillar proteins are described in Table 1.

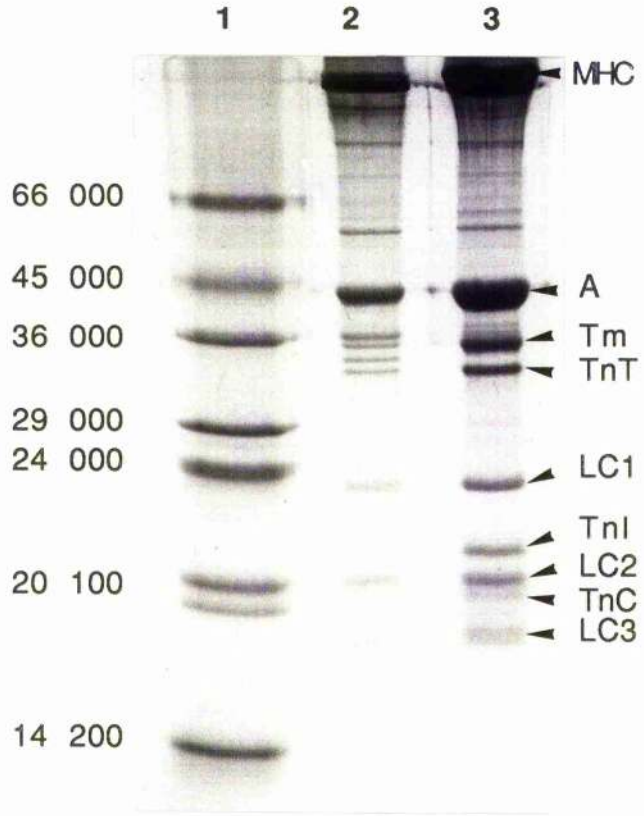


Figure 1. 13% SDS polyacrylamide gel of red and white muscle myofibrils from adult plaice, run together with molecular weight markers. Gel was stained with Coomassie blue G250.
Lane 1: molecular weight markers; Lane 2: red muscle myofibrils; Lane 3: white muscle myofibrils. MHC: myosin heavy chain; A: actin; Tm: tropomyosin; TnT: troponin T; LC1: myosin light chain 1; TnI: troponin I; LC2: myosin light chain 2; TnC: troponin C; LC3: myosin light chain 3.

Table 1

The apparent relative molecular masses of the myofibrillar components of red and white muscle fibres.

Protein	Red muscle	White muscle
Myosin heavy chain	200 000	200 000
Actin	44 000	44 000
Tropomyosin	40 000-39 000	39 000
Troponin T	38 000-36 000	36 000
Myosin light chain 1	29 500	29 500
Troponin I	23 000	23 000
Myosin light chain 2	21 500	21 000
Troponin C	20 500	20 500
Myosin light chain 3	18 000	18 000

Results

Identification of the Myofibrillar Components

The individual myofibrillar components were identified using a variety of techniques. Both myosin heavy chain and actin were characterised by their intense staining relative to other bands in the gel, they were identified on the basis of their unchanging apparent relative molecular masses when run on 13% SDS polyacrylamide gels (Crockford 1989).

Regulatory Proteins

In order to differentiate between tropomyosin and troponin T, SDS gels were run in the presence and absence of 8 M urea (Sender 1971). When urea is present tropomyosin runs with an apparent molecular weight greater than that of actin (Fig. 2). The apparent molecular weight of troponin T is unchanged in the presence of urea and it has a more basic pI than tropomyosin. Troponin T and troponin I are both well separated from the other myofibrillar proteins on non-equilibrating pH gradient electrophoresis (NEPHGE) IEF gels, both have basic isoelectric points whereas the rest of the myofibrillar proteins are acidic or neutral (Fig. 3a and b). Troponin I can be distinguished from troponin T on the second dimension SDS gels because it migrates with a significantly lower molecular mass (Wilkinson *et al.* 1984, Imai *et al.* 1986). It was difficult to ascertain whether isoforms of troponin T and troponin I present in red muscle were different from isoforms in white muscle. Both red and white muscle isoforms of troponin T and troponin I were more basic than the ampholytes currently available and had a tendency to run very close to the end of the IEF first dimension, sometimes right off the end of the tube gel. Troponin C was identified using its distinctive colour when stained with "Stains All" (Campbell *et al.* 1983) and also by its position on alkali-urea gels. Calcium binding proteins will stain blue with the cationic carbocyanine dye "Stains All"

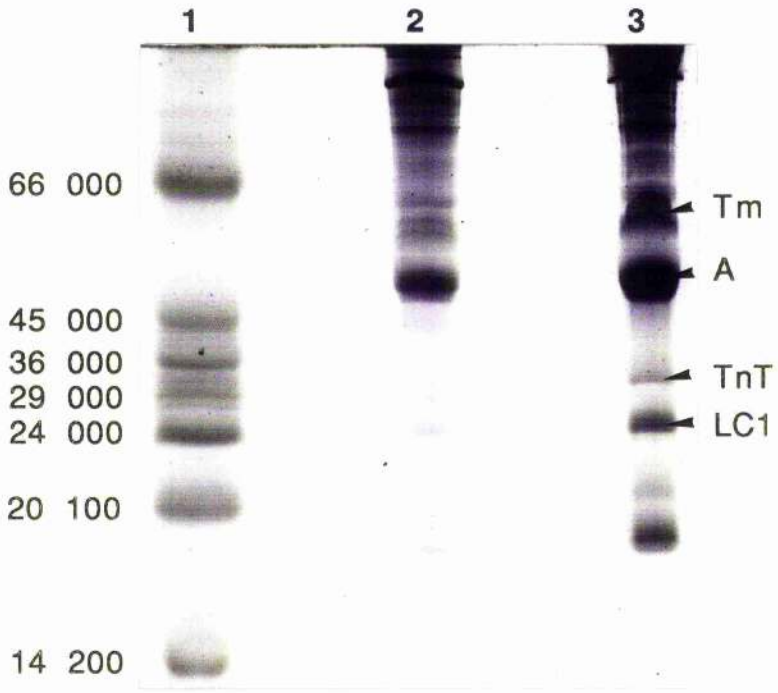
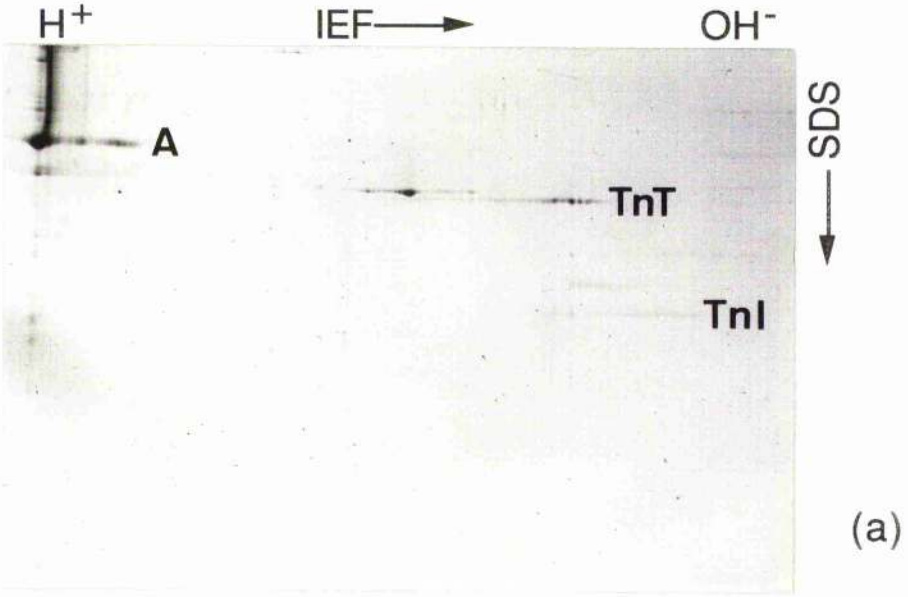


Figure 2. 8M urea, 13% SDS polyacrylamide gel of red and white muscle myofibrils from adult plaice, run together with molecular weight markers. Gel was stained with Coomassie blue G250.
Lane 1: molecular weight markers; Lane 2: red muscle myofibrils; Lane 3: white muscle myofibrils.
Tm: tropomyosin; A: actin; TnT: troponin T; LC1: myosin light chain 1.

Figure 3. Two-dimensional PAGE of basic myofibrillar proteins from adult muscle fibres. First dimension NEPHGE IEF, second dimension 15% SDS PAGE. Gels were stained with Coomassie blue G250.
(a) Red muscle fibres. (b) White muscle fibres.
A: actin; TnT: troponin T; TnI: troponin I.



(a)

(b)

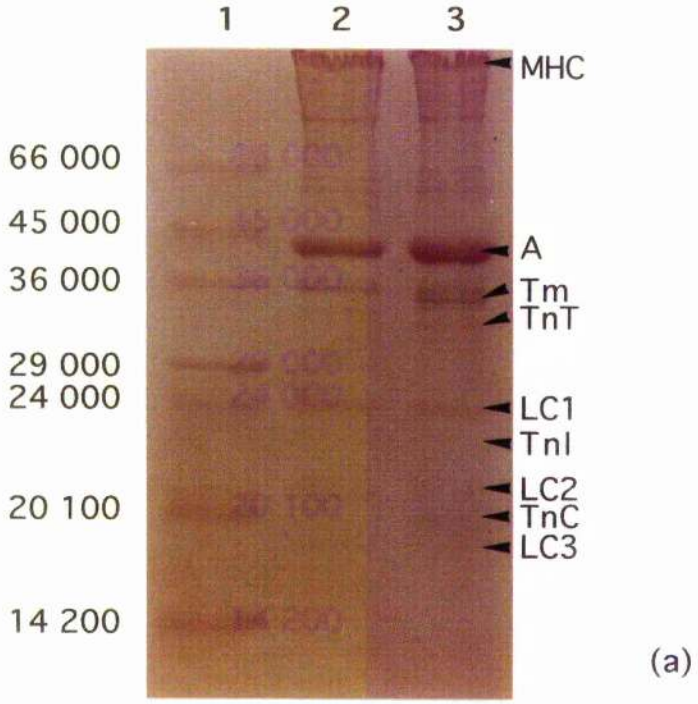
whereas other proteins will stain pink or red (Fig. 4a and b). The blue staining results from the interaction of individual dye molecules at ionic sites within the molecule.

Myosin Sub-unit Composition

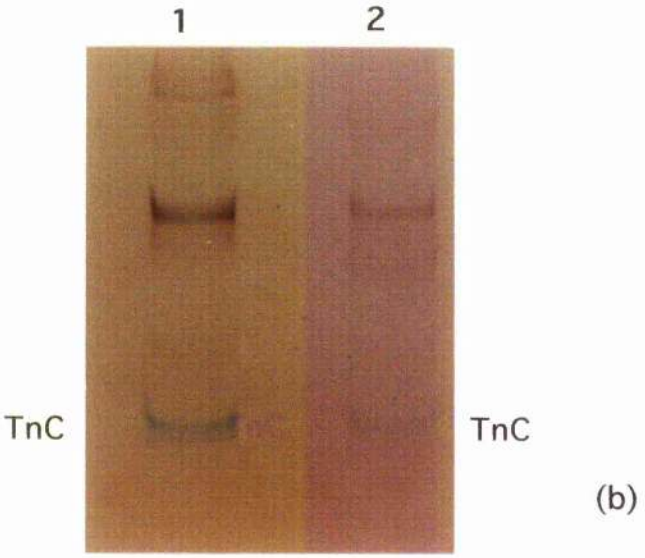
The first step in establishing the identities of myosin light chain 1, myosin light chain 2 and myosin light chain 3 was to isolate myosin from the rest of the myofibrillar components. Two methods were tried, since that of Huriaux and Focant (1990), originally used for the purification of myosin from eels, *Anguilla anguilla*, was found to be inadequate for the purification of plaice muscle myosin (Fig. 5a). At the end of the process actin and tropomyosin were still major contaminants (Fig. 5a). Method 2 (Giambalvo and Dreizen 1978), gave less contamination by actin but the buffers were not very effective at dissolving plaice myosin and yields were very low. The yield was improved by implementing several modifications taken from Margossian and Lowey's (1982) method of purifying myosin from rabbit skeletal muscle. It proved very difficult to obtain myosin completely free from any contaminants, but most of the thin filament proteins were removed (Fig. 5b and c). The relatively pure myosin was run on 10% alkali-urea gels, pH 8.9, in the absence of free calcium (Fig. 6). Huriaux and Focant (1974) have described in detail the use of alkali-urea gels in separating proteins according to their relative pI, and the expected mobility of the light chains. By using alkali-urea gels as a first dimension and transferring the separated bands to SDS gels it should be possible to distinguish light chain 1, light chain 2 and light chain 3 both by relative pI and molecular weight.

Red muscle myosin run on alkali-urea gels separated into more than three bands (Fig. 6). When these bands were transferred to SDS gels (Fig. 7) bands 3 and 4 appeared to correspond to light chain 1 (Fig. 7, lanes 4 and 5), band 5 to light chain 2 (Fig. 7, lane 8) and bands 6, 7 and 8 seemed to

Figure 4. (a) 13% SDS polyacrylamide gel of red and white muscle myofibrils from adult plaice, run together with molecular weight markers. Gel was stained with 'Stains All' Lane 1: molecular weight markers; Lane 2: red muscle myofibrils; Lane 3: white muscle myofibrils.
(b) 10% alkali-urea (pH 8.9) polyacrylamide gel of myofibrils from adult red and white muscle fibres. Gel was stained with 'Stains All'.
Lane 1: white muscle myofibrils; Lane 2: red muscle myofibrils.
MHC: myosin heavy chain; A: actin; Tm: tropomyosin; TnT: troponin T; LC1: myosin light chain 1; TnI: troponin I; LC2: myosin light chain 2; TnC: troponin C; LC3: myosin light chain 3.

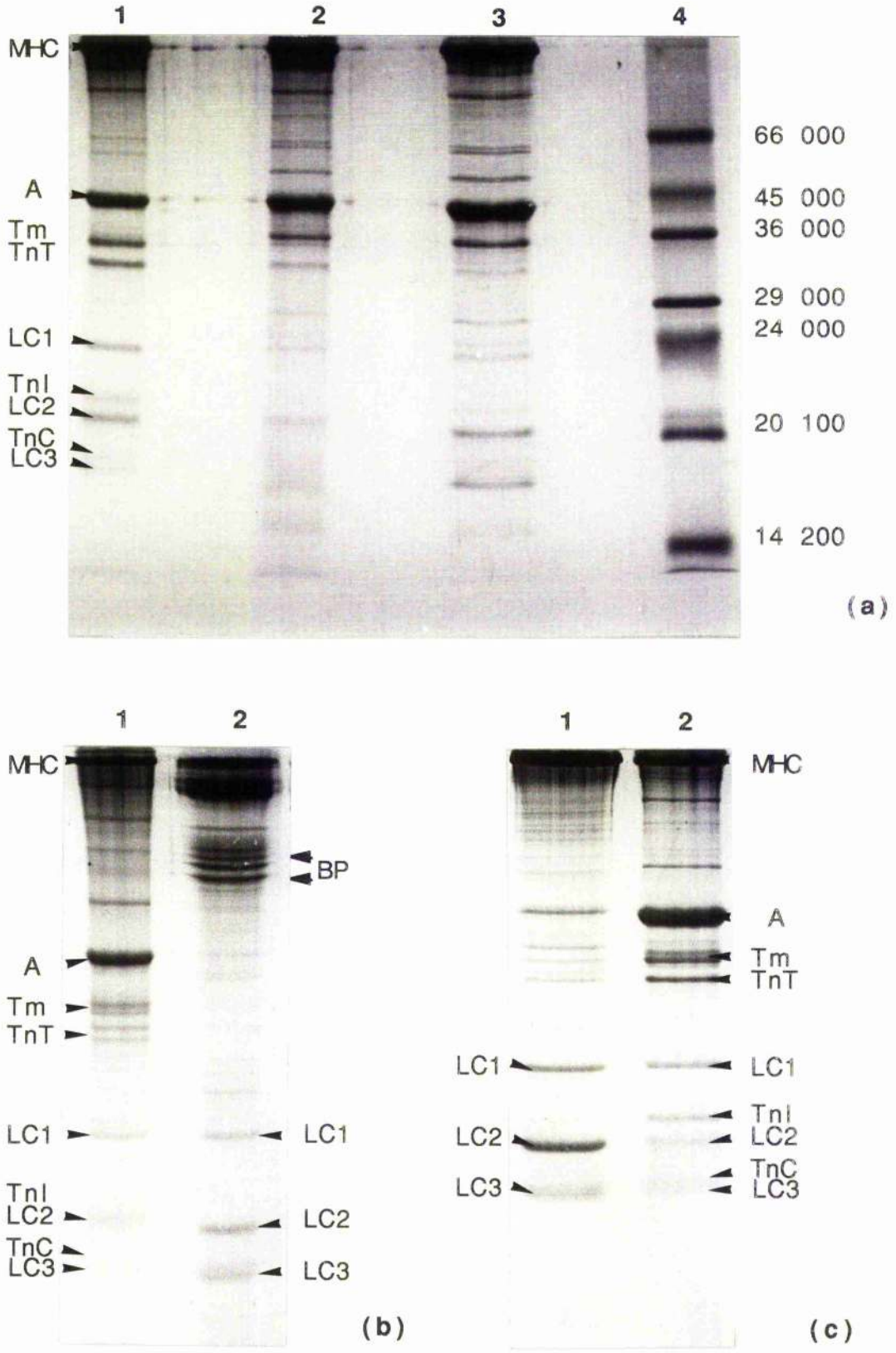

1
2
3
MHC
66 000
45 000
A
36 000
Tm
TnT
29 000
24 000
LC1
TnI
LC2
20 100
TnC
LC3
14 200

(a)


1
2
TnC
TnC

(b)

Figure 5. (a) 13% SDS polyacrylamide gel of 'myosin' prepared from white muscle using the method of Huriaux and Focant (1990). Gel was stained with Coomassie blue G250.
Lane 1: white muscle myofibrils; Lane 2: 'myosin' sample before dialysis; Lane 3: 'myosin' sample after dialysis; Lane 4: molecular weight markers.
(b) 13% SDS polyacrylamide gel of myosin purified from red muscle fibres using method 2. Gel was stained with Coomassie blue G250.
Lane 1: red muscle myofibrils; Lane 2: myosin purified from red muscle fibres.
(c) 15% SDS polyacrylamide gel of myosin purified from white muscle fibres using method 2. Gel was stained with Coomassie blue G250.
Lane 1: myosin purified from white muscle fibres; Lane 2: white muscle myofibrils.
MHC: myosin heavy chain; BP: breakdown products; A: actin; Tm: tropomyosin; TnT: troponin T; LC1: myosin light chain 1; TnI: troponin I; LC2: myosin light chain 2; TnC: troponin C; LC3: myosin light chain 3.


1
2
3
4
MHC
A
Tm
TnT
LC1
TnI
LC2
TnC
LC3
66 000
45 000
36 000
29 000
24 000
20 100
14 200
(a)
1
2
MHC
BP
A
Tm
TnT
LC1
TnI
LC2
TnC
LC3
LC1
LC2
LC3
(b)
1
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MHC
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TnI
LC2
TnC
LC3
(c)

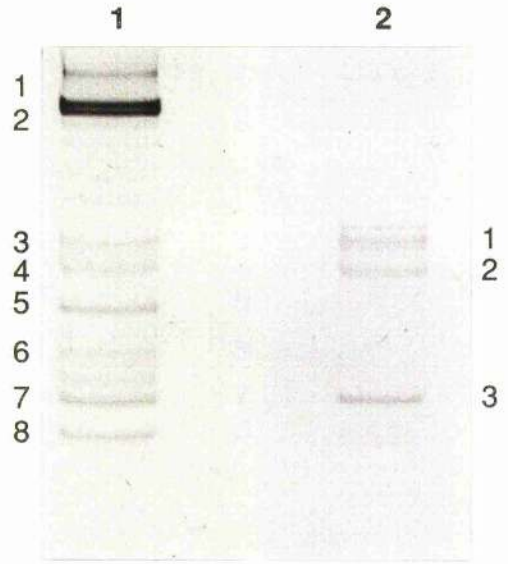
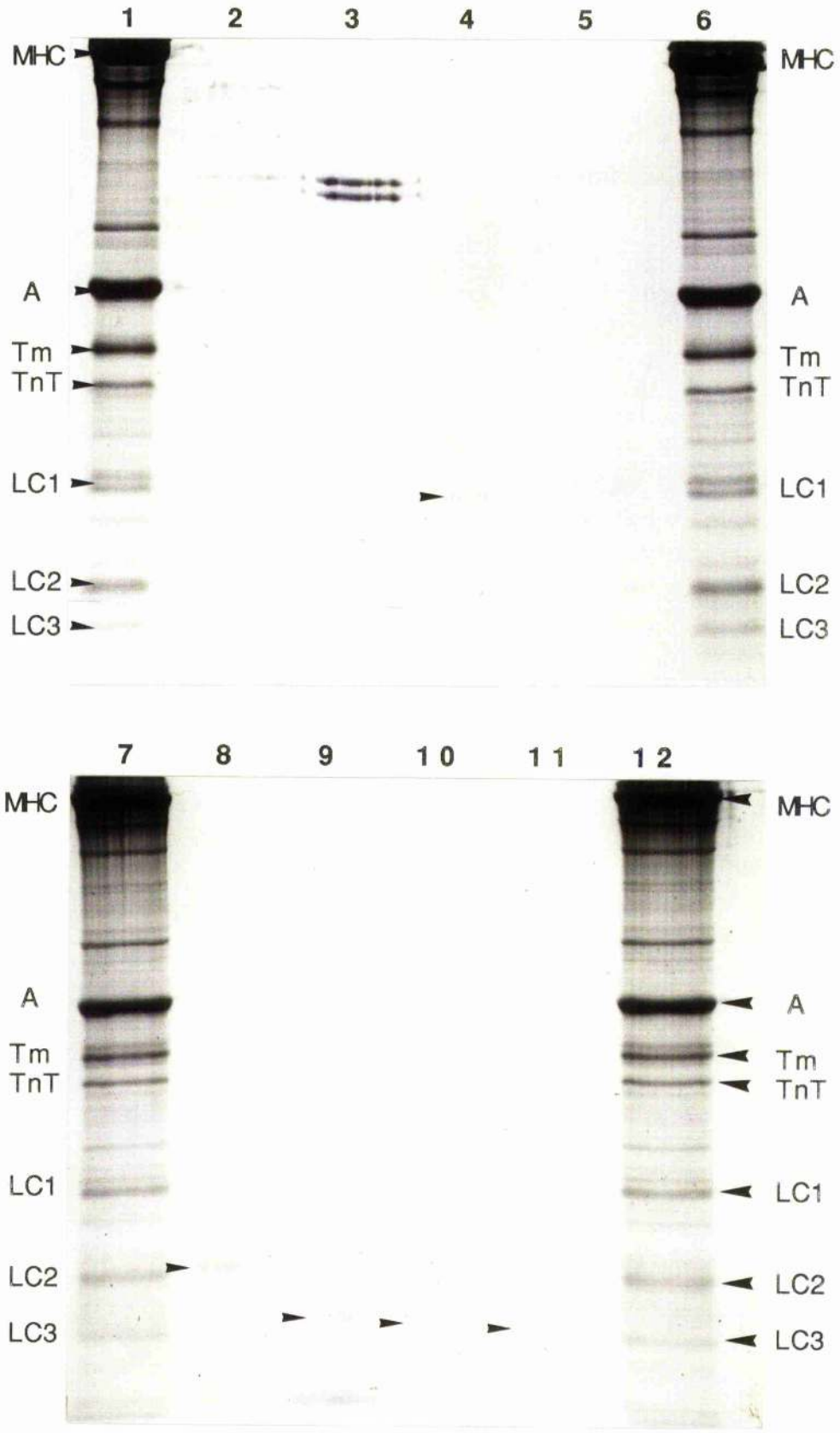


Figure 6. 10% alkali-urea polyacrylamide gel (pH 8.9) of purified myosin from red and white muscle fibres. Gel was stained with Coomassie blue G250.
Lane 1: red muscle myosin; Lane 2: white muscle myosin. Bands are numbered in the order in which they were cut out and run on 13% SDS polyacrylamide gels.

Figure 7. 13% SDS polyacrylamide gels of the bands cut out from a 10% alkali-urea (pH 8.9) polyacrylamide gel. Bands were from red muscle myosin purified using method 2 (Lane 1, figure 6). Gels were stained with Coomassie blue G250. Red muscle myofibrils were run on either side of each gel to act as standards. The arrows indicate the light chains identified within each of the bands after separation on alkali-urea gels (Figure 6). Each of the light chains was identified by its apparent molecular weight in relation to the red muscle myofibrils.

Lane 1: red muscle myofibrils; Lane 2: band 1 from figure 6, LC1; Lane 3: band 2 from figure 6, BP; Lane 4: band 3 from figure 6, LC1 (indicated by arrow); Lane 5: band 4 from figure 6; Lane 6: red muscle myofibrils; Lane 7: red muscle myofibrils; Lane 8: band 5 from figure 6, LC2 (indicated by arrow); Lane 9: band 6 from figure 6, LC3 (indicated by arrow); Lane 10: band 7 from figure 6, LC3 (indicated by arrow); Lane 11: band 8 from figure 6, LC3 (indicated by arrow); Lane 12: red muscle myofibrils.
MHC: myosin heavy chain; BP: breakdown products; A: actin; Tm: tropomyosin; TnT: troponin T; LC1: myosin light chain 1; LC2: myosin light chain 2; LC3: myosin light chain 3.


1
2
3
4
5
6
MHC
MHC
A
A
Tm
TnT
Tm
TnT
LC1
LC1
LC2
LC2
LC3
LC3
7
8
9
10
11
12
MHC
MHC
A
A
Tm
TnT
Tm
TnT
LC1
LC1
LC2
LC2
LC3
LC3

have the same molecular mass as light chain 3 (Fig. 7, lanes 9, 10 and 11). White muscle myosin ran as three bands on the alkali-urea gel first dimension (Fig. 6); band 1 was apparently light chain 1 (Fig. 8, lane 2), band 2 a mixture of light chain 1 and light chain 2 (Fig. 8, lane 3), and band 3 ran with the same molecular weight as light chain 3 (Fig. 8, lane 4). With the exception of light chain 2, both the fast and slow light chains appear to run very similarly on alkali-urea gels. Further confirmation of this was provided by running complete myofibrils on alkali-urea gels (Fig. 9) to see if the light chains ran identically when the other myofibrillar proteins were present. Band 4 from the red myofibrils (Fig. 9) seemed to consist of a combination of tropomyosin and light chain 1 (Fig. 10, lane 5). This band migrated to the same point as band 5 of the white myofibrils which consisted of tropomyosin, light chain 1 and light chain 2 (Fig. 11, lane 8). The heavier molecular weight bands in the red myofibrils- bands 1, 2 and 3 (Fig. 9), consisted of actin and another heavier protein, possibly C-line protein (Fig. 10, lanes 2, 3 and 4). The heavier bands from the white myofibrils- bands 1, 2, and 3 (Fig. 9), consisted of actin and troponin T (Fig. 11, lanes 2, 3 and 4).

Red muscle myosin light chain 2 (Band 6, Fig. 9 and Lane 9, Fig. 10) was more basic than white muscle light chain 2 (Bands 6 and 7, Fig. 9), but there only appeared to be one red light chain 3 (Band 7, Fig. 9) which had the same apparent relative molecular mass as white myosin light chain 3 (Band 8, Fig. 9 and lane 11 Fig. 11). The most basic band in the alkali-urea gel (Red myofibrils band 8, white myofibrils band 9, Fig. 9) appeared to be troponin C (Fig. 11, lane 12). Troponin C was not present in sufficient quantities in the red myofibrils to stain on the second dimension SDS gel (Fig. 10, lane 11).

The discrepancies between the alkali-urea gels of complete red myofibrils and purified red muscle myosin indicated the presence of multiple isoforms of either light chain 2 or light chain 3, making further investigation necessary.

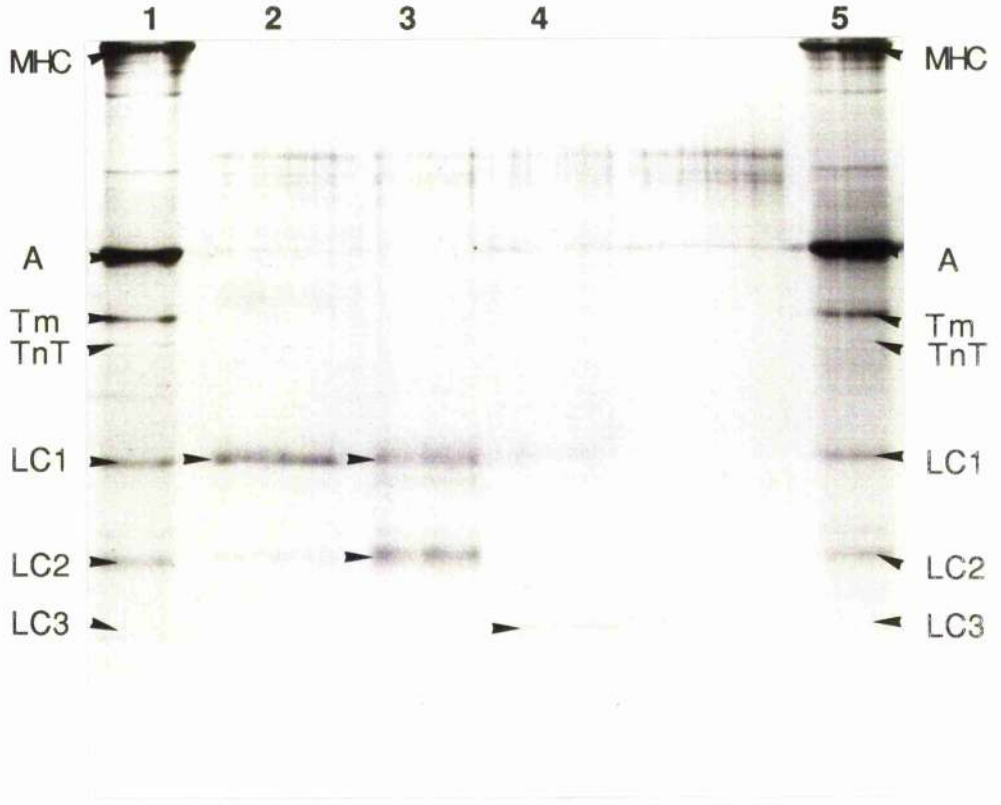


Figure 8. 13% SDS polyacrylamide gel of the bands cut out from a 10% alkali-urea (pH 8.9) polyacrylamide gel. Bands were from white muscle myosin purified using method 2 (Lane 2, figure 6). Gel was stained with Coomassie blue G250.
Lane 1: white muscle myofibrils; Lane 2: band 1 from figure 6, LC1 and LC2; Lane 3: band 2 from figure 6, LC1 and LC2; Lane 4: band 3 from figure 6, LC1 and LC3; Lane 5: white muscle myofibrils.
MHC: myosin heavy chain; A: actin; Tm: tropomyosin; TnT: troponin T; LC1: myosin light chain 1; LC2: myosin light chain 2; LC3: myosin light chain 3.

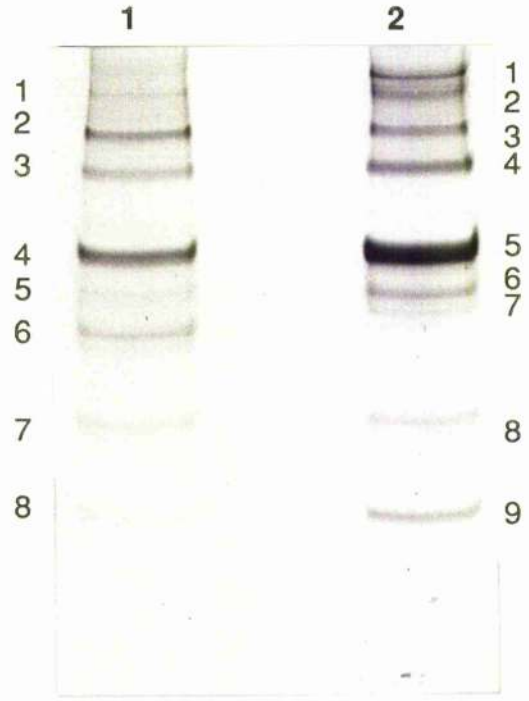
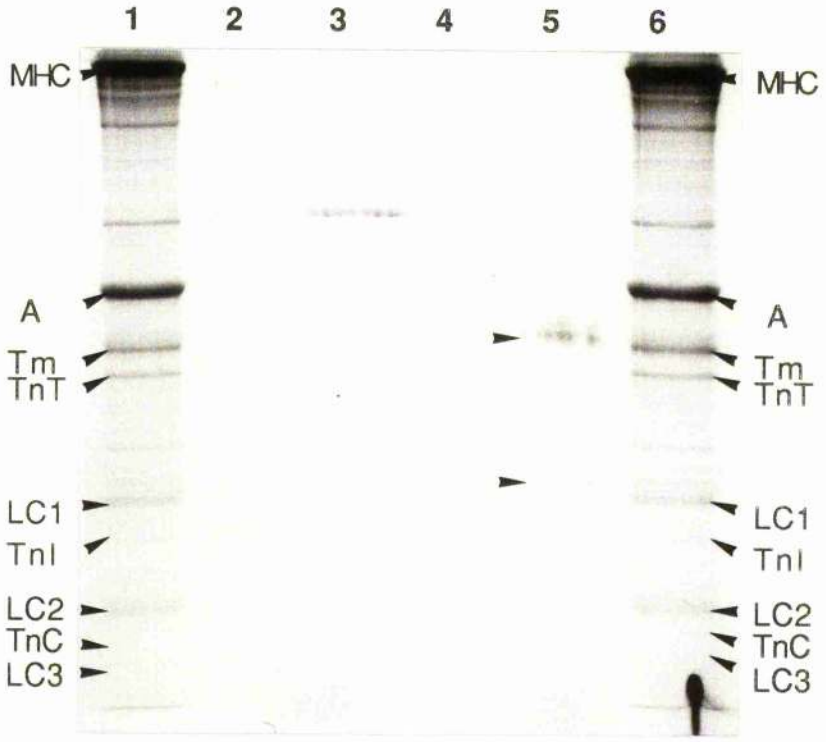


Figure 9. 10% alkali-urea (pH 8.9) polyacrylamide gel of myofibrils from adult plaice red and white muscle fibres. Gel was stained with Coomassie blue G250.
Lane 1: red muscle myofibrils; Lane 2: white muscle myofibrils. Bands are numbered in the order in which they were cut out and run on 13% SDS polyacrylamide gels.

Figure 10. 13% SDS polyacrylamide gels of the bands cut out from a 10% alkali-urea (pH 8.9) polyacrylamide gel. Bands were from red muscle myofibrils (Lane 1, figure 9). Gels were stained with Coomassie blue G250.
Red muscle myofibrils were run on either side of each gel to act as standards. The arrows indicate the major components identified within each of the bands after separation on alkali-urea gels (Figure 9). Each myofibrillar component was identified by its apparent molecular weight in relation to the red muscle myofibrils.

Lane 1: red muscle myofibrils; Lane 2: band 1 from figure 9; Lane 3: band 2 from figure 9; Lane 4: band 3 from figure 9, A; Lane 5: band 4 from figure 9, Tm (indicated by arrow), LC1 (indicated by arrow); Lane 6: red muscle myofibrils; Lane 7: red muscle myofibrils; Lane 8: band 5 from figure 9, LC1 (indicated by arrow), LC2 (indicated by arrow); Lane 9: band 6 from figure 9, LC2 (indicated by arrow); Lane 10: band 7 from figure 9; Lane 11: band 8 from figure 9; Lane 12: red muscle myofibrils.
MHC: myosin heavy chain; A: actin; Tm: tropomyosin; TnT: troponin T; LC1: myosin light chain 1; TnI: troponin I; LC2: myosin light chain 2; TnC: troponin C; LC3: myosin light chain 3.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2
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6
MHC
MHC
A
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Tm
TnT
Tm
TnT
LC1
TnI
LC1
TnI
LC2
TnC
LC3
LC2
TnC
LC3

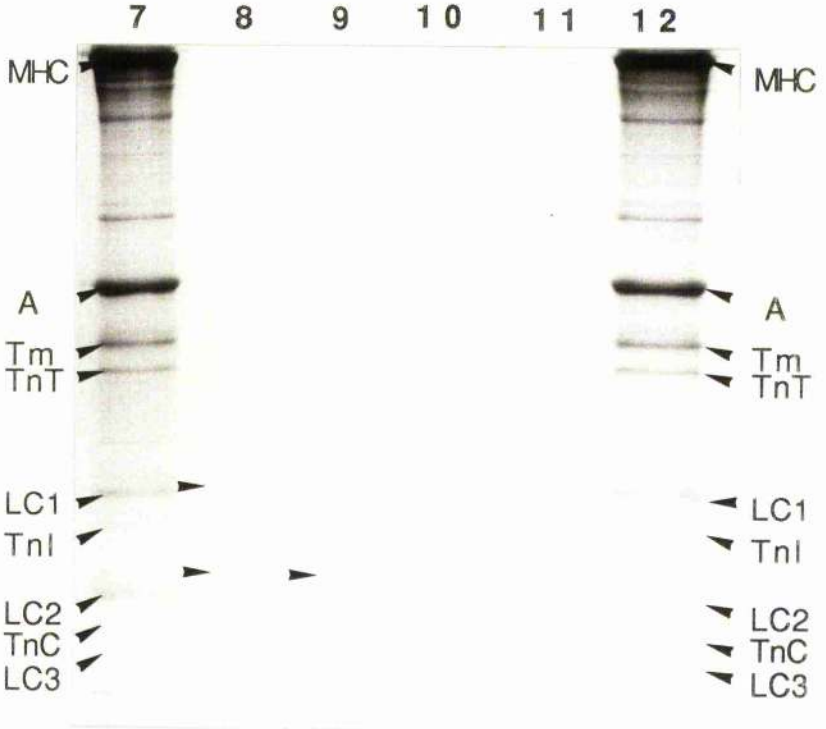
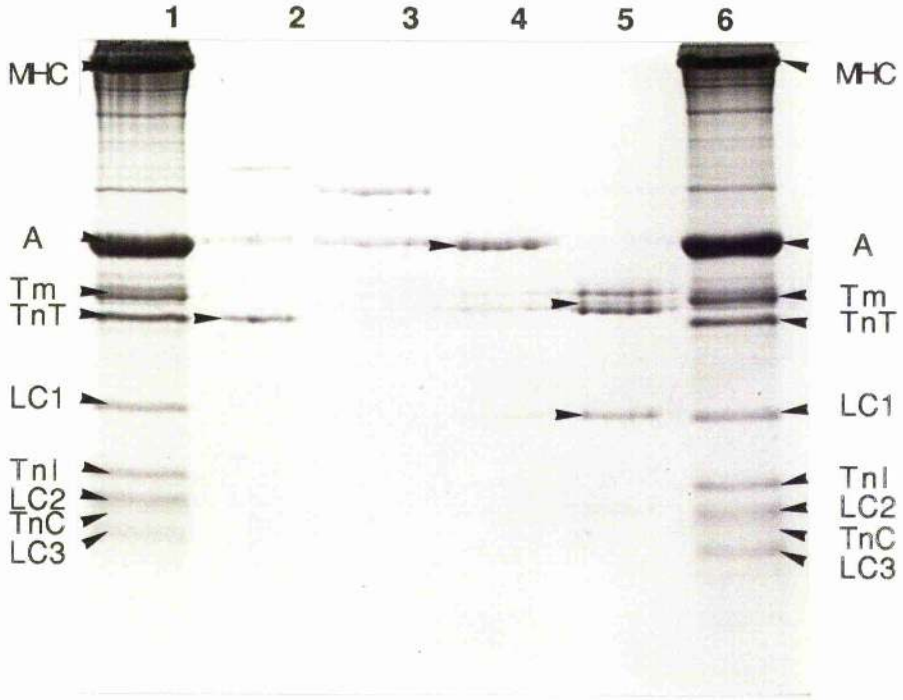

7
8
9
10
11
12
MHC
MHC
A
A
Tm
TnT
Tm
TnT
LC1
TnI
LC1
TnI
LC2
TnC
LC3
LC2
TnC
LC3

Figure 11. 13% SDS polyacrylamide gels of the bands cut out from a 10% alkali-urea (pH 8.9) polyacrylamide gel. Bands were from white muscle myofibrils (Lane 2, figure 9). Gels were stained with Coomassie blue G250.
White muscle myofibrils were run on either side of each gel to act as standards. The arrows indicate the major components identified within each of the bands after separation on alkali-urea gels (Figure 9). Each myofibrillar component was identified by its apparent molecular weight in relation to the white muscle myofibrils.

Lane 1: white muscle myofibrils; Lane 2: band 1 from figure 9, TnT (indicated by arrow); Lane 3: band 2 from figure 9; Lane 4: band 3 from figure 9, A (indicated by arrow); Lane 5: band 4 from figure 9, Tm (indicated by arrow), LC1 (indicated by arrow); Lane 6: white muscle myofibrils; Lane 7: white muscle myofibrils; Lane 8: band 5 from figure 9, Tm (indicated by arrow), LC1 (indicated by arrow), LC2 (indicated by arrow); Lane 9: band 6 from figure 9, LC2 (indicated by arrow); Lane 10: band 7 from figure 9, LC2 (indicated by arrow); Lane 11: band 8 from figure 9, LC3 (indicated by arrow); Lane 12: band 9 from figure 9, TnC (indicated by arrow); Lane 13: white muscle myofibrils.
MHC: myosin heavy chain; A: actin; Tm: tropomyosin; TnT: troponin T; LC1: myosin light chain 1; TnI: troponin I; LC2: myosin light chain 2; TnC: troponin C; LC3: myosin light chain 3.


1
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6
MHC
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Tm
TnT
LC1
TnI
LC2
TnC
LC3
MHC
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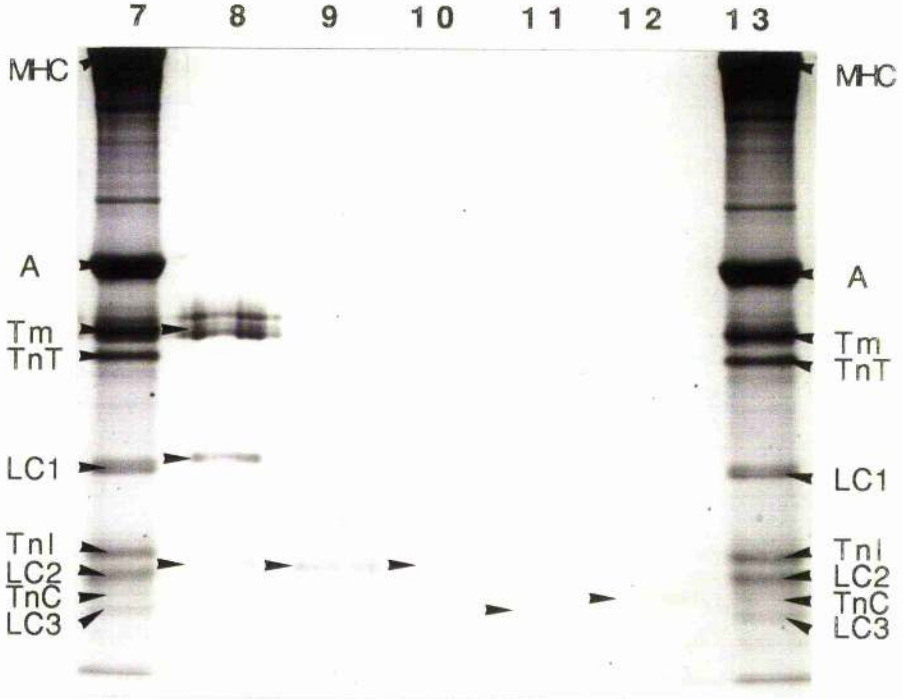
TnT
LC1
TnI
LC2
TnC
LC3


7
8
9
10
11
12
13
MHC
A
Tm
TnT
LC1
TnI
LC2
TnC
LC3
MHC
A
Tm
TnT
LC1
TnI
LC2
TnC
LC3

Myosin light chains can also be identified by their characteristic migration in the neutral to acid pH range on two dimensional PAGE gels, first dimension IEF, second dimension SDS (Rowlerson *et al.* 1985, Martinez *et al.* 1990).

Red myofibrils (Fig. 12a) appear to contain two isoforms of light chain 1, both of the same molecular weight but different pI. There are four isoforms of light chain 2, three of them with the same molecular weight but of different pI and one isoform with the same molecular weight as light chain 3 but different pI. Only one isoform of light chain 3 was observed. White myofibrils appeared to contain just one isoform of each light chain (Fig. 12b).

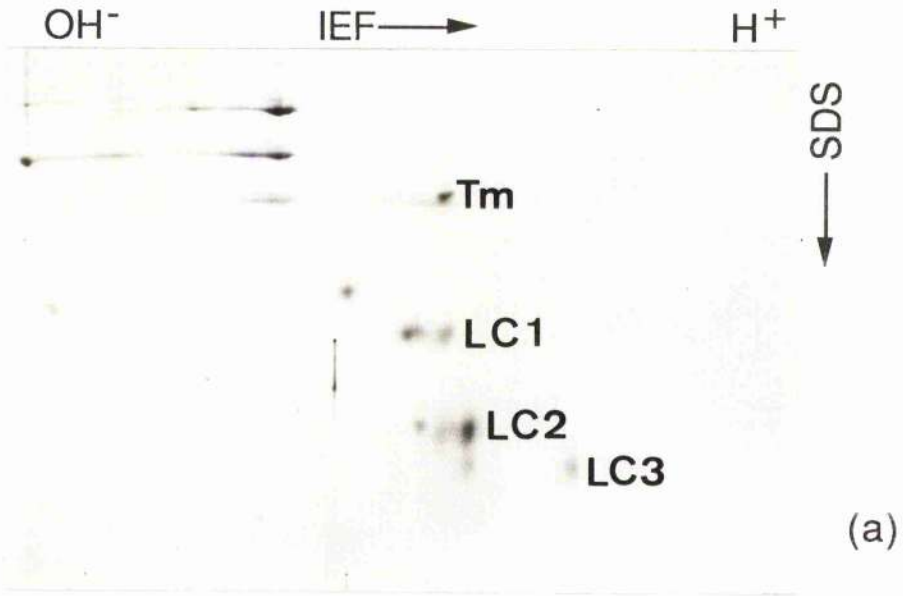
Plaice myotomal muscle contains at least five distinct fibre types (see Chapter 4). These fibre types are likely to each have their own distinct myosin light chain isoforms, if the bulk samples of red muscle fibres also contained some tonic or superficial white fibres; this could account for the differences between red muscle light chain composition as shown by alkali-urea gels and IEF gels.

Myosin Light Chain Composition of Identified Fibre Types

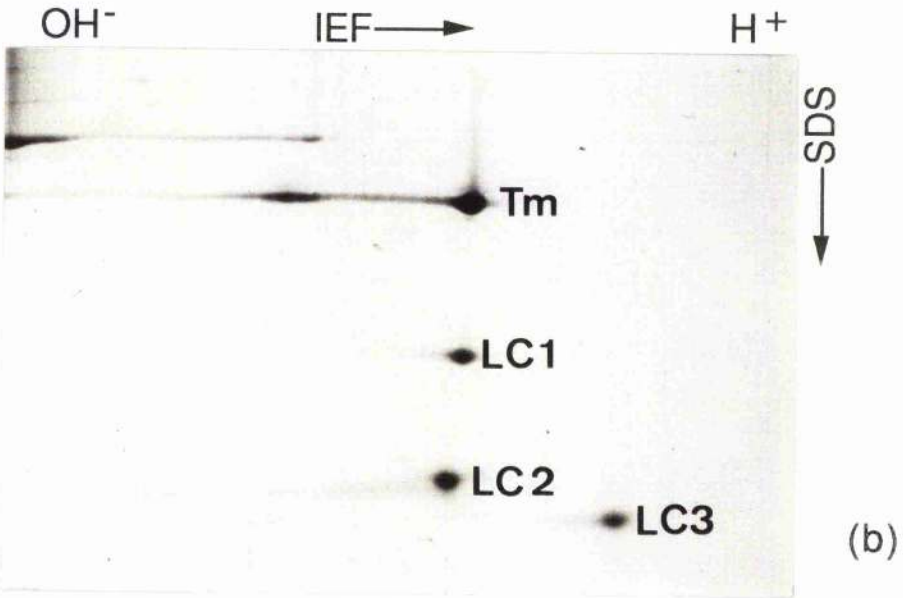
In order to ascertain the myosin light chain composition of the different fibre types, small fibre bundles (not more than eight fibres) identified by their different light scattering properties under dark field illumination, were dissected from various regions of the myotome. Each fibre type appeared to have its own pattern of myosin light chains.

Tonic fibres (Fig. 13a) appear to have one isoform of light chain 1 (MW 29 500), four light chain 2 isoforms (MW 21 500 and 18 000) and one isoform of light chain 3 (MW 18 000). Red fibres (Fig. 13b) consist of two light chain 1 isoforms (both

Figure 12. Two-dimensional PAGE of acidic myofibrillar proteins, stained with Coomassie Brilliant Blue G250. First dimension isoelectric focusing (IEF) and 15% acrylamide SDS PAGE in second dimension.
(a) Red muscle fibres from adult plaice.
(b) White muscle fibres from adult plaice.
Tm: tropomyosin; LC1: myosin light chain 1; LC2: myosin light chain 2; LC3: myosin light chain 3.



(a)



(b)

Figure 13. Two-dimensional PAGE of acidic myofibrillar proteins, stained with Coomassie Brilliant Blue G250. First dimension isoelectric focusing (IEF) and 15% acrylamide SDS PAGE in second dimension. Bundles of single fibre types identified by their different light scattering properties under dark field illumination, were dissected from various regions of the myotome.

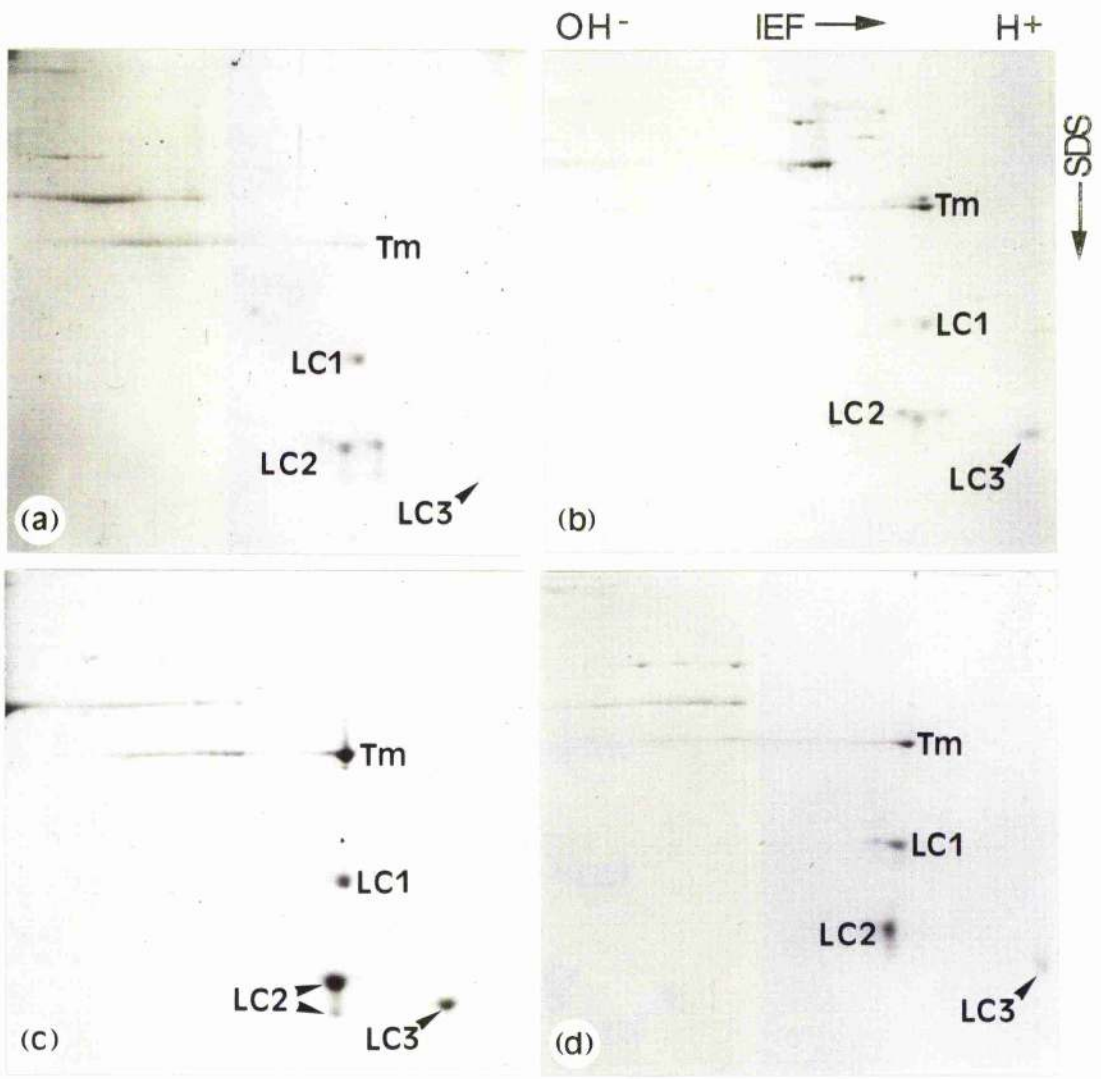
(a) Tonic muscle fibres.

(b) Red muscle fibres.

(c) Superficial white muscle fibres.

(d) Deep white muscle fibres.

Tm: tropomyosin; LC1: myosin light chain 1; LC2: myosin light chain 2; LC3: myosin light chain 3.


OH⁻
IEF
H⁺
SDS
Tm
LC1
LC2
LC3
(a)
(b)
(c)
(d)

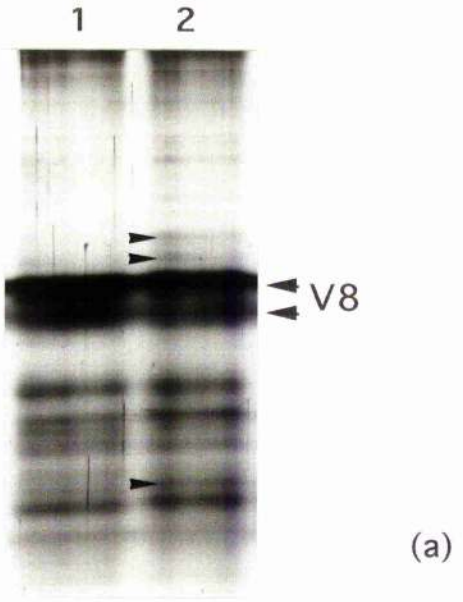
MW 29 500), three light chain 2 isoforms (MW 21 500) and one light chain 3 isoform (MW 18 000). Superficial white fibres (Fig. 13c) have apparently the same light chain composition as deep white fibres (Fig. 13d) but in addition possess an extra isoform of light chain 2 (MW 18 000).

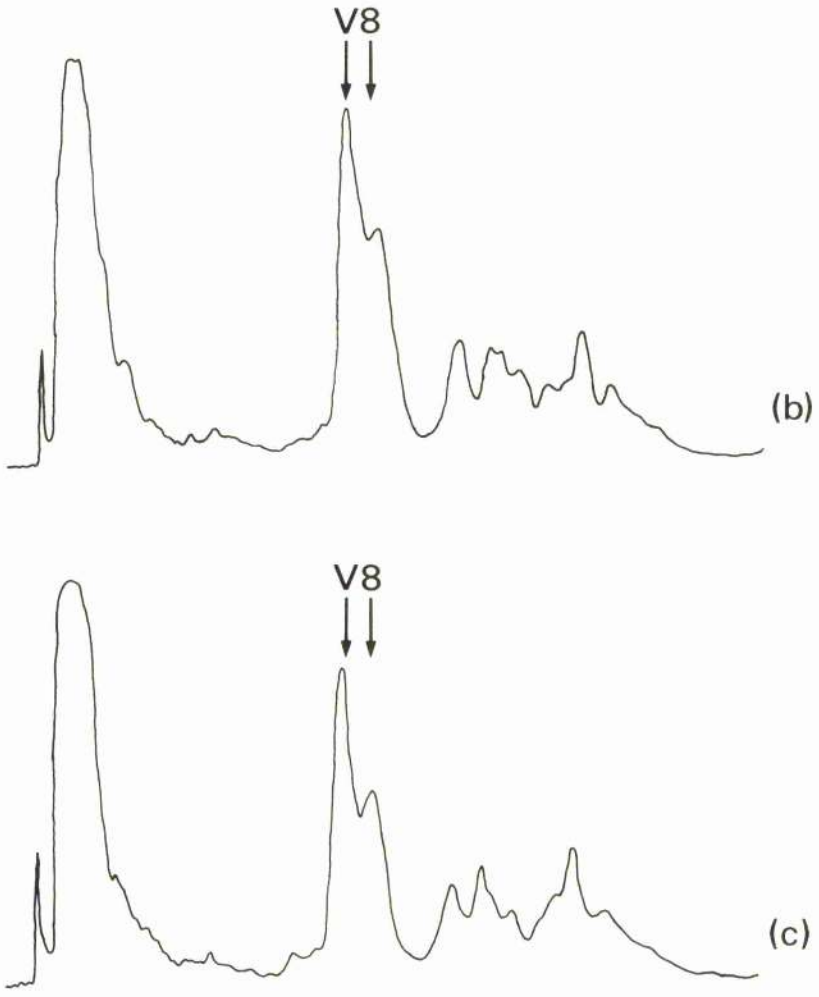
The same isoform of myosin light chain 3 was present in each of the four fibre types. Tonic, superficial white and deep white fibres all appeared to have light chain 1 in common. Red muscle fibres contained two myosin light chain 1 isoforms, one of which could correspond to the light chain 1 isoform present in tonic, superficial white and deep white fibres. The major difference between fibre types were the isoforms of myosin light chain 2 present.

Peptide mapping

V8 peptide maps of red and white muscle myosin heavy chains were very similar (Fig. 14). V8 cleaves proteins at the COOH site of aspartic acid and glutamic acid, giving breakdown products related to the amino acid sequence of the heavy chain component.

Figure 14. (a) Peptide maps of electrophoretically purified myosin heavy chains digested with *Staphylococcus aureus* V8 protease and run on a 15% SDS PAGE gel which was silver stained. Lane 1: myosin heavy chain from adult red muscle; Lane 2: myosin heavy chain from adult white muscle. The arrows indicate differences in the maps of the different myosin heavy chains.
(b) Densitometer trace of Lane 1.
(c) Densitometer trace of Lane 2.
V8: *Staphylococcus aureus* V8 protease.


1
2
V8
(a)


V8
(b)
V8
(c)

Discussion

Analysis of Myofibrillar Proteins

Thin Filament Proteins

SDS gels of plaice red and white myofibrils showed two isoforms each of tropomyosin and troponin T in red muscle fibres but only one isoform of each in the white myofibrils. Mammalian muscle fibres contain isoforms of the troponin complex specific to slow and fast fibre types (Schachat *et al.* 1987) and have an alpha-tropomyosin to beta-tropomyosin ratio specific to fibre type (Salviati *et al.* 1982). Previous work on teleosts has indicated the presence of red and white tropomyosin and troponin T isoforms (Focant *et al.* 1976, Crockford 1989) but their specific properties are as yet unclear. In rabbit fast skeletal muscle fibres, response to Ca^{2+} is determined by the tropomyosin and troponin T isoforms present (Schachat *et al.* 1987, Briggs *et al.* 1987). The troponin T isoforms present in the fast fibres have also been correlated with their shortening velocity (Greaser *et al.* 1988).

No clear differences could be distinguished between troponin I from plaice red and white fibres, both have the same apparent molecular weight on 13% SDS PAGE. Red muscle troponin I appears to have a slightly more basic pI, although this was difficult to determine because of the limitations of the technique used. In mammals troponin I from fast skeletal muscles has various properties including, the ability to inhibit specifically the magnesium stimulated ATPase of actomyosin and the formation of a complex with troponin C which is Ca^{2+} dependent and stable at high concentrations of urea. The inhibitory property of troponin I is highly specific for actin-activated myosin ATPase and is not shown with the Ca^{2+} stimulated ATPase of either myosin or actomyosin (Perry 1979). Different isoforms, specific to each fibre type, have been identified in mammalian slow skeletal, fast skeletal and

cardiac muscle (Syska *et al.* 1974, Greaser and Gergely 1973). Perry (1985) suggested that troponin I isoforms may differentially alter the Ca^{2+} binding characteristics of troponin C.

Troponin C was identified both on SDS PAGE and alkali-urea gels by its characteristic blue colour when stained with 'Stains All' (Campbell *et al.* 1983). Plaice red and white troponin C both have apparently the same molecular weight and run with the same charge on alkali-urea gels. Troponin C has a high Ca^{2+} binding affinity and can neutralise the inhibitory activity of troponin I on the actomyosin ATPase (Perry 1979). Calcium ions binding to troponin C are thought to open a hydrophobic cavity, which is the site of interaction with troponin I. In the absence of Ca^{2+} the inhibitory region of troponin I would preferentially bind to actin (Grabarek *et al.* 1992), preventing it from interacting with myosin and activating ATPase.

Thick Filament Proteins

Myosin light chain sub-unit composition

Electrophoretic analysis of the red and white muscle fibres of adult plaice showed that both fibre types contained myosin light chain 1, 2 and 3. In all the teleost species previously studied red muscle has been characterised as having only two myosin light chains (Rowlerson *et al.* 1985, Karasinski and Kilarski 1989, Focant *et al.* 1976, Huriaux and Focant 1985, Huriaux *et al.* 1990, Martinez *et al.* 1990), similar to mammalian slow muscle (Lowey and Risbey 1971). In addition to possessing three myosin light chain sub-units, multiple isoforms of light chains 1 and 2 were also present in the red fibres. Bulk preparations of red fibres contained four isoforms of light chain 2 whereas preparations made from 3-5 fibres contained only three light chain 2 isoforms. Within the red muscle there could be several fibre types not distinguishable

by mATPase staining, each fibre type containing different single isoforms of light chain one and two. Staron and Pette (1987) demonstrated that histochemically typed single fibres may contain up to fifty four possible isomyosins.

The myosin light chain composition of the superficial fibres differs from that of the deep white fibres solely by the presence of an extra isoform of light chain 2 in the superficial white fibres. Deep white muscle showed a pattern of light chains typical of fast muscle fibres (Focant and Huriaux 1976). The three myosin light chains are divided into two classes, light chain 1 and 3 are termed the alkali light chains because it is possible to remove them from myosin by treatment with guanidine-HCl or alkaline conditions. Removal of light chain 1 and light chain 3 results in a complete loss of ATPase activity (Weeds and Lowey 1971). Cross-hybridisation experiments using rabbit myosin subfragment 1 have shown that the alkali light chains have a role in the regulation of ATPase activity (Wagner and Weeds 1977).

Myosin light chain 2 is classed differently to light chains 1 and 3, it can be removed from myosin by treatment with dithiobis-(nitrobenzoic acid) (DTNB), and so is termed the DTNB light chain. It is possible to phosphorylate myosin light chain 2 both *in vitro* and *in vivo* and it has been linked to a specific Ca^{2+}-dependent kinase and a specific Ca^{2+}-independent phosphatase (Barany and Barany 1980). The role of myosin light chain 2 during the contraction of fish muscle fibres is unclear. Results obtained by Yancey and Johnston (1982) suggest that myosin light chain 2 phosphorylation is not an integral part of the contraction-relaxation cycle in fish muscle.

Myosin heavy chain sub-unit composition

The peptide maps of myosin heavy chains from plaice red and white fibres were very similar. Scapolo and Rowlerson (1987) suggested that the greater the difference in the primary structure of the heavy chains the greater the variation in peptide maps, but this is difficult to quantify. The advantage of cutting out all the myosin heavy chains from the different fibre types in one band is that all the samples then experience exactly the same conditions during digestion. Any differences in peptide maps will be solely a result of structural differences and not because of varying degrees of digestion. To what extent the contractile properties of a fibre are modified by only small structural differences in myosin heavy chain is not clear. The unloaded contraction speed of rabbit muscle fibres is closely correlated with the myosin heavy chain isoform present (Reiser *et al.* 1985). Studies with amphibian skeletal muscle fibres have shown differences in the contractile properties of two fibre types with the same myosin light chain components but different myosin heavy chains (Lannergren 1987). Histochemical typing of muscle fibres for myofibrillar ATPase has always been correlated with the presence of distinct myosin heavy chain isoforms in other vertebrates (Salviati *et al.* 1982). Plaice red and white muscle fibres while histochemically different, have very similar myosin heavy chains, the major difference between the two fibre types appears to be the presence of multiple isoforms of myosin light chain 2 in red muscle fibres. Analysis of single muscle fibres in mammals have shown that different myosin heavy chain isoforms are frequently present in the same fibre (Betto *et al.* 1986, Staron and Pette 1987).

Non-typical fibre type distribution in the plaice

The red myotomal muscle fibres of adult plaice differ drastically from those of other teleosts in that they contain three myosin light chains rather than the characteristic two

previously observed in teleost red muscle fibres (Rowlerson *et al.* 1985, Karasinski and Kilarski 1989, Huriaux *et al.* 1990, Martinez *et al.* 1990). Plaice red muscle fibres also appear to contain an alkali-stable ATPase more characteristic of pink muscle fibres (Scapolo and Rowlerson 1987, Johnston *et al.* 1975). Pink muscle fibres from all other teleost species studied, have contained three myosin light chains, similar to white fibres (Rowlerson *et al.* 1985, Scapolo and Rowlerson 1987). Both the myosin light chain composition and the reaction when staining for mATPase suggest that the red fibres present in the myotomes of adult plaice are actually a fast red (pink) fibre type (Johnston 1983a), rather than the more usually observed slow red fibre type. Biochemical analysis of plaice muscles by Priede and Holliday (1980) and the actual swimming behaviour of plaice observed by Greer Walker *et al.* (1978) tend to support this proposal. Priede and Holliday (1980) calculated that glycogen stores in plaice white muscle were sufficient to allow fish to swim at 2 body lengths/s for 1.2 hours before exhaustion. Plaice have been observed swimming at 0.9-2.0 body lengths/s for 0.5 hours with periods of rest between phases of activity (Greer Walker *et al.* 1978). Duthie (1982) suggested that these rest periods were necessary to allow repayment of the oxygen debt because anaerobic muscles were used to provide propulsion.

Chapter 6

Developmental transitions in myosin sub-unit composition from larval inner to adult fast muscle in plaice, *Pleuronectes platessa* L.

Introduction

During the development of skeletal muscle fibres in both birds and mammals there is a sequential expression of myosin isozymes (Whalen 1981, Gauthier 1990, Hoh *et al.* 1988, Hoh and Hughes 1989, Bandman *et al.* 1982). Myosin heavy chain isoforms specific to different developmental stages and muscle fibre types have been identified (Bandman *et al.* 1982, Whalen *et al.* 1979, Crow and Stockdale 1986), as have specific myosin light chain isoforms (Barton *et al.* 1985, Barton *et al.* 1989). The sequential expression of myosin heavy chain isoforms in single fibre types appears to be strongly correlated with their velocity of shortening throughout development (Reiser *et al.* 1988). In addition to the presence of specific embryonic and neonatal myosin isoforms, adult isoforms eg. cardiac myosin, may be transiently expressed in a non-specific manner by developing muscle fibres (Barton *et al.* 1985). Isoforms of the myofibrillar protein components may either be produced by different genes transiently expressed (Periasamy *et al.* 1984) or may be the products of an alternative splicing mechanism together with differential transcription, or they could result solely from differential transcription (Barton and Buckingham 1985, Breibart *et al.* 1985).

Developmental transitions of myosin isoforms have also been identified during the differentiation of teleost locomotory fibres. In the zebrafish, *Brachydanio rerio*, the first myofibrils produced in the embryo are immunologically similar to myofibrils from adult slow muscle fibres (Van Raamsdonk *et al.* 1978). All the embryonic cells initially produce 'redlike'

myofibrils for a short period, these are apparently broken down and replaced by 'whitelike' myofibrils in the inner fibres, but continue to be produced in the superficial fibres. In both the superficial and inner muscle fibres of the sea bass, *Dicentrarchus labrax*, there is a developmental transition in myosin sub-unit composition. This transition from an early larval form to a late larval form and the subsequent expression of the isoforms typical of adult red and white muscle have been examined using isoform-specific anti-bodies to mullet myosin and the appearance of histochemical mATPase activity (Scapolo *et al.* 1988). The transition from the early to the late larval isoform in the inner fibres occurs rapidly and relatively early during the larval period, whereas the transition in the superficial fibres from the early to the late isoform is a gradual process. The adult red isoform of myosin is found to occur earlier in the superficial red muscle, compared with the appearance of the corresponding adult white myosin. Embryonic, larval and adult myosin and parvalbumin isotypes have also been analysed in the barbel, *Barbus barbus* (Focant *et al.* 1992). The myosin isoforms present show a sequential transition from embryonic to larval to adult forms, characterised by specific heavy chains but with the light chain component differing only by the LC1:LC3 ratio. The sarcoplasmic parvalbumins also showed developmental transitions in their isotype distribution, closely reflecting the developmental stage.

A study of the myofibrillar protein composition of myotomal muscles in Atlantic herring, *Clupea harengus*, also showed changes linked to development (Crockford and Johnston 1993). The inner larval muscle fibres contained myosin heavy chain, troponin T and troponin I isoforms not present in either of the adult muscle fibre types. This sequential expression of development specific isoforms of the myofibrillar proteins was almost certainly linked to the changing functional requirements of the muscle during growth.

Plaice, *Pleuronectes platessa*, undergo various structural and locomotory changes during the passage from larvae to adult. The aim of this study was to characterise the myosin sub-unit composition of the major myotomal muscle fibre type in larval, juvenile and adult stages of the lifecycle.

Materials and Methods

Developmental Stages

Both wild and laboratory reared fish were investigated (Tables 1 and 2). Laboratory reared fish (incubation temperature 8°C) were sampled at hatching and then at 6, 10 and 20 weeks post-hatching (Table 1). Metamorphosis was complete at the time of the 10 week sample.

Juvenile stages covering the same size range as laboratory reared juveniles and a larger size class were trawled from Dunstaffnage Bay, Argyll and St. Andrews Bay, Fife, during late June and early July (Table 2). The larger juveniles (> 10 cm) had probably hatched as larvae in the previous spring and so were approximately 1 year old. Adult plaice, standard length 26.2 ± 2.9 cm (mean ± SD, n = 10) were trawled from St. Andrews Bay.

Gel electrophoresis

Myofibrils were prepared from fast muscle fibres using the method described in Chapter 2. Inner muscle fibres were isolated from the larvae by first removing the head and yolk-sac/gut and stripping off the skin and adjacent layer of superficial fibres with jewellers forceps which left just the inner fibres. Samples of white muscle were dissected from the juvenile and adult fish using a Zeiss Stemi SV6 dissecting microscope, taking care to avoid contamination with red

muscle fibres. Small bundles of superficial white fibres were dissected under dark field illumination.

Samples of known protein concentration were prepared for electrophoresis by centrifuging the necessary volume of myofibrils and dissolving the pellet in either IEF or SDS sample buffer. Gel electrophoresis was carried out as described in Chapter 2. The myosin light chains were identified by their migration patterns on acid to neutral pH IEF gels as the first dimension, with 14% SDS gels as the second dimension.

Peptide maps of the electrophoretically purified myosin heavy chains were prepared using V8 protease (Sigma). Gels were stained with Coomassie brilliant blue G250 (Neuhoff *et al.* 1988) and silver (Bloom *et al.* 1988).

Table 1

Ages and lengths, at sampling, of plaice reared in the laboratory at 8°C.

Age when sampled (Post hatching)	**Total Length** (mm) (mean ± SD, n = 10)
Hatching	6.6 ± 0.7
6 weeks	10.5 ± 0.3
10 weeks (Metamorphosis)	12.2 ± 1.4
20 weeks (Juvenile)	19.1 ± 3.8

Table 2

Developmental stages and standard lengths of juvenile plaice trawled from Dunstaffnage Bay, Argyll and St. Andrews Bay, Fife.

Developmental Stage	**Standard length** (cm) (mean ± SD, n = 10)
0-group	2.4 ± 0.6
1-group	10.4 ± 3.4
Adult	26.2 ± 2.9

n = Total number of individuals

Results

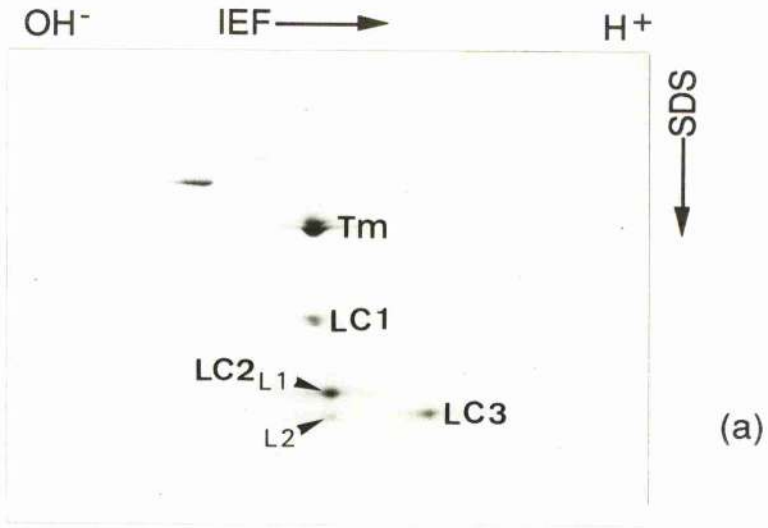
Myosin Light Chain Composition

Myosins isolated from the inner muscle fibres of larvae and the fast muscles of juveniles and adults contained two alkali (LC1 and/or LC3) and two phosphorylatable light chain (LC2) components. The apparent relative molecular masses (M_r) of myosin LC1 and LC3 from the inner muscle of larvae were 29,500 and 18,000 respectively. Identical isoforms of LC1 and LC3 were present in fast muscle fibres of larval, juvenile and adult stages. By contrast, there were marked changes in the composition of the phosphorylatable light chains at metamorphosis and during the juvenile stages. Myosins isolated from the inner muscle fibres of newly hatched plaice larvae contained two isoforms of LC2 which had different relative molecular masses M_r ($LC2_{L1}$, M_r = 21,000 and $LC2_{L2}$, M_r = 18,000) (Fig. 1a) but had a similar pI. LC2 composition was unchanged during the larval period (Fig. 1b). However, at the end of metamorphosis (Fig. 1c), in addition to $LC2_{L1}$ and $LC2_{L2}$, the inner muscle fibres expressed two additional isoforms of LC2 which were characteristic of the entire fast muscle mass of 0-group and 1-group plaice (LC_{F1}, M_r = 21,500 and $LC2_{F2}$, Mr = 18,000) (Figs. 2a, b, 3a). $LC2_{L1}$ and $LC2_{L2}$ were distinguished from $LC2_{F1}$ and $LC2_{F2}$ by their more acidic pI (Fig. 1c). The superficial fast muscle fibres of mature fish also expressed both LC_{F1} and $LC2_{F2}$ (Fig. 3b) whereas myosin from the deep fast muscle of adult fish only contained $LC2_{F1}$ (Fig. 3c).

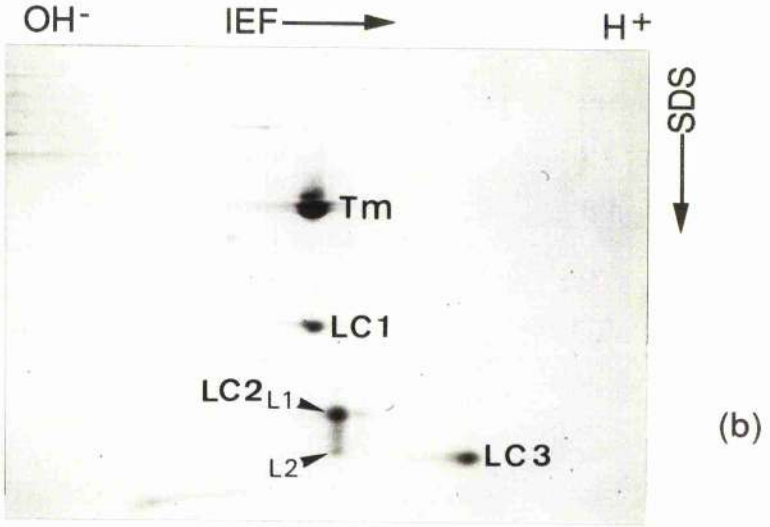
Myosin Heavy Chain Sub-unit Composition

Peptide maps of the myosin heavy chains digested with V8 protease showed that the myosin heavy chain composition of the inner muscle fibres also changed with development (Fig. 4).

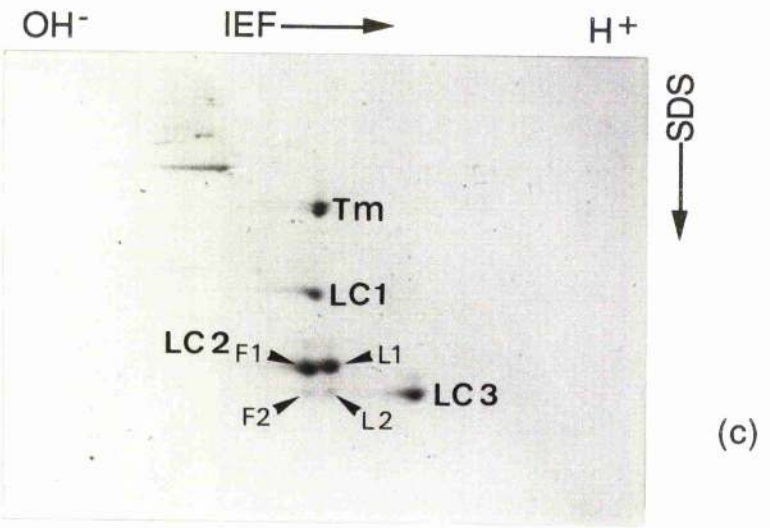
Figure 1. Two-dimensional PAGE of acidic myofibrillar proteins, stained with Coomassie Brilliant Blue G250. First dimension isoelectric focusing (IEF) and 15% acrylamide SDS PAGE in second dimension.
(a) 1 day old larval inner muscle.
(b) Inner muscle from larvae at 6 weeks post hatching.
(c) Inner muscle from juveniles that had completed metamorphosis (10 weeks post hatching).
Tm: tropomyosin; LC1: myosin light chain 1; LC2: myosin light chain 2; LC3: myosin light chain 3.


OH⁻
IEF
H+
SDS
Tm
LC1
LC2
L1
L2
LC3

(a)

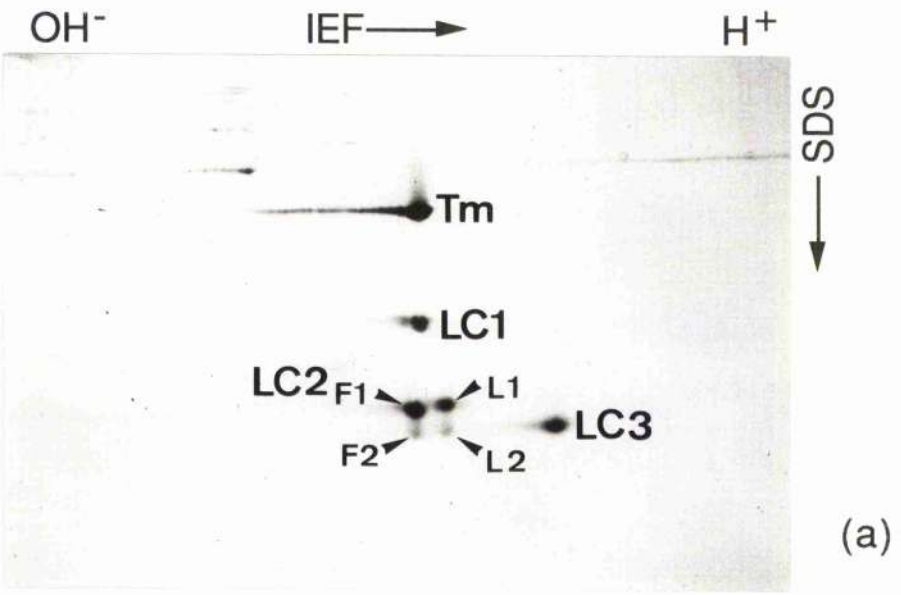

OH⁻
IEF
H+
SDS
Tm
LC1
LC2
L1
L2
LC3

(b)


OH⁻
IEF
H+
SDS
Tm
LC1
LC2
F1
L1
F2
L2
LC3

(c)

Figure 2. Two-dimensional PAGE of acidic myofibrillar proteins, stained with Coomassie Brilliant Blue G250.
(a) Inner muscle fibres from laboratory reared fish at 20 weeks post hatching.
(b) Inner muscle fibres from 0-group juveniles caught in the wild.
Tm: tropomyosin; LC1: myosin light chain 1; LC2: myosin light chain 2; LC3: myosin light chain 3.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IEF
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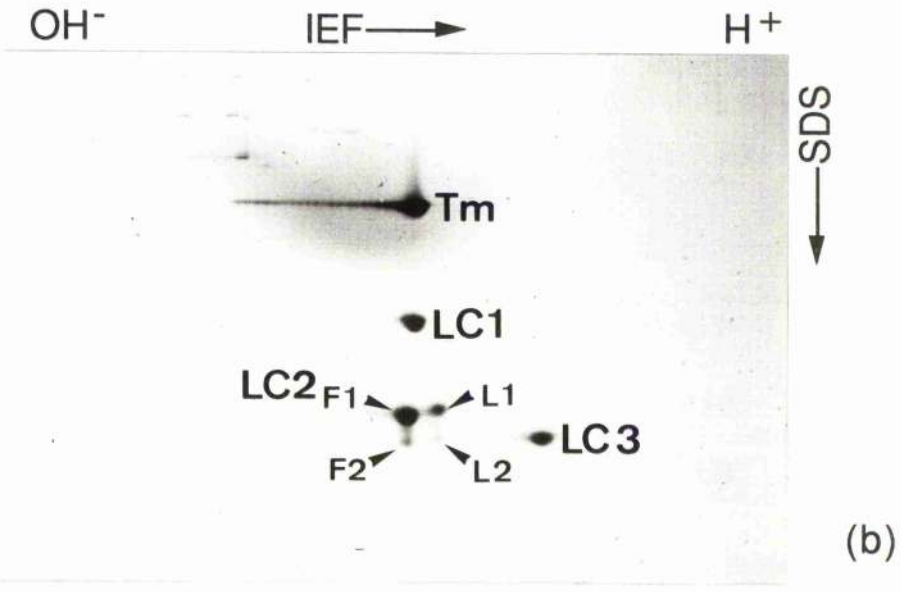
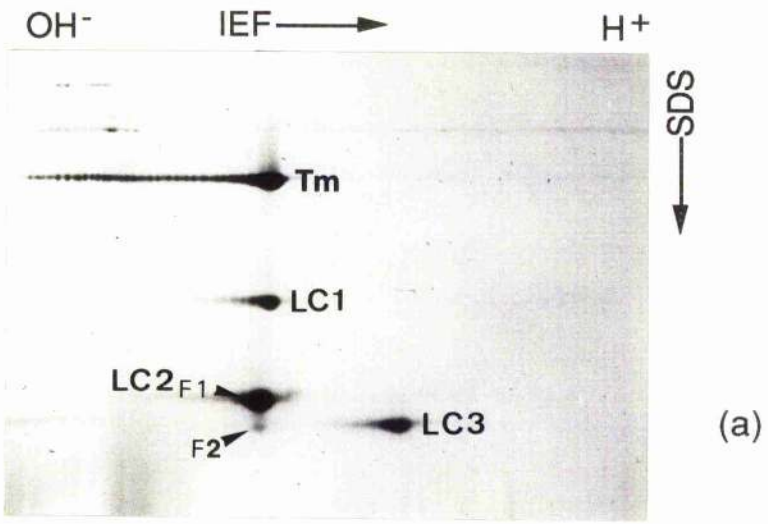
Tm
LC1
LC2
F1
L1
LC3
F2
L2

(a)

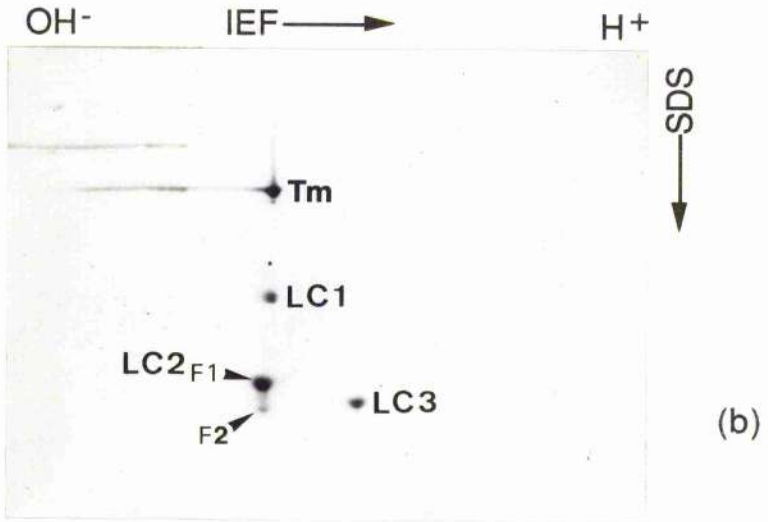

OH⁻
IEF
H⁺
SDS
Tm
LC1
LC2
F1
L1
LC3
F2
L2

(b)

Figure 3. Two dimensional PAGE of acidic myofibrillar proteins, stained with Coomassie Brilliant Blue G250.
(a) Inner muscle fibres from wild 1-group juveniles.
(b) Superficial white fibres dissected from adult fish.
(c) Deep white muscle fibres from adult plaice myotomes.
Tm: tropomyosin; LC1: myosin light chain 1; LC2: myosin light chain 2; LC3: myosin light chain 3.


OH⁻
IEF
H⁺
SDS
Tm
LC1
LC2F1
F2
LC3

(a)


OH⁻
IEF
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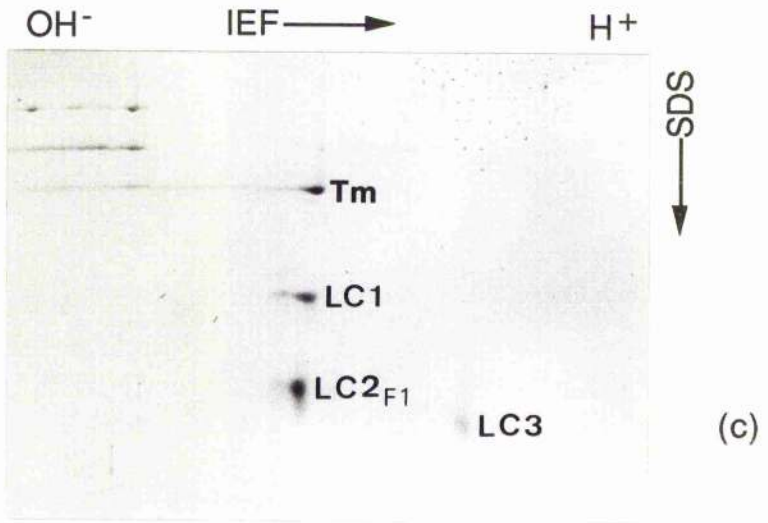
SDS
Tm
LC1
LC2F1
F2
LC3

(b)


OH⁻
IEF
H⁺
SDS
Tm
LC1
LC2F1
LC3

(c)

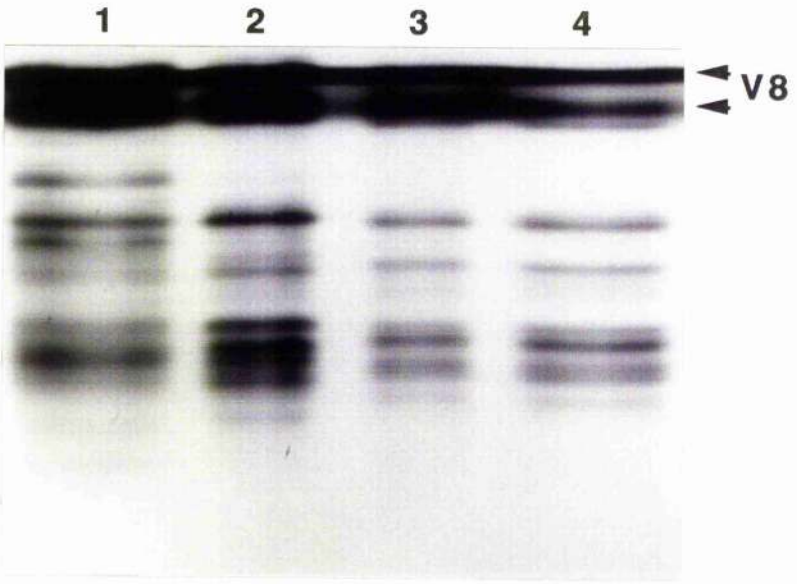


Figure 4. Peptide maps of myosin heavy chains from adult deep white muscle (lane 1), 1-group juvenile inner muscle fibres (lane 2), inner muscle fibres from larvae that had completed metamorphosis (lane 3), and inner muscle fibres from larvae at 6 weeks post hatching (lane 4), digested with *Staphylococcus aureus* V8 protease. Peptides were separated on 15% acrylamide SDS PAGE gels and stained with silver.

Peptide maps of inner muscle myosin heavy chains from larvae at six weeks of age and larvae after the completion of metamorphosis were similar but those of 1-group juveniles and the deep white muscle of adults varied considerably (Fig. 4).

Discussion

During the development from larvae to adult, plaice express developmental isoforms of myosin light chain 2 and the myosin heavy chain sub-units. In contrast the alkali light chains of larval inner muscle fibres were apparently similar throughout development. The inner muscle fibres of newly hatched plaice larvae contained two unique isoforms of myosin light chain 2, easily distinguished by their pI (Fig. 1a). Neither of these isoforms were present in adult deep white or adult superficial white fibres. At metamorphosis both the larval and the juvenile isoforms of light chain 2 were present in the inner muscle fibres, and continued to be expressed by both the 0-group wild juveniles and the 20 week old laboratory reared fish. However 1-group juveniles had by this stage ceased to express the larval myosin light chain 2 isoforms. Two types of white fibres could be distinguished in adult plaice under dark field illumination and by their mATPase staining (see Chapter 4). The superficial white fibres showed the same pattern of myosin light chains as the juvenile inner fibres, a possible indication that superficial fibres are an undifferentiated stock of precursor fibres as suggested by Bone (1966) and Mosse and Hudson (1977).

A minimum of three different myosin heavy chain isoforms were identified in the fast muscle fibres of plaice during their development from larval inner muscle fibres to adult deep white fibres. Larvae before and after the completion of metamorphosis had similar V8 peptide maps of the myosin heavy chain component from the inner muscle fibres, but the deep white muscle fibres of 1-group juveniles and sexually mature adults both expressed different myosin heavy chain isoforms.

Histochemical studies of the swimming muscle of turbot, *Scophthalmus maximus*, also suggest the sequential expression of a range of myosin isoforms during the first few months of

life, with the pH sensitivity of the myofibrillar ATPase activity continually changing over this period (Calvo and Johnston 1992). Molecular biology studies performed on carp, *Cyprinus carpio*, have indicated a minimum of 28 myosin heavy chain isoforms expressed in different tissues and/or stages of development (Gerlach *et al.* 1990).

The ATPase activity of myosin and the maximum velocity of fibre shortening are generally recognised to be linked to the myosin heavy chain composition (Barany 1967, Reiser *et al.* 1988). Variations in the light chain 3 content of single fibres have also been quantitatively correlated with contractile velocity, suggesting a modulatory role for the alkali light chains (Greaser *et al.* 1988, Sweeney *et al.* 1986). Exactly how myosin light chain 2 composition influences the mechanical properties of muscle fibres in fish is unclear (Yancey and Johnston 1982). Studies on the role of myosin light chain 2 during the contraction of mammalian muscle fibres have shown that phosphorylation increases isometric force and the rate of force production at submaximal levels of calcium activation (Sweeney and Stull 1990). Phosphorylation of myosin light chain 2 may underlie the increased rate and extent of force production associated with isometric twitch potentiation in intact muscle fibres by increasing the rate constant describing the transition from non-force-generating cross bridges to force-generating states (Sweeney and Stull 1990). Metzger *et al.* (1989) suggested that phosphorylation of myosin light chain 2 modulates the movement of the cross-bridges away from the thick filament backbone and towards the thin filament. Hofmann *et al.* (1990) concluded that myosin light chain 2 may modulate the number of cross-bridges formed during Ca^{2+} activation and also the rate of cross-bridge detachment during shortening by influencing the conformation of the S1-S2 hinge region of the myosin sub-unit.

Periasamy *et al.* (1984) found that myosin heavy chain isoforms expressed throughout the development of rat skeletal

muscle are the products of different genes transiently expressed during development. In birds and mammals, myosin light chain isoforms appear to be produced from a single gene by an alternative splicing mechanism and/or differential transcription (Barton and Buckingham 1985). However, the myosin alkali light chains of the grey mullet, *Mugil capito,* show a large number of amino acid sequence differences which suggest that in teleosts, light chain 1 and light chain 3 are the products of two different genes (Dalla Libera *et al.* 1991).

Regulation of the developmental contractile isoforms of myosin appears to be influenced by both myogenic and neurogenic factors (Hoh 1991). Weydert *et al.* (1987) demonstrated that the divergence of mammalian primary fibres into fast and slow fibre types was independent of innervation. However the later development of muscle fibres and many of the changes in contractile protein isoform expression that occur during development appear to be either directly or indirectly linked to the innervation of the muscles (Van Horn and Crow 1989). Nerves may also regulate the pattern of protein synthesis within the muscle fibres (Bandman *et al.* 1982). Thyroid hormones have also been shown to play a part in the developmental modulation of myosin expression (Gardahaut *et al.* 1992).

Developmental changes in the expression of myosins may plausibly reflect changes in the functional characteristics of the muscles as the fish grows. Larval and adult plaice have very different modes of swimming (Batty 1981), resulting from an increase in Reynolds number as the larvae grow and inertial forces gradually become more important than viscous forces (Batty 1984). In the larvae, speed of the propulsive wave v and tail beat frequency are not firmly linked to the swimming speed at cruising speeds as is the case in adult plaice. The ratio of swimming speed u to body wave speed v increases with increasing speed in larval plaice whereas in the adults it has been found to decrease slightly with increasing

speed (Batty 1981). In larvae *u:v* is much lower (0.2-0.4) than in adult fish (0.6-0.8) showing that propeller efficiency is greater in the adult fish (Batty 1981). Myofibrillar ATPase activity declines with length in the plaice (Witthames and Greer Walker 1982). The decline was related to a decrease in the *in vitro* muscle contraction rate, appearing to be a scale rather than an age effect (Witthames and Greer Walker 1982). In contrast the glycolytic enzyme activity of the white muscle fibres increases with length (Somero and Childress 1982). The relative metabolic cost of swimming is dramatically affected by body size (Webb 1977), linked to a change in the relationship between scaling of anaerobic metabolism and maximum aerobic metabolism. Aerobic-anaerobic interactions are important because power requirements during burst and sprint swimming are partially provided by aerobic metabolism and because the recovery period following an anaerobic episode is also dependent on aerobic metabolism (Goolish 1991). Studies by Hinterleitner *et al.* (1987), El Fiky *et al.* (1987), El Fiky and Wieser (1988), suggest that the swimming of early larvae is almost entirely aerobic. As the larvae increase in size their weight-specific aerobic capacity declines, because of decreasing surface area and an increase in the amount of time taken for blood to circulate.

Scaling effects on muscle contractile properties have only been studied in a few species and only for relatively large juvenile and adult fish. For example, in the Atlantic cod (*Gadus morhua*) the cycle frequency required for maximum power output during cyclical contractions decreases with fish length (Anderson and Johnston 1992), but under optimal conditions of strain and stimulation the maximum power output of fast muscle fibres is relatively scale independent for fish measuring between 10 cm and 60 cm standard length (Anderson and Johnston 1992). Williams and Brown (1992) found no change in maximum escape swimming speed following metamorphosis and settling in a related flatfish species, *Pleuronectes americanus.* Certainly, in *Pleuronectes platessa*

body length rather than metamorphosis would appear to be the main determinant of myosin composition in the presumptive fast muscle mass of juvenile fish.

Chapter 7

General Discussion

The metamorphosis from a pelagic larva to a benthic juvenile is one of the most striking features of the plaice life cycle. This change in lifestyle is associated with drastic changes in appearance, the left eye migrates over to the right hand side of the skull, and body shape alters from bilaterally symmetrical to asymmetrical. These dramatic changes in external morphology are not however, accompanied by any change in muscle fibre distribution in the myotomes. After metamorphosis is complete, plaice retain the arrangement of muscle fibre types characteristic of larvae (Chapter 4).

In contrast, metamorphosis in herring larvae is associated with a shift from larval to adult muscle fibre types (Batty 1984). Herring larvae complete metamorphosis by 40 mm total length and remain pelagic throughout their life cycle (Batty 1984). Immunocytochemical studies of herring at different developmental stages have shown that the expression of myosin light chain 3 is switched off in the superficial muscle fibres during metamorphosis (Johnston and Horne 1993). Adult slow fibres, which do not contain myosin light chain 3, form externally to the larval superficial muscle (Johnston and Horne 1993).

Larvae of the Japanese flounder, *Paralichthys olivaceus,* which have a similar life cycle to plaice, exhibit dramatic changes in both swimming behaviour (Fukuhara 1986) and muscle structure during metamorphosis (Yamano *et al.* 1991). Metamorphosis in the Japanese flounder is characterised by a behavioural change from slow swimming pelagic larvae to fast swimming benthic juveniles (Fukuhara 1986). Fukuhara (1986) correlated change in swimming style with morphological change in the finfold, while Yamano *et al.* (1991) linked the transition in swimming styles to change in muscle morphology.

Before metamorphosis, the myotomal muscles of *Paralichthys olivaceus* were characterised by large numbers of vacuoles and basophilic sarcoplasm. After metamorphosis myofibrils were more abundant and the vacuolar structure had completely disappeared (Yamano *et al.* 1991). Another species of flounder, *Pleuronectes americanus*, shows no change in escape response parameters during metamorphosis (Williams and Brown 1992). The muscle structure of *Pleuronectes americanus* has not been examined and so no correlation can be made between locomotory requirements and muscle morphology.

One of the major findings of this study was that in plaice developmental isoforms of myosin were expressed sequentially. The myosin sub-unit composition of the inner muscle fibres changed during development (Chapter 6), even though the distribution of muscle fibres remained unaltered in juvenile plaice after the completion of metamorphosis. The myosin heavy chain component was similar in plaice before and after metamorphosis, but myosin light chain sub-unit composition of the inner muscle fibres varied (Chapter 6). After the completion of metamorphosis plaice inner muscle fibres simultaneously expressed both larval and adult isoforms of myosin light chain 2. A similar transition from larval to juvenile isoforms of troponin T was observed in the Japanese flounder, *Paralichthys olivaceus* (Yamano *et al.* 1991) where it was linked to the changes in swimming style described earlier. Why the isoforms of myosin light chain 2 expressed should alter during plaice development is unclear. It is not known if myosin light chain 2 composition does alter the contractile properties of plaice muscle. In mammals, myosin light chain 2 appears to modulate cross-bridge formation (Metzger *et al.* 1989, Hofman *et al.* 1990). Phosphorylation of mammalian myosin light chain 2 apparently increases the transition from non-force-generating cross-bridges to force-generating states, thereby increasing isometric force and rate of force production (Sweeney and Stull 1990). The role of myosin light chain 2 during the contraction of teleost muscle fibres is

unclear. Phosphorylation of light chain 2 does not seem to form an integral part of the excitation-contraction cycle in fish muscle (Yancey and Johnston 1982).

The myosin heavy chain sub-unit composition of plaice inner muscle fibres, although unaffected by metamorphosis, did alter throughout development from juvenile to adult (Chapter 6). In birds and mammals, the myosin heavy chain has been strongly linked with change in velocity of muscle shortening during development (Reiser *et al.* 1988). The developmental stages at which transitions in myosin heavy chain were observed in plaice tend to suggest that muscle fibre structure is linked to length increases and changing Reynolds Number rather than to external appearance and behaviour.

Influence of temperature on muscle fibre development

Temperature influenced both the relative timing of muscle fibre differentiation in the plaice embryo (Chapter 3) and the ultrastructure of the muscle fibres in newly hatched plaice larvae (Chapter 4). How muscle fibre structure at hatching affects the subsequent survival of the larvae is unclear. Plaice larvae reared at 5°C had slower escape velocities and swimming speeds (measured as body lengths/second) than plaice larvae hatching at 12°C (Gibson personal communication). Batty and Blaxter (1992) showed that the muscle contraction time of an initial C-start made by a newly hatched larvae was temperature-dependent. Batty *et al.* (1993) observed no differences in locomotor performance of larvae reared at a particular temperature compared with larvae transferred to that temperature from another temperature with a 2 hour acclimation period. The contraction time of adult plaice muscle was also dependent upon ambient temperature and not upon acclimation temperature (Wardle 1980).

Studies on other teleost species have shown that muscle fibre structure continues to be influenced by temperature throughout the juvenile and adult stages of the lifecycle (Johnston and Lucking 1978, Johnston and Maitland 1980, Heap *et al.* 1987, Calvo and Johnston 1992). In another flatfish species, *Scophthalmus maximus*, temperature continued to influence muscle structure after metamorphosis (Calvo and Johnston 1992). Relatively small differences in rearing temperature were sufficient to affect the distribution of muscle fibre types and energy storage levels in larval and juvenile fish (Calvo and Johnston 1992). This study concentrated upon the ways in which temperature influenced muscle fibre structure of plaice embryos and newly hatched larvae. Temperature may be capable of influencing hypertrophic and hyperplastic growth in plaice during the larval period and after the completion of metamorphosis as seen in juvenile turbot (Calvo and Johnston 1992). Temperature may also affect the contractile protein composition of plaice muscle fibres, at all stages of the life cycle. Common carp, *Cyprinus carpio,* which are regularly exposed to large fluctuations in temperature; have been shown to express different isoforms of myosin after hot- (30°) and cold-acclimation (10°C) (Watabe *et al.* 1992). The myosin light chain 2 sub-unit composition of plaice larvae at hatching was independent of temperature (not illustrated) but myosin heavy chain composition remains to be investigated, as do any links between temperature and the myosin isoforms expressed by muscle fibres.

As plaice grow and develop the temperature at which they show maximum growth increases (Hovenkamp and Witte 1991). In the normal course of development, juvenile plaice are exposed to temperatures lethal to larvae (Hovenkamp and Witte 1991). Herzig and Winkler (1986) observed an apparent correlation between the range of temperatures tolerated by teleost larvae and the temperatures at which the adults would spawn. Plaice eggs were successfully incubated at

temperatures ranging from 5-12°C during this study. Adult plaice and eggs would not normally experience seawater temperatures of 12°C around the British Isles during the spawning season. Near surface sea temperatures recorded in the North Channel between Ireland and the West of Scotland over the past 40 years have ranged from 4.8-9.8°C during March (Jones and Jeffs 1991), with temperatures averaging 7.2°C during the spawning season. The distribution of plaice does, however, extend south into the Mediterranean (Wimpenny 1953), where sea surface temperatures could reach 12°C during the breeding season. The extent to which thermal history of the larvae makes a significant contribution to physiological variation in the adults is as yet unknown.

Future Studies

Possible areas of further study include the influence of temperature on muscle development and structure in juvenile and adult plaice, and the effect muscle structure has upon swimming performance at different temperatures. Additional research is also needed to investigate in greater detail the links between muscle structure and mode of swimming during development. The development of antibodies specific to the myosin isoforms expressed during development would enable closer examination of muscle fibre differentiation in plaice embryos, larvae and juveniles. If, in addition, plaice swimming was recorded at each developmental stage on high-speed video, changes in muscle fibre structure could then be studied in conjunction with any hydrodynamic differences. Another potential area of investigation is to determine the mechanism by which plaice regulate myosin sub-unit composition, muscle fibre formation and distribution of fibre types. In mammals and birds, thyroid hormones appear to contribute to the regulation of myosin isoform expression

(Russell *et al.* 1988, Gardahaut *et al.* 1992) which suggests that thyroid hormones may also be linked with the regulation of the contractile protein isoforms produced at the different stages of the teleost life cycle (Chapter 6). Smolting of salmon parr is influenced to a certain extent by thyroid hormone (Hoar 1988). Thyroid hormones do not trigger smolting in salmon, instead thyroid hormones appear to enhance smolting characteristics that are regulated endogenously, or by other hormonal factors (Hoar 1988). The thyroid hormones appear to influence metamorphosis itself in *Paralichthys olivaceus*, the Japanese flounder (Inui and Miwa 1985). Treatment of flounder larvae with thyroid hormone induces precocious metamorphosis, whereas treatment with thiourea inhibits metamorphosis (Inui and Miwa 1985). Significant quantities of thyroid hormones are consistently found in teleost eggs (Tagawa *et al.* 1990). Levels of thyroid hormone fluctuate within the eggs, possibly as they are utilised and metabolised by the developing embryo (Tagawa *et al.* 1990). The biochemical and morphological changes in muscle structure which occur during the metamorphosis of *Paralichthys olivaceus* are regulated by thyroid hormones (Yamano *et al.* 1991). Thyroxine precociously induces the larval muscle to become the juvenile phenotype, while thiourea inhibits the developmental changes (Yamano *et al.* 1991).

Muscle fibre innervation has also been linked with the control of contractile protein isoform expression in mammals (Condon *et al.* 1990). Van Raamsdonk *et al.* (1984) suggested that innervation was unlikely to regulate the differentiation of the different fibre fibre types within the myotomes of embryonic zebrafish, *Brachydanio rerio*. Within the zebrafish embryo formation of the various types of muscle fibres precedes the differentiation of the spinal cord cells, and the spinal cord sensory neurones develop before the motorneurones (Van Raamsdonk *et al.* 1984). Development of the muscle fibres could be myogenically regulated. Each fibre type could form through the differential expression of the genes coding

for the muscle fibre proteins, as suggested by Hoh *et al.* (1988). Muscle fibre development in the plaice could be regulated by a combination of hormones, innervation or myogenic factors making it an extremely interesting area for future research.

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Appendix

Fixatives (Mahoney 1966)

Formol-Saline

Formalin	10 ml
Sodium chloride	0.9 g
Distilled water to	100 ml

Bouin's Fluid

Picric acid (saturated solution)	75 ml
Formalin	25 ml
Glacial acetic acid	5 ml

Zenker's Fluid

Mercuric chloride	5 g
Potassium dichromate	2.5 g
Sodium sulphate	1 g
Distilled water to	100 ml

Immediately before use 5 ml of glacial acetic acid was added.

Heidenhain's "Susa"

Mercuric chloride	4.5 g
Sodium chloride	0.5 g
Trichloroacetic acid	2 g
Glacial acetic acid	4 ml
Formalin	20 ml
Distilled water to	100 ml

Neutral buffered formalin

Formalin	100 ml
Disodium hydrogen phosphate	11.7 g
Sodium dihydrogen phosphate	7.2 g
Distilled water to	1 litre

Buffers

Phosphate buffer

Disodium hydrogen phosphate	100 mM
Sodium dihydrogen phosphate	100 mM
pH 7.2	

Phosphate buffered saline (PBS)

Sodium chloride	160 mM
Disodium hydrogen phosphate	10 mM
Sodium dihydrogen phosphate	10 mM
pH 7.2	

Cacodylate buffer

Sodium cacodylate	200 mM
pH 7.2 using 1N HCl	

Histological Stains

Ehrlich's Haematoxylin

Haematoxylin powder	10 g
Absolute alcohol	500 ml
Glycerol	500 ml
Distilled water	500 ml
Glacial acetic acid	50 ml
Aluminium potassium sulphate	in excess

Ripen at room temperature in a flask stoppered with cotton wool. Shake flask periodically and place in sunlight if possible. Ripening period at least 6 weeks.

Eosin

Eosin	0.5%
in distilled water.	

Toluine Blue

Toluine blue	0.5%
in Borax solution	0.25%

Electrophoresis

Electrophoretic equipment used

Gels were run using a Biorad Protean Cell and the Biorad Mini-Protean II dual slab cell with a Mini 2-D electrophoresis module which transforms the Mini-Protean II cell into a miniature 2-D electrophoresis cell.

Vertical slab gels were used for both SDS PAGE and alkali urea PAGE applications. Gels 160 mm x160 mm x1.5 mm were cast and run on the Protean Cell, well formers were used which allowed a maximum of either 100 or 150 µl of sample to be loaded.

Mini-gels used were 70 mm in length x 80 mm wide and either 0.75 mm gel thickness or 1 mm gel thickness (2nd dimension IEF). Sample application wells contained a maximum volume of 30 µl.

IEF gels were cast in capillary tubes 1 mm diameter, placed within a larger casting tube. They were then attached to molded sample reservoirs which would contain upto 100 µl of sample.

Equipment cleaning

Before use all electrophoresis equipment was soaked overnight in a solution of Decon and then cleaned with more Decon and paper towels. It was rinsed extensively in tap water to completely remove the Decon and then rinsed in deionised Milli Q water and left to drain. Equipment was dried ready for use with clean paper towels. Acetone was used to degrease the glass plates, these were first wiped with acetone soaked paper towels and then rinsed with a stream of acetone,

this removed any dust that may have settled. Once acetone had evapourated from the plates they were ready to assemble.

Preparation of Gels

All slab gels were prepared and cast in the same way, only the reagents differed depending upon the type of gel.

Stock solutions of all gel components were prepared and stored for convenience. The required volumes of these were accurately measured out, mixed together and diluted with Milli Q water to the final volume, less that of the TEMED and ammonium persulphate necessary for polymerisation. The solution was degassed in a large vessel connected to a vacuum pump before the TEMED and ammonium polysulphate were added gently and mixed in carefully to keep the introduction of oxygen to minimum.

The difference in size between the two slab systems meant that gels were poured differently. Mini-gels could be poured using a 1 ml Gilson automatic pipette. The larger gels were poured by first placing the mixture in a large 100 ml syringe with the plunger removed and a tap fitted, and then controlling the flow between the plates so that no air bubbles were trapped. During filling gel holder cassettes were rocked gently, which helped to prevent eddy formation.

Gels without a stacking gel were filled to the top of the glass plates and well formers inserted. Polymerisation occurred within 15 minutes, however mini-gels were left for at least 45 minutes before using and large gels for 2 hours.

Those gels requiring a stacking gel were filled to the necessary depth with resolving gel and overlaid with isobutanol. They were then left to polymerise for 30 minutes before the stacking gel was poured on top. The isobutanol was first poured off, the gel surface washed twice with Milli Q

water and then a small volume of gel solution without the polmerising agents was used to equilibrate the surface of the resolving gel to the stacking gel. This was poured off prior to putting on the actual gel. The well formers were then placed in position. The stacking gel should polymerise within 15 minutes from pouring but were usually left to polmerise for 30 minutes. The total length of polymerisation time for the resolving gel was 1 hour for mini-gels and 2 hours for the large gels. The well forming combs were left in place until the gel was ready to be used, they were then carefully removed and the wells washed twice with electrode buffer.

IEF gels for the Mini-Protean II 2-D cell were cast in the following way;- Capillary tubes, 1 mm in diameter, are sold together with a larger casting tube, 10 mm in diameter. The large tube was soaked in Decon and rinsed the same way as the glass plates for the slab gels were, but not rinsed in acetone. One end was covered with parafilm and the tube was placed parafilmed end downwards in a clamp stand. The gel solution was prepared by combining all the reagents, except TEMED and ammonium polysulphate, and dissolving them by gentle aigitation and warming in a 25°C water bath. When high temperatures are used to dissolve urea there is a loss of buffering capacity because of "forced ageing" of the Ampholytes. The gel solution was degassed on a vacuum pump before the TEMED and ammonium polysulphate were added. The gel mixture was pipetted into the casting tube and the capillary tubes stood upright within the tube so that the liquid moved into them by capillary action. Special care was taken when placing the small tubes within the larger one not to trap any air bubbles. More gel mixture was poured down the side of the casting tube until it reached the required level within the capillary tubes. Tube gels polymerised within 20 minutes and were ready to use 1 hour after pouring. Before use capillary tubes were removed from the casting tube and any excess gel was cleaned off before the tubes were fitted into the sample reservoirs and the tube adapter.

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