University of St Andrews

Contractile Protein Isoforms in two Tilapiine Fishes (*Oreochromis niloticus* and *O. andersoni*) and their F1 Hybrid

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Contents

Declaration

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

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

Certificate

I hereby declare that K. Eric Wommack has spent three terms engaged in research work under my direction, and that he has fulfilled the conditions of General Ordinance No. 2 (Resolution of the University Court No. 1, 1967) and that he is qualified to submit the accompanying thesis for the Degree of Master of Science.

Summary

Chapter 1

Polymorphism of the contractile proteins of skeletal and cardiac muscle in vertebrates is briefly reviewed. Specia attention placed on the limited findings concerning contra protein isoforms in fish. Background information on the structure and function of each protein is also presented. Current knowledge and theories on the genetic mechanism responsible for the production of contractile protein isofo are summarized. Particular reference is made to genetic studies on variation within fish populations. Finally, stu on the adaptation of contractile proteins for function at different temperatures are reviewed. The aim of the pre study was to characterize the contractile proteins in fast muscle of two *Oreochromis* species (*Oreochromis niloticus*, and *O. andersoni*) which are known to have very different force-temperature relationships.

Chapter 2

Electrophoretic techniques were utilized to obtain information on the apparent molecular weight and relative charge of the contractile proteins of fast, skeletal muscle from *O. niloticus*, *O. andersoni*, and their F1 hybrid. Among the three study animals isoform differences were exhibited in myosin light chain 1 and troponin I. The two isoforms of myosin light chain

1, LC-1a and LC-1b, had apparent molecular weights (Mr) of 23.5kD and 22kD, respectively. *O. niloticus* contained only LC-1b. In *O. andersoni* myosin light chain 1 expression showed intraspecific variation, with individuals exhibiting either both isoforms or only LC-1a. Similarly, in the F1 hybrid myosin light chain 1 expression varied intraspecifically as individuals exhibited either both isoforms or only LC-1b. Myosin light chain 1b in *O. andersoni* appeared to have a different isoelectric point (pI) than LC-1b in *O. niloticus.* Troponin I (Tn-I), Mr of 21kD, appeared in both parental species and the hybrid. In addition, *O. andersoni* and the hybrid contained another troponin I isoform, troponin Ix (Tn-Ix). Troponin I and Tn-Ix differ markedly in molecular weight with Tn-Ix having an Mr of 22.7kD. In the presence of 5mM Ca^{+2} Tn-Ix appears to have a greater binding affinity for troponin C.

Chapter 3

Myosin heavy chains were electrophoretically purified from fast muscle of *O. andersoni* and analysed for intraspecific isoform variation using the technique of peptide mapping by limited proteolysis. Intraspecific variation in myosin heavy chain isoform expression was observed; however, this variation was not correlated to isoform variation in myosin light chain 1.

Chapter 4

The possible implications of the major findings of this work are discussed. Proposals are made for future work to further investigate the polymorphic character of the contractile proteins in *O. andersoni*, *O. niloticus*, and their F1 hybrid.

Chapter 1

General Introduction

Polymorphism of Contractile Proteins

The physiological properties and anatomical organization of vertebrate skeletal muscle illustrates a wide range of variation. Yet, on an ultrastructural level the basic organization of the myofibrils into myosin thick and actin thin filaments is highly conserved throughout all muscle. This is the essential paradox of comparative muscle physiology which has prompted investigation of differences in the contractile proteins as the source of variation. Two proteins which are similar in function and higher structure, but differ in primary amino acid sequence and possibly activity are defined as isoforms. Comparative molecular physiology of muscle is largely concerned with identifying contractile protein isoforms and investigating the physiological ramifications of isoform differences.

Myosin:

Myosin, which accounts for 46% of the myofibrillar protein mass, is the major protein of the myofibrils (Huxley and Hanson, 1957). The myosin molecule (480kd) is polymeric in structure consisting of two heavy chains (200kD each) and four light chains (16-30kD each). These subunits are readily dissociated
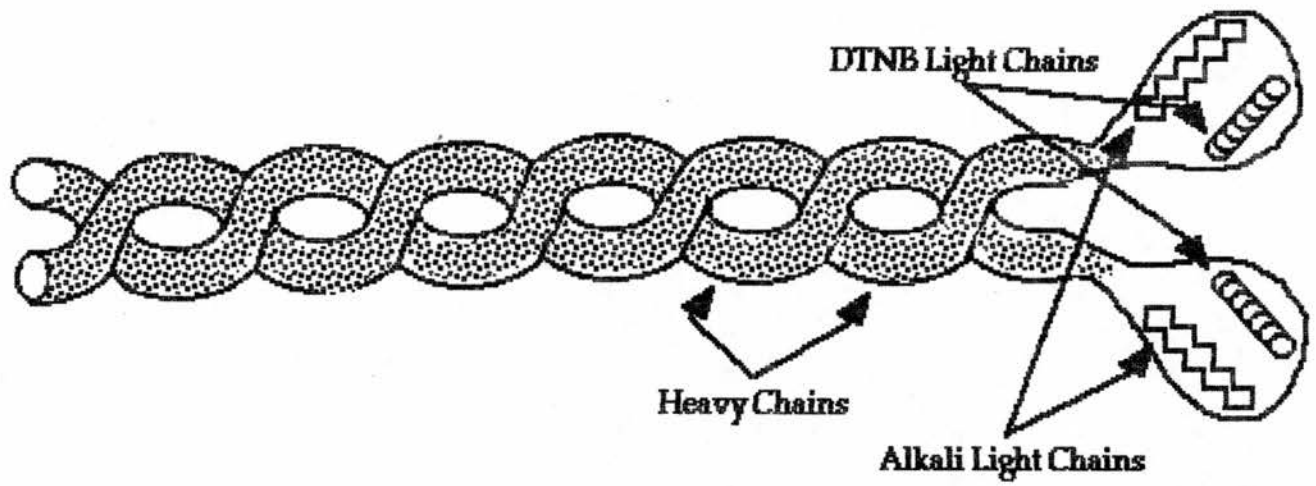
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Figure 1.0 Schematic model of Myosin

in 0.1% sodium dodecyl sulfate (SDS) or 6M Urea (fig. 1.0). The myosin heavy chain (MHC) is largely alpha helicial in secondary structure and is essential for the two functions of myosin. The C terminal half of MHC combines with neighboring MHC's to form the thick filament, while the globular N-terminal half protrudes from the thick filament in order to bind with actin and perform the ATPase function. Functional regions of the MHC have been identified by first fractionating myosin with proteolytic enzymes and then analysing the proteolytic fragments (Lowey *et. al.*1969). Using this method the N-terminal has been identified as the S-1 subfragment or S-1 head. Rushbrook *et. al.* (1987) confirmed the existence of isomyosins, isoforms of myosin, between dystrophic and normal chickens by differences in S-1 subfragments.

Myosin light chains are associated with the globular subfragment 1 (S1) end of the myosin heavy chain. Bound to each S1 head is one 5',5'-dithobis-(2-nitrobenzoic acid) (DTNB) light chain (LC-2), which undergoes phosphorylation, and one alkali light chain either LC-1 or LC-3 (Weeds, and Lowey, 1971; and, Dow and Stracher, 1971). The number designation of the myosin light chains refers to their molecular weights in decreasing order LC-1> LC-2> LC-3. Perrie and Perry (1970) speculated that the existence of isomyosins was a result of differences in the myosin light chains. Holt and Lowey (1977) later proved this point using affinity chromotography. The difference between the isolated isomyosins depended on the alkali light chain content of the two S1 subfragments. Three combinations

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were possible: a homodimer of LC-1; a homodimer of LC-3; or a heterodimer containing one LC-1 and one LC-3.

Non-denaturing electrophoretic separation, using the pyrophosphate system of Hoh (1975), has provided evidence for fibre type isomyosins in chick (Hoh *et. al.* 1976) and frog (Lannergren and Hoh, 1984). This method also allows measurement of ATPase activity of the isolated isomyosins (Hoh *et. al.* 1976). Native isomyosin separation is limited by the inherent difficulties of resolving highly similar large molecules; furthermore, it reveals no information on which myosin subunit isoforms are responsible for the observed variation. In order to overcome the latter limitation Billeter *et. al.* (1981); and Biral *et. al.* (1984) combined native and dissociating techniques to identify isomyosins in human and rabbit (d'Albis *et. al.*, 1979) skeletal muscles. Most isomyosin research has concentrated on analysing heavy and light chain subunits all of which have been shown to exist as isoforms.

Myosin Heavy Chain isoforms (MHC):

In general the structure of MHC is highly conserved as evidenced from comparison of cloned mRNA's (Kavinsky *et. al.*, 1983). However, MHC isoforms have been shown to exist in numerous studies (reviewed by Swynghedauw,1986; and Syrovy, 1987). The importance of myosin heavy chain in myosin function is emphasized as isomyosins are defined by the MHC isoform they contain (Whalen, 1985).

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Within mammalian skeletal muscle eight MHC isoforms have been uncovered: embryonic and neonatal MHCs (Whalen, *et. al.* 1979; and Hoh and Yeoh, 1979), two in fast muscle (Salviati, *et. al*, 1982; and Staron and Pette, 1987b), two in slow muscle of chicken (Rushbrook and Stracher, 1979), and two distinct superfast muscle MHC isoforms in cat jaw (Rowlerson, *et. al.* 1981) and bovine extraocular muscle (Sartore, *et. al.* 1987). Cardiac muscle is much less heterogenous exhibiting only two MHC isoforms, alpha and beta MHC (Sartore *et. al.*, 1978; Hoh *et. al.*, 1978) of which alpha is identical to slow MHC in skeletal muscle (Lompre *et. al.* 1984).

Myosin Light Chains (LC):

Each myosin light chain exists in isoforms according to fibre or tissue type. Myosin light chains 1, 2, and 3 of fast muscle have molecular weights of 21kD, 18-19kD, and 16.5kD, respectively. These proteins are easily resolved using denaturing electrophoretic techniques. Slow muscle also contains three light chains of molecular weights 27.5kD, 26.5kD, and 17.5kD; however, LC-1s and LC-2s comigrate in SDS electrophoresis and are not resolved (Sreter, *et. al.* 1972). Light chains of cardiac muscle appear as either ventricular isoforms LC-1v (26-28kD) and LC-2v (20-21kD) or atrial isoforms LC-1a (24-26kD) and LC-2a (26.5kD) (Sartore, *et. al.* 1978). All of these light chains are distinct with the exception of the Hoh *et. al.* (1978) finding that LC-1v is identical to LC-1s in rat. One other cardiac myosin light chain isoform has been

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observed during development. Whalen *et. al.* (1981) found a specific myosin light chain (LCemb) in fetal atria and ventricles which strongly resembled adult LC-1a.

Studies into the role of the light chains in muscle physiology have revealed confusing and contradictory results. Dow and Stracher (1971) found that LC-1 and LC-2 were essential for full activity of myosin ATPase. Whereas, Weeds and Lowey (1971) found LC-1 and LC-3 to be essential for myosin ATPase activity. In later studies Wagner and Weeds (1977) cross hybridized subfragment-1, containing the alkali light chains, from fast myosin onto slow myosin and visa versa. They found that the alkali light chain present affected the actin activated ATPase. Wagner and Giniger (1981) challenged this conclusion by finding no significant difference in the actin binding or ATPase properties of myosin devoid of alkali lightchains.

Actin (Act):

Among all the contractile proteins actin is the most highly conserved exhibiting no isoforms between slow and fast fibre types. In vitro actin exhibits two forms: G or globular actin (43-48kD); and F or filament actin. F actin is the polymeric form which is spontaneously polymerizes from G actin through the hydrolysis of ATP. Actin is also the main protein of the thin filament comprising 21% of the total myofibrillar protein mass (Huxley and Hanson, 1957).

Vandekerchove and Weber (1979) in comparing amino acid sequences between bovine cardiac and rabbit skeletal muscle

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found only 4 out of 375 amino acid residues were different. Actin isolated from fast and slow muscle was found to activate myosin ATPase equally (Gergely *et. al.* 1965). This evidence suggests that the role of actin in the contractile mechanism is similar in all muscle types. Biochemical variation in the properties of the thin filament is due to isoform differences in the calcium regulatory proteins (Dhoot and Perry, 1979).

Calcium Regulatory Proteins:

The four proteins responsible for Ca^{+2} regulation in the myofibril are divided into two groups; tropomyosin, and the troponin complex. All have been shown to exist as isoforms; however, troponin isoforms generally correlate to muscle or fibre type. These proteins have been shown to be associated with the thin filament each constituting 5% of the total myofibrillar proteins (Ohtsuki *et. al.*, 1986).

Tropomyosin (Tm):

Tropomyosin, first isolated and described by Bailey (1948), is a filamentous protein of nearly 90% alpha helix secondary structure (Ooi *et. al.*, 1962). One Tm molecule is formed from two 33kD subunits, alpha Tm and beta Tm, which exist in different ratios depending on muscle fibre type (Bronson and Schachat, 1982). Mak *et. al.*(1980) found the two

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Tm isoforms to differ at 39 amino acid residues producing only slight differences in molecular weight and charge. Structurally, *in vivo* the two Tm subunits coil about one another and form head to tail filaments which are then located in the groove of the actin helix (Ebashi *et. al.*, 1969; and Flicker *et. al.*, 1982). Two other Tm isoforms, gamma Tm and delta Tm, were identified in rabbit soleus muscle using two dimensional electrophoresis (Heeley *et. al.*, 1983). The existence *in vivo* of heterodimer and homodimer combinations of the four Tm isomers is not proven. *In vitro* assembly of alpha and beta subunits into either Tm heterodimer or Tm homodimers all showed similar ability to induce Ca^{+2} sensitivity of actomyosin ATPase activity (Cummins and Perry, 1973). While Tm is not necessary for ATPase activity in actomyosin (Lehman and Szent-Gyorgyi, 1972); it is essential in order to produce Ca^{+2} sensitive Mg^{+2} ATPase of actomyosin in the presence of troponin. In the absence of functional differences between the Tm isoforms it is hypothesized that Tm variation is probably related to its interaction with the troponin complex. There is, for instance, evidence suggesting different binding affinities between troponin-T and alpha and beta tropomyosin (Perry, 1985).

Troponin Complex:

Troponin-T (Tn-T):

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The highly basic protein, Troponin-T, is named for its primary function of binding to tropomyosin. Tn-T occurs in isoforms of molecular weights ranging from 31kD to 36kD making it the largest of the troponin complex. Electron microscopy studies of the troponin complex have revealed conflicting evidence as to the placement of Tn-T. Flicker *et. al.* (1982) proposed that Tn-T comprised the filamentous tail region of the complex. Ebashi and Ohtsuki (1983) on the basis of similar techniques found inconclusive evidence for the microstructure of the troponin complex.

In comparison to isoforms of the other troponins, the isoforms of Tn-T present a complex picture of differentiation. Perry and Cole (1974) first reported the existence of two different Tn-T isoforms from leg and breast fast muscle of the chicken. This finding of Tn-T fast isoforms was later confirmed by Wilkinson (1977) who found no similar differences in fast isoform expression of troponin I or C. Later studies using sensitive electrophoretic techniques of single isolated rabbit muscle fibres have found two and three fast isoforms of Tn-T. The combinations of Tn-T and Tm isoforms in fast muscle were expressed in a continuum without respect to traditional histochemical fibre type (Schachat *et. al.*, 1985). Schachat *et. al.* (1987) later correlated the different Tn-T and Tm combinations to differences in the force Ca^{+2}/isometric tension relation, thus illustrating the effect of isoform expression on a particular contractile property of single muscle fibres.

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Similarly, Greaser *et. al.* (1988) found that three fast isoforms of Tn-T were expressed in different proportions in fast fibres. The relative proportions of these isoforms correlated to higher Ca^{+2} sensitivity of tension development in single fibres.

Troponin-I (Tn-I):

. Schaub and Perry (1969) and Hartshorne and Mueller (1968) were the first to identify the inhibitory factor protein as part of the troponin complex. Thus troponin-I has been named for its ability to inhibit Mg^{+2} ATPase activity of actomyosin especially in the presence of tropomyosin (Wilkinson *et. al.*, 1972). Tn-I isoforms are all basic proteins of molecular weights ranging from 21kD to 24kD. Dhoot *et. al.*, (1978) found fibre type specific Tn-I isoforms to be a property of muscle from a range of mammalian sources. They found immunoperoxidase staining on the basis of Tn-I localization to be a precise means of typing muscle fibres.

Wilkinson and Grand (1978) determined the amino acid sequence of four Tn-I isoforms from rabbit cardiac, slow and fast skeletal muscle, and chicken fast muscle. In the actin binding sites the Tn-I isoforms were highly similar; whereas, the Tn-C binding sites, in the N-terminal region, showed considerable sequence differences. The divergence in the Tn-C

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sites suggests isoform dependent differences in Tn-I/Tn-C interaction. Perry and Cole, (1974) found troponin-I from skeletal muscle to contain *in vitro* phosphorylation sites; however, these sites are probably unimportant *in vivo* as they will be complexed with Tn-C. Furthermore, they found no correlation between the Ca^{+2} sensitivity of actomyosin and the phosphorylation state of the troponin complex. Cardiac Tn-I was found to have an additional 26 residues which contained a major phosphorylation site at serine 20 (Grand *et. al.*, 1976). Phosphorylation of this site was found to be under hormonal control; and, to be inversely related to changes in the Ca^{+2} sensitivity of cardiac actomyosin (Perry, 1983; and England, 1983).

Troponin-C (Tn-C):

The calcium ion binding component of the troponin complex, troponin- C, is a highly soluble acidic protein of around 18kD molecular weight. Fast and slow isoforms of Tn-C exist; but, are not as tissue specific as Tn-I since slow and cardiac forms are identical Grand *et. al.*, (1976). Wilkinson (1980) argued that slow and cardiac Tn-C's are produced from a single gene as their amino acid sequences were identical. The fast isoform and the slow/cardiac isoform also differ in number of Ca^{+2} binding sites. There are two sites in the former and one in the latter Tn-C isoform (Potter and Johnson, 1982). This

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difference is functionally relevant as the binding of Ca^{+2} to Tn-C initiates myofibril contraction (Syrovy, 1987). While the Tn-C isoforms in slow and cardiac muscle are identical, their actual Ca^{+2} binding properties *in vitro* are probably determined by the difference in slow and cardiac Tn-I isoforms (Perry, 1985).

In summary the components of the troponin complex, and tropomyosin interact in the following model as proposed by Ohtsuki *et. al.* (1986) from the wealth of experimental evidence. Tn-T binds to tropomyosin, on the actin filament, and to troponin I. In the Ca^{+2} free state (relaxation) Tn-I binds strongly to actin and tropomyosin and weakly to troponin C, thereby, inhibiting myosin-actin interaction and preventing myosin ATPase activity. The binding of Ca^{+2} to Tn-C (contraction) strengthens the troponin I/ troponin C bond and weakens the troponin I bonds to actin and Tm. Calcium ions thus elimininate Tn-I's inhibition of myosin-actin interaction. Troponin T is required for Ca^{+2} sensitivity of contraction as it is the link holding Tn-I and Tn-C to the thin filament in the presence of Ca^{+2}.

Polymorphism of contractile proteins in fish muscle

In comparision to higher vertebrates there is relatively little information on the characteristics of muscle proteins of fish. The earliest studies concentrated on the instability of

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fish myosin. In two separate studies Connell (1960) and Hamoir (1960) both found that isolated preparations of fish muscle myosin were much more labile than similar mammalian preparations. This instability is characterized by rapid aggregation and loss of ATPase activity (Connell, 1963). In later studies Johnston (*et. al.*, 1975; 1980; Johnston and Walesby, 1977; 1979) found that the relative instability of fish myosin and loss of ATPase activity was inversely related to the temperature environment of the animal. He postulates that in skeletal muscle of ectotherms from colder environments myosins assume "...a more open molecular structure..." (Johnston *et. al.*, 1975).

Of the constituent components of myosin, the myosin light chains have been the most extensively studied in fish. Thus far only fast and slow isoforms of myosin heavy chain have been illustrated in skeletal muscle of roach, *Rutilus rutilus* (Karasinski and Kilarski, 1989) and carp *Cyprinus carpio* (Crockford, 1989). Among the myosin light chains fish exhibit the same isoforms as higher vertebrates; however, fish myosin light chains show a high degree of interspecific variation especially in the alkali light chains (Focant *et. al.*, 1976; Huriaux and Focant, 1985; Rowlerson *et. al.*, 1985).

Concerning the calcium regulatory proteins of fish fast muscle isoforms are similar to mammalian fast muscle isoforms (Johnston and Walesby, 1979 and Crockford, 1989). Johnston and Walesby (1979) observed that the thermal environment of a fish

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directly correlated to the stability of Ca^{+2} sensitivity of actomyosin ATPase activity over a range of assay temperatures. In essence the actomyosin from tropical fish maintained Ca^{+2} sensitivity over a wider range of temperatures than temperate or Antarctic fish. They also observed considerable variation of the Ca^{+2} regulatory proteins in particular troponin C.

Isolated cod tropomyosin had similar properties to isolated preparations from higher vertebrates (Odense *et. al.*, 1969). However evidence for the existence of both alpha and beta tropomyosin isoforms has been contradictory. Both Dabrowska and Szpancenko (1977) and Focant *et. al.*, (1981) found only alpha Tm in actomyosin preparations while Crockford (1989) found both alpha and beta Tm isoforms in myofibrillar preparations.

Molecular Genetics of Contractile Proteins

Experimental evidence on the structural genes of the contractile proteins is begining to indicate that most contractile protein isoforms actually arise from single genes (Swynghedauw, 1986 for review). So far it is known that a pool of about 22 structural genes code for all the contractile proteins in rabbit. In any one instance only 8 of these genes need to be expressed in order to synthesize a myofibril (Dhoot and Perry, 1979). It is this diversity of isogenes which lends

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credence to the Pette and Staron (1988) view of a continuum of isoform expression in muscle fibres rather than defined isoforms for specific fibre types.

Molecular genetic techniques have identified the mechanisms of isoform expression in certain contractile proteins. MHC and actin are both encoded by multigene families. Genes coding for MHC and actin are placed into either the sarcomeric gene family or the smooth muscle/non-muscle gene family. Within the sarcomeric MHC family of the rat seven MHC genes have been cloned. These seven genes correlate to the MHC isoforms already discovered in direct protein studies. Each of these isogenes encodes for MHC isoforms specific to developmental stage or muscle type (see review by Emerson and Bernstein, 1987).

Detailed sequence analysis of the sarcomeric MHC family has revealed three common features in these genes. First, the portion of the MHC molecule involved in filament assembly is highly conserved. Second, specific MHC isoform differences are observed in the final four amino acid residues of the COOH-terminal and the entire 3' non translated sequence. Finally, each gene contains many introns which are apparently uncorrelated to the functional regions of MHC (Nadal-Ginard *et. al.*, 1982; and Wydro *et. al.*, 1983). Within the sarcomeric actin gene family there are between 10 and 20 gene sequences; however, only skeletal and cardiac actin isoforms are expressed. This excess of sequences is probably due to pseudogenes which resemble

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functional genes but are no longer transcribed (Minty *et. al.*, 1982).

While it is generally thought that contractile protein isoforms arise from isogenes other mechanisms have been implicated in the production of myosin alkali light chains, troponin T, and tropomyosin. Myosin light chains 1 and 3 of fast muscle are markedly different in molecular weight; however, the first 141 amino acid residues from the COO- end are identical in the two proteins. (Periasamy *et. al.*, 1984). Nabeshima *et. al.* (1984) proved that LC-1 and LC-3 are encoded by the same gene. The differences between the two proteins is a result of alternative transcription of the LC-1/LC-3 gene to produce two different precursor RNAs.

Skeletal and smooth muscle exhibit two isoforms of alpha tropomyosin. Two studies (Pearson-White and Emerson, 1987; and Ruiz-Opazo and Nadal-Ginard, 1987) have found that both alpha Tm isoforms are encoded by one gene; and, are the result of alternative splicing of precursor RNAs to form isoform specific mRNAs. Similarly, alternative splicing of precursor RNAs has been implicated in production of troponin T isoforms in chicken fast skeletal muscle (Wilkinson *et. al.*, 1984) and human slow skeletal muscle (Gahlmann *et. al.*, 1987).

Genetic variation in fish

The study of genetic variation has been greatly enhanced through the use of gel electrophoresis of native proteins (Ayala

and Kieger, 1984). In brief, this method allows the researcher to assay the soluble enzymes from tissue homogenates of a large number of individuals. Staining techniques,which utilize specific enzyme-substrate reactions, are then applied to the gel to identify isoenzymes. From this information the allelic character (genotype) of a particular gene locus is inferred for each individual studied (Ayala and Kieger, 1984).

Native gel electrophoresis has quantified the level of genetic variability within wild fish populations of cod (Mork *et. al.*, 1985), herring   (Ryman *et. al.*, 1984), and the Atlantic eel (Voellestad, 1986). Powers and Place (1978) were able to link genotypic variation in the killfish, *Fundulus heteroclitus*, to clinal variation in temperature across its range. Later, Powers and Haedrich (1981) estimated the amount of enzyme polymorphism in *F. heteroclitus* and fish from other more stable environments. Polymorphism estimates correlated well to environmental variability in *F. heteroclitus*, but were not significant in other species.

Electrophoretic evidence has proven invalable in the study of the morphologically very similar Tilapiian species. McAndrew and Majumdar (1984) estimated, from electrophoretically determined allelic frequencies the evolutionary relationships of the three closely related cichlid genera: *Tilapia, Sarotherodon*, and *Oreochromis*. Established interspecific isoenzyme patterns have proven useful in identifying aquacultural stocks of Tilapiian fish species

(McAndrew and Majumdar, 1983). Such involved electrophoretic identification for cultured Tilapia is essential as they readily produce interspecific and intergenic hybrids (McAndrew, personal communication).

Pasdar and co-workers have, in two separate genetic studies, utilized the ability of sunfish of the genus *Lepomis* to form fertile F1 hybrids. Measurement of four specific enzyme activities in muscle homogenates found the values for the hybrid to be intermediate to those of the parents. They suggest that the coordinated response of enzyme activities in the hybrid within a tissue type could be due to either regulatory gene elements or "...the pleiotropic effect of changes at a single gene exerting effects at many developmental levels. (Pasdar, *et. al.* 1984)." Intermediate enzyme activities did not correlate to intermediate growth rate as sunfish F1 hybrids showed heterosis through greater growth rates than parental species. Therefore, alterations in the hybrid gene regulatory mechanisms exert different effects on molecular and morphological traits (Pasdar *et. al.*, 1984). However, heterosis of growth rate is not a ubiquitous feature of hybrid fish since it was not observed in interspecific F1 hybrids of the *Oreochromis* genus (McAndrew and Majumdar, 1989).

Initially, it would seem natural that the essential enzyme driving the contractile machinery, myosin ATPase, should be examined in a manner similar to the enzymes used for previous genetic studies. However, the unstable and insoluble nature of

myosin, especially in fish (Connell, 1959), and insolubility of other contractile proteins severely limits native electrophoretic techniques (Syrovy, 1984). Therefore, direct information on the isoform composition of contractile proteins is reliant on denaturing electrophoretic techniques.

Temperature and contractile properties of skinned muscle fibres

Physiological tests on the contractile properties of single muscle fibres are necessary to elucidate the possible functional role of contractile protein isoforms. Removal of the sarcolemma from single muscle fibres through either mechanical or chemical skinning allows the direct study of intact myofilaments (Johnston, 1983). Skinned single fibres, while revealing information on the characteristics of the cross bridge properties of muscle, do not indicate the locomotory performance of the animal (Johnston & Altringham, 1988). However, for the study of the effects of temperature on contractile properties, skinned fibres provide an ideal system as any temperature compensation by motor neurons or muscle membranes is effectively removed (Johnston & Brill, 1984). Previous studies using skinned single fibres of fish have related differences in contractile properties to: environmental temperature (Johnston & Brill, 1984; Johnston & Altringham,

1988; Johnston & Altringham, 1985; Altringham & Johnston, 1985b); acclimation temperature (Johnston *et. al.*, 1985; Altringham & Johnston, 1985a); fibre type (Altringham & Johnston, 1981; Altringham & Johnston, 1985); phosphate concentration (Altringham & Johnston, 1985c); and osmoregulatory solutes (Altringham *et. al.* 1982).

ATPase activity of myofibrils and actomyosin is a proven test of the metabolic requirements of different muscle fibre types in and between species (Johnston, Frearson, and Goldspink, 1972 and 1973). ATPase activity measurements when combined with contractile property data provides strong evidence for the adaptational effects of temperature. Johnston and Altringham (1985 & 1988) established the relationship of isometric force generation (Po), ATP utilization, and unloaded maximum contraction velocity (Vmax) in fish from Antarctic, temperate, and tropical temperature environments. They found that measurements of Po at environmental temperatures were similar; but, Po at 0^0c increased with respect to the temperature environment of the fish. Po values from 0^0c measurements illustrated capacity adaptation of the cross bridge machinery in fish from cold environments. Unlike isometric force, Vmax showed no correlation to thermal environment and was hypothesized to be a function of fibre type. Finally, ATP utilization in isometrically contracting fibres was highly similar across a range of experimental temperatures. The economy of contraction expressed as Po/ molecule ATP myosin

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$S1^{-1}$ s^{-1} increased in the order Antarctic >temperate > tropical species.

Previous experiments done by Ian Johnston, Gabriel Matungi, and Paul Harrison have tested the physiological criteria of ATPase activity and isometric force on two closely related tropical freshwater fish. *Oreochromis niloticus* is native to the Nile valley and equatorial regions of Africa, where annual water temperatures range between 25^0c and 35^0c. *O. andersoni* is native to the region of southern Africa below 15^0 south latitude. This region is cooler exhibiting annual water temperatures 16^0c to 28^0c (Balarin, 1979).

High performance liquid chromatography (HPLC) measurements, done by Paul Harrison, of ATPase utilization in isometrically contracting single skinned fibres (fig. 1.2) show similar myosin ATPase activities between *O. niloticus* and *O. andersoni* over a range of temperatures. However, the isometric tension (Po) generated by these fibres (fig. 1.3) diverged signifigantly at temperatures below $12-15^0c$ with *O. andersoni* > *O. niloticus* (Johnston and Matungi, pers. comm.). Therefore, the economy of contraction (Po/ isometric ATPase) at 0^0c was 50% higher in *O. andersoni*. The Po and economy of contraction measurements in the *O. niloticus- O. andersoni* F1 hybrid resembled those of *O. niloticus*. These physiological results indicate that *O. andersoni* is the more cold tolerant species. This finding illustrates the power of economy of contraction

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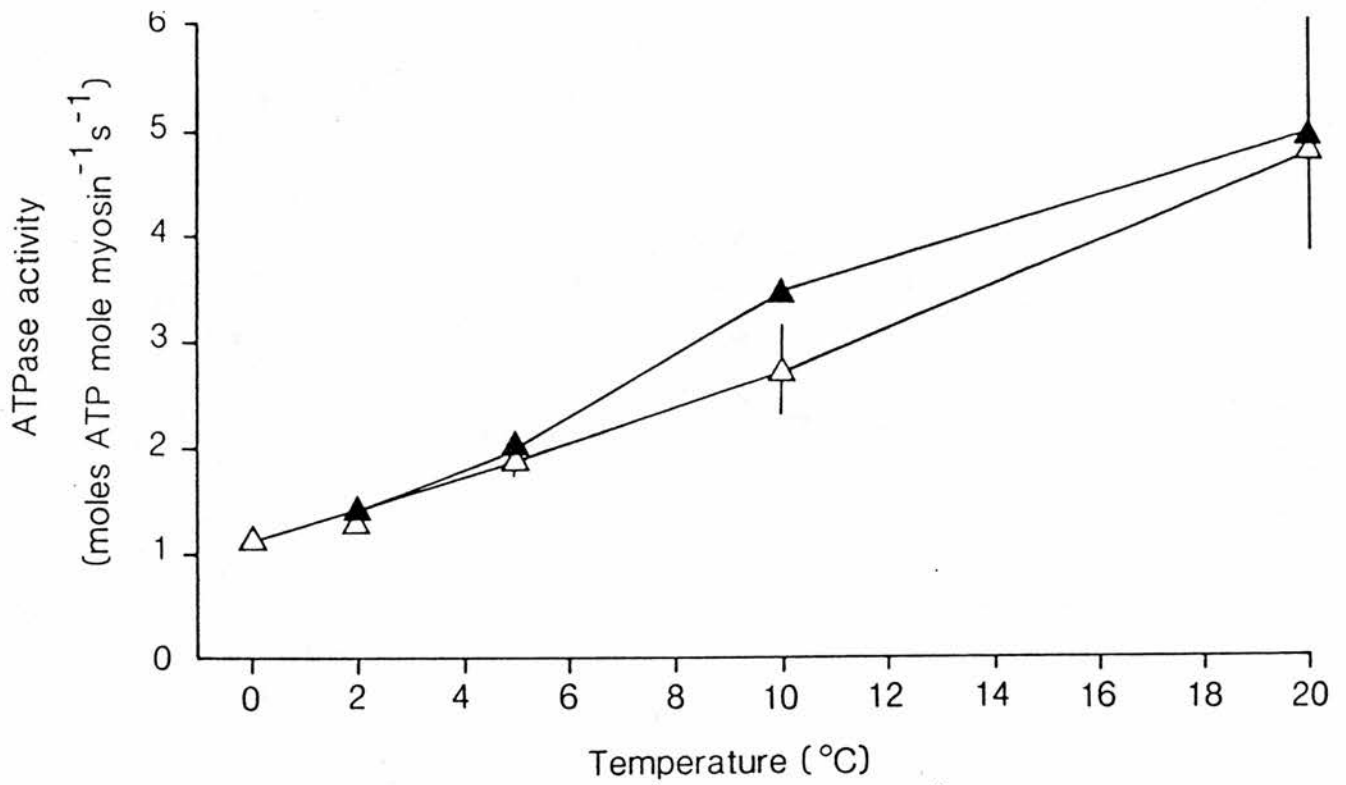
Figure 1.2 ATPase activity for isometrically contracting skinned fibres from *Oreochromis andersoni* (open triangles) and *O. niloticus*. Error bars represent mean ± standard error of 6-10 fast muscle fibres/species. (courtesy of P. Harrison).
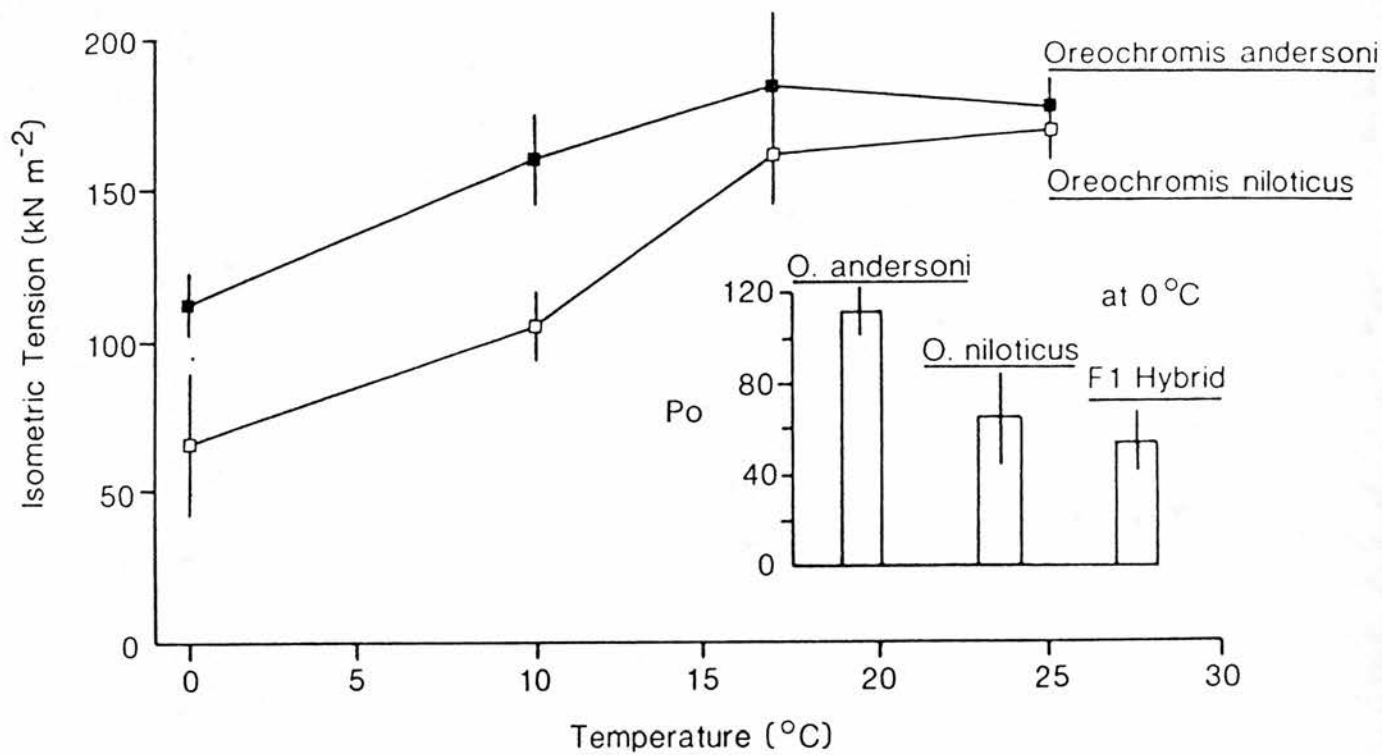
Figure 1.3   Isometric tension (Po) generation by skinned fibres from pure strain *O. niloticus* and *O. andersoni*.   Inset shows Po for hybrid offspring measured at 0°C.

measurements in delineating capacity adaptation in closely related fish of similar temperature environments.

The objectives of this study are to use electrophoretic techniques in order to characterize the myofibrillar proteins of *O. andersoni* and *O. niloticus*; and, to identify any intraspecific protein isoforms within species and interspecific protein isoforms between species. The inheritence of isoforms will be analyzed through the comparison of F1 hybrid proteins with those of the *O. andersoni* and *O. niloticus* parental strains.

Chapter 2

Polymorphism of Contractile Proteins in *Oreochromis niloticus, O. andersoni*, and their F1 Hybrid

Introduction

It is well established that the contractile proteins of fish are similar to those of higher vertebrates (see Johnston, 1983 for review). The key difference between fish muscle and mammalian muscle is the anatomical separation of fibre types in myotomal muscle (Bone, 1966; and Johnston, 1981 review). Another advantage of fish is that a large proportion of their body mass (40%-60%) is composed of locomotor muscle (Bone, 1978). This distinct division and quantity makes the myotomal muscle of fish ideal for sampling muscle tissue of homogeneous fibre type composition. This is especially true for fast muscle which makes up 90% of fish skeletal muscle (Johnston, 1981). Fast muscle was used for this comparative study.

Comparative studies of fish fast muscle, utilizing electrophoretic techniques, have established striking

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intergenic variation in the expression of the myosin alkali light chains (Rowlerson, *et. al* 1985; Perzanowska, Gerday, and Focant, 1978; Focant, and Huriaux, 1976; Focant, Huriaux, and Johnston, 1976; Huriaux, and Focant, 1985). This variability, however, is not correlated to lifestyle or locomotory characteristics. Intraspecific isoform variation in myosin light chains and troponin-I of carp has been implicated in temperature acclimation; however, the function of these isoforms in the acclimatory response is unknown (Crockford and Johnston, 1989; and Johnston, 1979). Indeed, the true function of the alkali light chains remains unclear.

In comparison to the alkali light chains the DTNB light chain (also referred to as light chain 2 or P-light chain) is less intergenically variable (Huriaux and Focant, 1985; Focant, Huriaux, and Johnston, 1976) and is capable of reversible phosphorylation *in vivo* (Barany and Barany, 1980). This phosphorylation is not thought to be involved in the contraction-relaxation cycle of fish fast muscle fibres (Johnston, 1982).

In an electrophoretic survey of carp fast muscle myofibrils Crockford (1989) reported isoforms of Tn-T and Tm consistent with previous findings in mammals. He also discovered an extra minor troponin-I isoform found only in warm acclimated fish. In goldfish acclimated to different

temperatures, the cross-hybridization of cold Ca^{+2} regulatory proteins onto warm desensitized actomyosin altered the actomyosin ATPase activity to that of natural cold actomyosin (Johnston, 1979).

The three aims of this electrophoretic survey were to: (A) characterize the contractile proteins of *O. niloticus* , and *O. andersoni* ; (B) investigate possible intraspecific and/or interspecific variation in contractile protein expression; and (C) study the expression of parental isoforms in the fertile F1 hybrid of *O. niloticus*, and *O. andersoni* crosses. The findings will be discussed with reference to previous skinned fibre experiments; furthermore, possible genetic mechanisms of intraspecific variation will be proposed.

Methods

Tilapia breeding protocol:

Eggs collected from a single female were divided equally into four one litre incubation jars. Fertilization was initiated by mixing the semen of a single tilapia male into the incubation jar. The eggs hatched within 3-4 days and at 10 days the fry were moved to larger storage tanks. The fish examined were *Oreochromis andersoni*, *O. niloticus*, and their fertile F1 hybrid offspring from reciprocal mating pairs [*O. andersoni* female x *O. niloticus* male (AN hybrids); and *O. niloticus* female x *O. andersoni* male (NA hybrids)]. Parental species and hybrid and hybrid offspring are shown in plate A.

Preparation of Myofibrils:

Oreochromis andersoni, *O. niloticus*, and their fertile F1 hybrid were obtained from the Institute of Aquaculture at Stirling University. All of the fish sampled were of adult size (> 100g) with total body weights of: 415g $\pm$ 122, (n=14) hybrids; 148g $\pm$ 50 (n=17) *O. andersoni*; and 828g $\pm$ 264 (n=5) *O. niloticus*. The fish were stunned by a blow to the head, killed by decapitation and placed on ice. All succeeding steps were carried out on ice to minimize proteolysis and denaturation of myofibrillar proteins.

Plate A   Photographic illustration of parental species and
F1 hybrid fishes used in this study.

O.niloticus

O.andersoni x O.niloticus

— X —

F1

O.andersoni

F1

O.niloticus X O.andersoni

20 cm

Fast (white, glycolytic) muscle, from the dorsal epaxial region, was dissected carefully so as to avoid contamination from superficial slow (red, oxidative) muscle. After removal the muscle tissue was immediately frozen and stored at - 20ºC. The preparation of myofibrils used was an adaptation of Perry and Gray (1956). Two grams of frozen or fresh samples were minced finely with scissors and placed in 10 volumes of washing buffer(100mM NaCl, 10mM Tris-HCl, pH 7.2 20ºC). Minced samples in buffer were homogenized at high speed for 3 x 30s allowing one minute of cooling on ice between homogenizations. After the first homogenization the non-ionic detergent, Triton-X 100 (Sigma Chemical Co.), was added to a final concentration of 1% and left to stand for 30min. The addition of Triton-X insured the removal of soluble membrane proteins from the myofibrillar proteins (Solaro, Pang, and Briggs, 1971). The Triton-X treated homogenate was then centrifuged at 6000g for 10 minutes.

After centrifugation the supernatant was discarded and the pellet was resuspened with low speed homogenization, 2 x 15s, and centrifuged at 6000g for 10min. The pellet was washed another 4 times in this manner. Finally, the pellet was resuspened in 25vol. of washing buffer and centrifuged at 400g for 2min. The purified myofibrils, found in the top 1/3 of the supernatant, were carefully removed by pipette. The

collected fraction was centrifuged at 6000g for 10min. and resuspended in 5mls. of washing buffer. This final centrifugation and resuspention served to increase the protein concentration of the myofibrillar suspension.

Estimation of protein concentration:

The Lowry *et. al.* (1951) assay of protein concentration was found to be unnecessasarily time consuming for the estimation of myofibrillar protein concentration. The following method was developed to quickly estimate the protein content of the highly concentrated myofibrillar suspension. Prior to measuring the absorbance of the myofibrils, the spectrophotometer was zeroed at 280nm against 5% Sodium Dodecyl Sulfate (SDS) in washing buffer. By zeroing the spctrophotometer against this solution the absorbtion due to SDS was effectively removed from subsequent readings of protein concentration. After zeroing, a 0.625mg/ml solution of Bovine Serum Albumin (BSA, Sigma Chemical Co.) in 5% SDS buffer was read for absorbance at 280nm. This value was always around 0.6 OD.

One hundred microlitres of the myofibrillar suspension was diluted 4x in buffer to a final concentration of 5% SDS. This mixture was heated at 70°C for 3 min. Heating was necessary to promote full denaturation of the protein by the SDS. The dilute 5% SDS

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myofibrillar solution was read for absorbance at 280nm. The reading obtained was compared with the 0.625mg/ml BSA standard reading. Further dilution was usually necessary to lower the absorbance of the myofibrils to within 0.02 OD of the BSA standard. To estimate the protein concentration, the final number of dilutions necessary was multiplied by 0.625mg/ml. This estimation was used to prepare 2mg/ml myofibrillar protein samples for SDS polyacrylamide gel electrophoresis (PAGE).

To test the accuracy of this method known concentrations of BSA were assayed in triplicate using both the Maddy & Spooner (1970) adaptation of the Lowry et. al. (1951) method and the 5%SDS method. Calibration curves of each test are shown in figure 2.0. At concentrations below 2.5 mg/ml the standard error in the 5%SDS estimation compared favorably to that of the Lowry. The one disadvantage apparent in the calibration curves is the greater slope of the 5%SDS curve. It is known that spectrophotometer measurement error becomes signifigant for absorbance values greater than 1. This fact limits the concentration range of the 5%SDS test to samples below 1.5 mg/ml; whereas, the Lowry test is useful for samples up to 5 mg/ml. It was for these two reasons that a 0.625mg/ml BSA standard was chosen for the test.
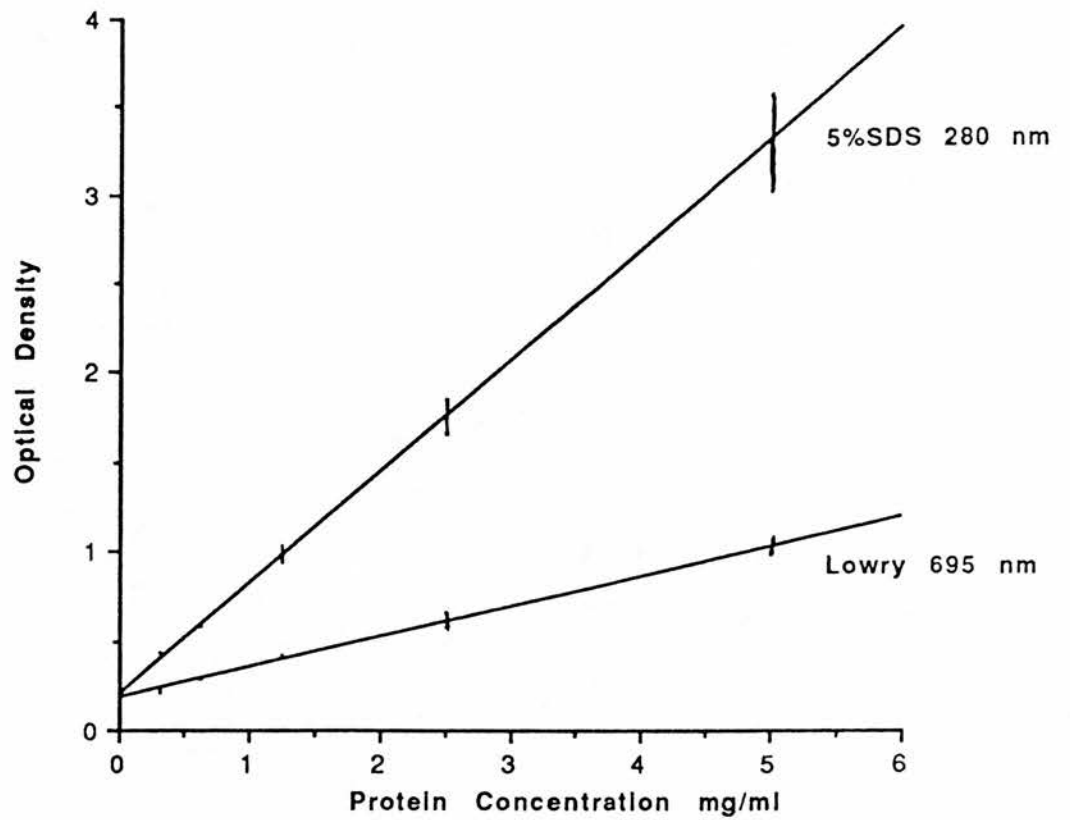
Electrophoretic Techniques

Figure 2.0 Comparison of calibration curves from Lowry and 5%SDS methods used for estimating protein concentration in bovine serum albumin standard solutions. Error bars for each point represent mean ± standard deviation.

Equipment cleaning and assembly:

All equipment for the Bio-rad Protian vertical slab gel unit was carefully and thoroughly cleaned by soaking in a bath of dilute Decon detergent. After soaking the equipment was lightly scrubbed using a paper towel, then rinsed with warm tap water. Finally, the equipment was rinsed with Milli-Q deionized, glass distilled water and left to dry. Prior to assembly, the glass gel plates were wiped clean with a paper towel soaked with acetone. This acetone rinse insured the removal of any grease not removed by washing alone.

Teflon spacers of widths either 1.5mm or 3mm were placed along the 160mm edges of the 160mm x 180mm glass plate. Another plate of identical dimensions was placed on top of the spacers and then both plates clamped together to form the gel cassette. Along one180mm side the plate edges were covered with a thin layer of silicone grease. This greased edge was then sealed against a rubber sealing strip on the gel casting stand.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

Polyacrylamide gels were made from the following stock solutions: acrylamide/bisacrylamide stock (30% acrylamide: 8% bisacrylamide); Resolving gel buffer stock

(3M Tris-HCl, pH 8.8 20°C); Stacking gel Buffer stock (0.5 M Tris-HCl, pH 6.8 20°C); and 10% SDS stock. Stock solutions were mixed according to the recipe in Hames (1981) based on the original SDS- discontinuous system of Laemmli (1970). All reagents used were of analytical quality obtained from Sigma Chemical Co..

Resolving gels were made to a final volume of 30mls by first mixing either 15mls (15% acrylamide gel) or 7.5mls(7.5% acrylamide gel) acrylamide/bisacrylamide stock; 9.45mls (15% acrylamide gel) or 16.95mls (7.5% acrylamide gel) ultrapure deionized-glass distilled water; and 3.75mls (375mM) resolving gel buffer stock. This mixture was degassed in an Erlenmyer flask under strong vacuum for 3min, then 0.3 mls (0.1%) of 10% SDS stock was added. The gel mixture was then degassed for another 3min. After degassing the polymerization catalysts TEMED (0.0015mls) and a 10 mg/ml stock solution of ammonium persulphate (1.5mls) were added in that order. It was necessary to make the ammonium persulphate solution just before adding it to the degassed gel mixture.

The gel mixture was poured in the gel cassette using a 100ml syringe with a small tap. The tap was placed in the gap between the glass plates and then opened. It was important to stop pouring half way to the desired level and swirl the mixture between the plates. Swirling removed any microbubbles caught during pouring and insured mixing

of the polymerization catalysts with the gel mixture. Pouring was then resumed up to the desired level (approx. 8cm). After swirling the full mixture the top of the gel was overlaid with 4mls of butan-2-ol to form an even gel surface. Polymerization of the acrylamide began within 15min and was complete by 2h.

Stacking gels (4% acrylamide gel) were mixed from the following: 2.5 mls acrylamide-bisacrylamide stock; 5mls stacking gel buffer stock; 11.3 mls ultrapure water. This mixture was then degassed twice adding 0.2mls of 10% SDS stock between degassings. Prior to pouring the stacking gel the butan-2-ol was poured off and any remaining butan-2-ol was rinsed away with at least four changes of ultrapure water. To equilibrate the surface of the resolving gel 2mls of stacking gel, without catalysts, was pipetted into the cassette, swirled across the resolving gel for 1min and poured off. TEMED (0.0015mls) and ammonium persulphate stock (1ml) were added to the gel mixture and the stacking gel was poured as described above. Immediately, after pouring a Teflon well former was wedged between the plates. Polymerization occurred within 15min and was complete in 45min.

After complete polymerization the well former was carefully removed and the wells rinsed with three changes of tank buffer (25mM tris,195mM glycine, 0.1% SDS; pH 8.3). The upper electrode chamber was clamped onto the

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top of the gel cassette and filled with tank buffer. Next, the casting stand was removed and the upper electrode attached to the gel cassette was loaded into the buffer tank. The gel was then ready for sample loading.

Sample preparation for SDS-PAGE:

Equal volumes of 10% SDS stock and myofibrils were mixed and left to dissolve for 15min at 70°C. Samples in 5% SDS were made to a final concentration of 5mg/ml protein in 63mM Tris-HCl; 1mM dithioerythritol; 2% 2-mercaptoethanol; 20% glycerol; 0.0025% bromophenol blue; pH 6.75 20°C. The samples were heated for 3min at 100°C and then stored at -20°C.

Prior to electrophoresis samples were thawed and then centrifuged for 10min at high speed to pellet any undissolved particulate matter. Samples were loaded into the wells using a glass micro syringe taking care not to pierce the bottom of the well. For the first 1/2h the gel was run at 10mA/gel and 50v. Current and voltage were then raised to 30mA/gel and 200v and left to run for 10h.

Alkali-Urea PAGE (AU-PAGE):

Alkali-urea PAGE being a continuous electrophoresis system requires only one buffer for the gel and tank. The buffer chosen was either, 0.5M glycine-NaOH, 50mM $CaCl_2$, pH 10 at 20°C, or 0.5 M glycine, 50 mM $CaCl_2$, pH 8.9 at

20ºC using 1M Tris. To make 50 mls of a 7.5% acrylamide gel required 12.5mls of acrylamide/bisacrylamide stock; 24g of urea (8M); 5mls of 10x concentration glycine/Ca⁺ buffer (5M Glycine, 500mM CaCl₂); all made up to 47mls with ultrapure water.

The mixture was heated using warm tap water to dissolve the urea. Once all the urea had dissolved the liquid was degassed under vacuum. As the solution cooled under vacuum the urea precipitated; therefore, it was necessary to redissolve the urea by gently warming the vacuum vessel with warm tap water. This vacuum/redissolving step was repeated twice. After redissolving the last time the solution was cooled to room temperature using the cold water tap. It was necessary to cool the solution before adding the polymerization catalysts, as the warm gel mixture would polymerize too quickly. Finally, TEMED and ammonium persulphate were added at 0.030mls and 3.125mls, respectively.

The gel cassette was filled as described above; however, as no stacking gel was required the plates were completely filled and a Teflon well former was wedged between the plates. Polymerization began within 15min and was complete within 1.5hrs. After complete polymerization the well former was removed and the wells rinsed as described earlier.

Sample Preparation for AU-PAGE:

One part myofibrils and 2 parts of a fresh solution of 12M Urea were mixed (final concentration 8M Urea) and allowed to stand for 1 hour at room temperature. After sitting for 1 hour the myofibrillar proteins were presumed to be fully denatured by the concentrated urea. Samples in urea were then mixed with gel buffer to the following concentrations: 50mM glycine- NaOH; 5mM $CaCl_2$; pH 10 at 20^oC. To test for the binding of troponin-I and troponin-C samples were either made to 5mM $CaCl_2$ (binding) or 10 mM EGTA (unbinding). Finally, glycerol was added to 20% concentration and the sample left at 25^oC for one hour. Just prior to loading, the samples were centrifuged for 10min at high speed.

Samples were run into the gel at a constant 50v for 1/2h, after which the voltage and current were raised to 400v and 50mA/gel. Gels were then left to run for another 7.5h.

Two dimensional Electrophoresis:

In the first dimension myofibrillar proteins were separated according to pI using AU-PAGE of pH 10 or 8.9. After electrophoresis the gel was quickly stained for 5 minutes in a comassie brilliant blue solution (40% methanol: 7% acetic acid: 53% ultrapure water: 0.25%

comassie brilliant blue (Sigma Chemical Co.)). The gel was then placed in ultrapure water and washed 5 times to normalize its pH. If necessary the gel could be stored in ultrapure water overnight at 4^0c.

Gel strips were cut away from the first dimension using a scalpel taking care not to tear the strip. The strips were then equilibrated in a test tube for 15 to 45min in sample buffer at 2X concentration containing 4% SDS. The SDS sample buffer was then poured off and 2mls of stacking gel solution (without catalysts) was added. This solution was allowed to equilibrate with the gel strip for 5min.. After the second equilibration the gel strip was ready to be loaded onto the second dimension.

The SDS resolving gel of the second dimension was prepared as described earlier and allowed to fully polymerize before loading the first dimension gel strip. Once the resolving gel was totally rinsed of butan-2-ol the gel strip was loaded edge on between the glass plates. This was done by laying the strip over the top edge of the plates, then gently working the 1.5mm edge between the plates first with the fingers (wearing protective latex gloves) then with a weighing spatula. The stacking gel was then poured, taking care not to trap bubbles underneith the gel strip, until the strip was fully enveloped. Finally, well formers were placed on either end of first dimension sample and the stacking gel was left to polymerize.

After polymerization the wells were rinsed and SDS-PAGE samples from the same fish as the first dimension were loaded into the wells. The gel was run as above; however, the initial run-in time was extended to 2h. The longer run-in time allowed all of the proteins of the first dimention to enter the stacking gel.

Staining Techniques

Coomassie Brilliant Blue:

Prior to staining, gels were fixed for 1h in fixing/destain solution (40% methanol, 7% acetic acid, 53% ultrapure water). Fixed gels were then stained overnight in coomassie brilliant blue solution (0.25% coomassie brilliant blue in fixing/destain solution). This solution was made by dissolving, with 1h of vigorous stirring, 2.5g of coomassie brilliant blue (Sigma Chemical Co.) in 400mls of methanol (BDH). Acetic acid (BDH) and water were then added in the percentages given above. This solution was then centrifuged at high speed for 1/2h, and filtered twice through a Whatman no.1 filter. After staining gels were placed back in fixing/destain solution to remove background stain and thus enhance the protein bands.

If electrophoresis was performed for preparative reasons such as peptide mapping or two dimensional electrophoresis the fixing/destain step was eliminated

from the procedure. Instead, gels were immediately stained for 5min and then washed in several changes of ultrapure water to remove methanol and acetic acid.

Silver Staining:

Reagents used in silver staining were obtained in a Silver Stain Kit from Sigma Chemical Co. The method used was based on Heukeshoven and Dernick (1985). No modifications were found necessary to the protocol given by Sigma in the kit.

"Stains-all" Staining:

The method followed was that of Campbell (1983). After electrophoresis gels were fixed overnight in 25% propan-2-ol. The next day the gel was washed at a temperature of 40°C 4 times with 25% propan-2-ol. Heating and multiple washings insured the removal of all the SDS which can interfere with the subsequent protein staining. Gels were stained in the dark for at least 48 h in the following solution: 0.0025% Stains-all, 25% propan-2-ol, 7.5% formamide, and 30mM Tris base pH 8.8. Afterwards gels were destained in the dark with 25% propan-2-ol. It was important to carry out all staining and destaining in the dark as Stains-all is light reactive. If the stain happens to fade from overexposure to light (more than 15 min) the gel can be restained in a fresh staining solution.

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Results

Sample Loading

In order to obtain gels suitable for analysis it was necessary to determine the ideal sample conditions for maximum resolution of the protein bands. In figure 2.1 myofibrils from a single *O. andersoni* sample were prepared at different concentrations with two different reducing agents. The amount of total protein loaded into each well was also varied to test for resolution. The best combination of reducing agents, protein concentration, and sample loading was judged to be; 2% 2-mercaptoethanol, 1mM dithioerithritol; 2 mg/ml; and 0.1 mg/well, respectively. This combination is seen in lane K. Samples reduced only by 1mM dithioerithritol contained distortion lines running through the protein banding pattern. The addition of 2-mercaptoethanol, shown in lanes A, C, E, G, I, K, greatly reduced this effect. A comparison of the 2-mercaptoethanol lanes shows little variation in the intensity and sharpness of the bands over the range of protein concentrations and sample loadings.

Myofibrillar protein isoforms in *O. andersoni*, *O. niloticus*, and the F1 hybrid
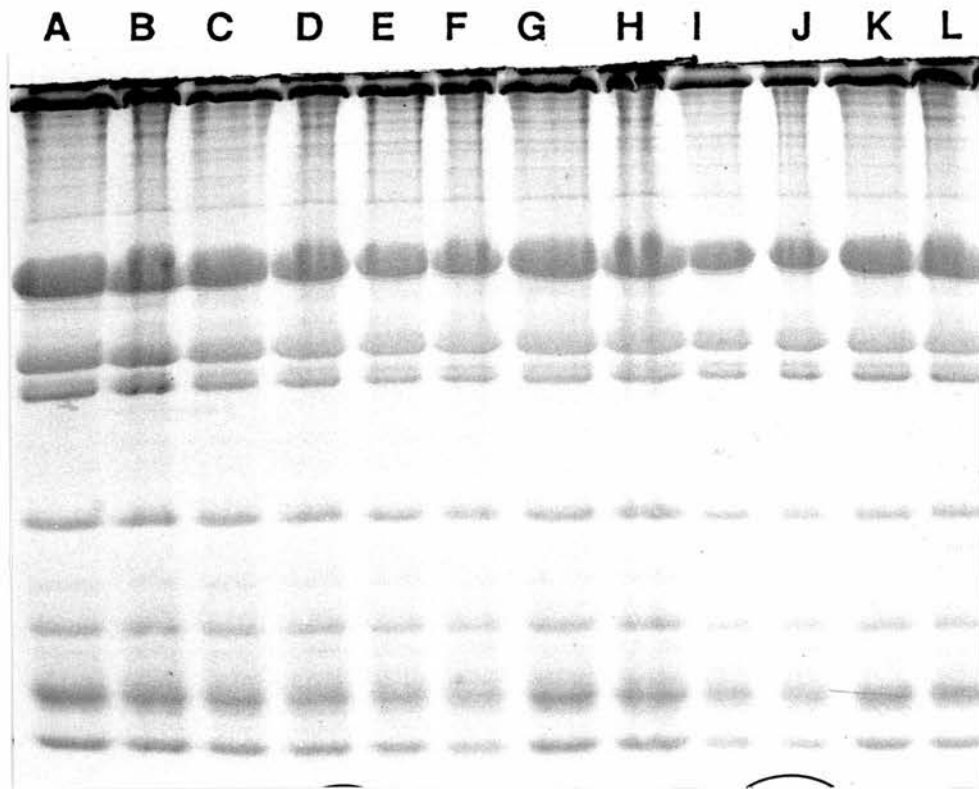
Figure 2.1 15% SDS-PAGE of a single sample to determine the effects of sample concentration and reducing agents on the quality of protein bands.

Key: Lanes A, C, E, G, I, & K all contained 2% 2-mercaptoethanol and 1mM dithioerithritol. All other lanes contained only 1mM dithioerithritol. Sample concentration & protein loading: lanes A & B 5mg/ml, 0.15mgs; lanes C & D 4mg/ml, 0.12mgs; lanes E & F 3mg/ml, 0.09mgs; lanes G & H 3mg/ml, 0.15mgs; lanes I & J 2mg/ml, 0.04 mgs; lanes K & L 2mgs/ml, 0.1mgs.

O. niloticus

The fast muscle myofibrillar proteins of five individual *O. niloticus* were analysed using 15% SDS-PAGE. All individuals exhibited the characteristic pattern shown in figure 2.2 lane G. The apparent molecular weight of each protein band was determined from the calibration curve shown in figure 2.3. To construct the curve the mobility of each standard protein (lane A, fig. 2.2) was measured relative to the tracking dye front. This relative mobility value was then plotted against the known log MW of the standards. Myosin (205 kD) and actin (45 kD) were identified by their apparent molecular weights and heavy staining indicating their high concentration. Myosin and actin are known to contribute 43% and 22% respectively, to the myofibril protein mass (Yates and Greaser, 1983). The apparent molecular weights of the other protein bands were: tropomyosin (34.5kD); troponin T (33kD); myosin light chain 1b (22kD); troponin-I (21kD); myosin light chain 2/troponin C (19-18kD); and myosin light chain 3 (15kD).

Identification of the protein bands in figure 2.2 was not based on comparison of apparent molecular weights with known values for the myofibrillar proteins. Such comparison identification is only possible if identical
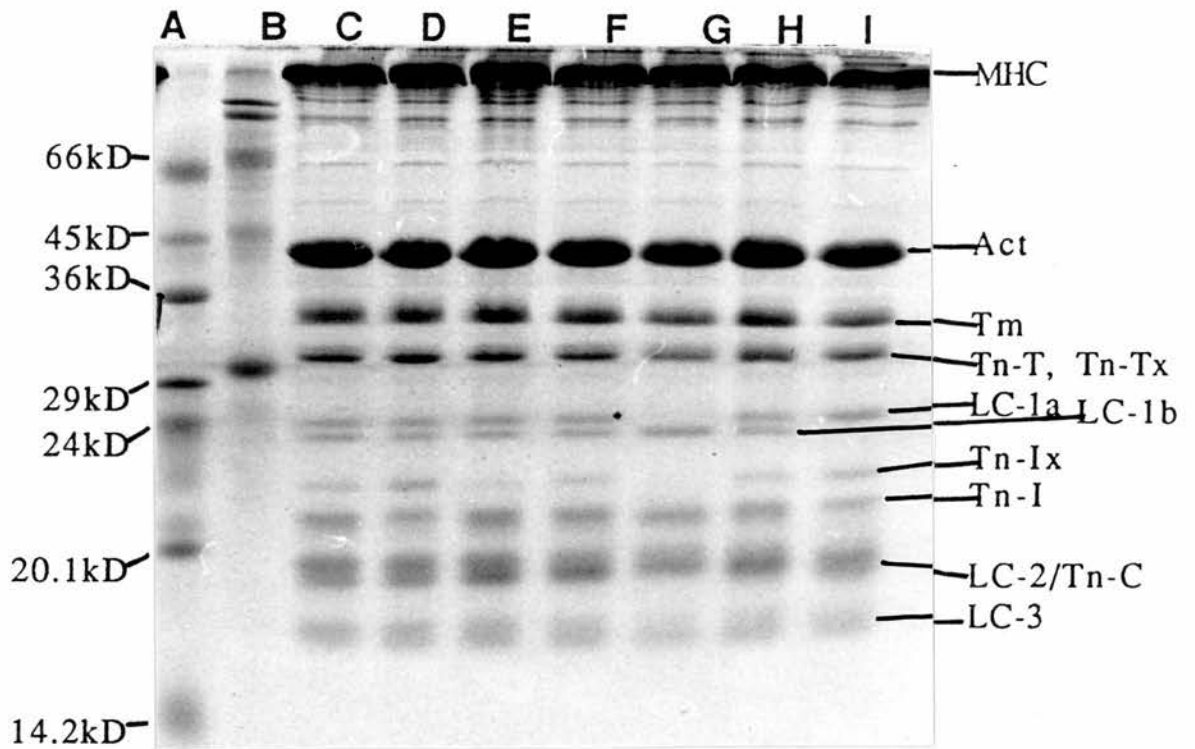
Fig 2.2 15% SDS-PAGE of *O. niloticus*, *O. andersoni*, and hybrid fast muscle myofibrils. Conditions of electrophoresis given in text. Key: (A) low molecular weight marker; (B) high molecular weight marker; (C, F, & G) mixture of *O. andersoni* and *O. niloticus* fast myofibrillar proteins; (D) AN hybrid; (E) NA hybrid; (G) *O. niloticus* (I) *O. andersoni*. (MHC) Myosin Heavy Chain; (Act) Actin; (Tm) Tropomyosin; (Tn-Tx) Troponin-Tx; (Tn-T) Troponin-T; (LC-1a) myosin Light Chain 1a; (LC-1b) myosin Light Chain 1b; (Tn-Ix) Troponin-Ix; (Tn-I) Troponin-I; (LC-2) myosin Light Chain 2; (Tn-C) Troponin-C; (LC-3) myosin Light Chain 3.
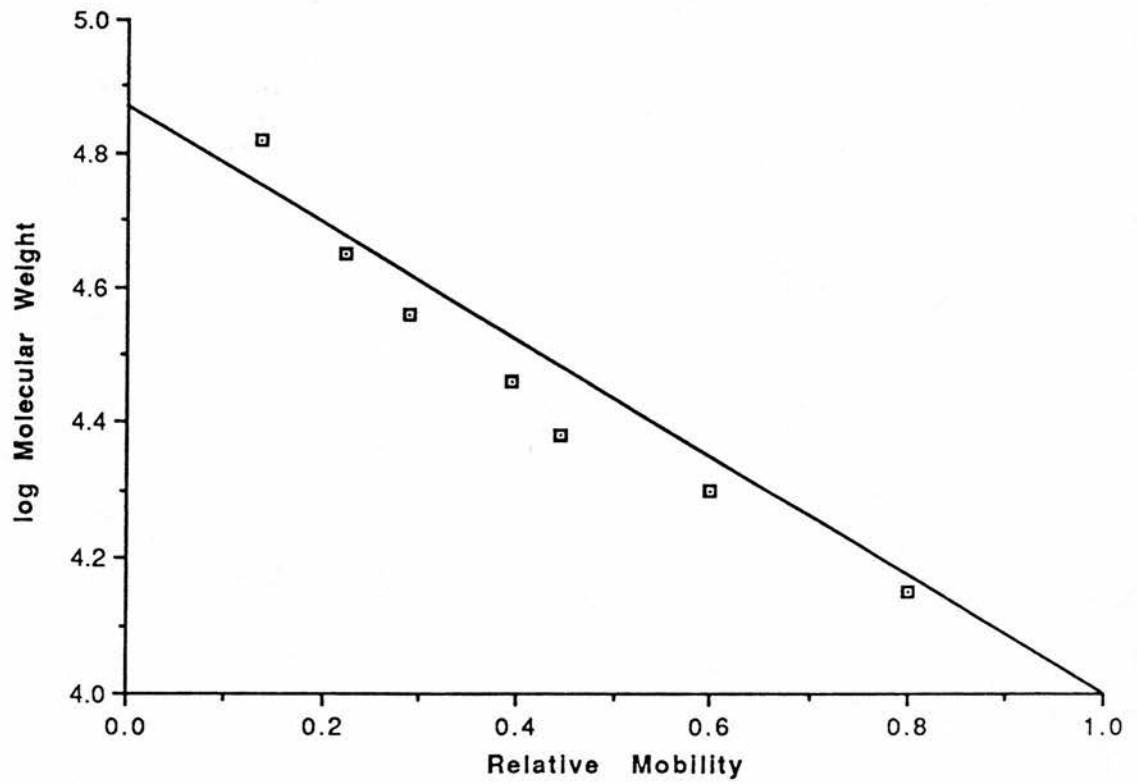
Figure 2.3 Calibration curve of relative mobility versus log molecular weight produced from low molecular weight marker proteins in figure 2.2 This curve was used to estimate the apparent molecular weight (Mr) of the myofibrillar proteins.

species are analysed using the same electrophoretic methods. The possible misintrepretation which can arise from cross species comparisons is illustrated by the observation that in carp and goldfish the SDS-PAGE apparent molecular weights of LC-2 and LC-3 are reversed (Focant and Huriaux, 1978 and 1985; and Crockford,1989). Focant and Huriaux (1978) also found that in different pH conditions the position of carp LC-3 changed while the positions of LC-1 and LC-2 were unaffected. To insure proper identification each protein band was judged on two other criteria: separation by charge and size on two dimensional electrophoresis; and, differential staining in coomassie brilliant blue, silver stain, and "Stains-all".

Figure 2.4, lane A is the banding pattern of *O. niloticus* fast muscle myofibrillar proteins separated according to relative pI in 10% AU-PAGE at pH 10. This lane was run on a 15%SDS-PA gel to produce the two dimensional electrophoretogram shown in figure 2.5a. This technique of two dimensional electrophoresis produced protein bands which appear to be spread across a range of pI's. The presumed cause of artifactual band spreading is the lack of a complete polymerization between the first dimension gel sample piece and the stacking gel of the second dimension. As the proteins migrated from the first into the second dimension, diffusion spread the proteins
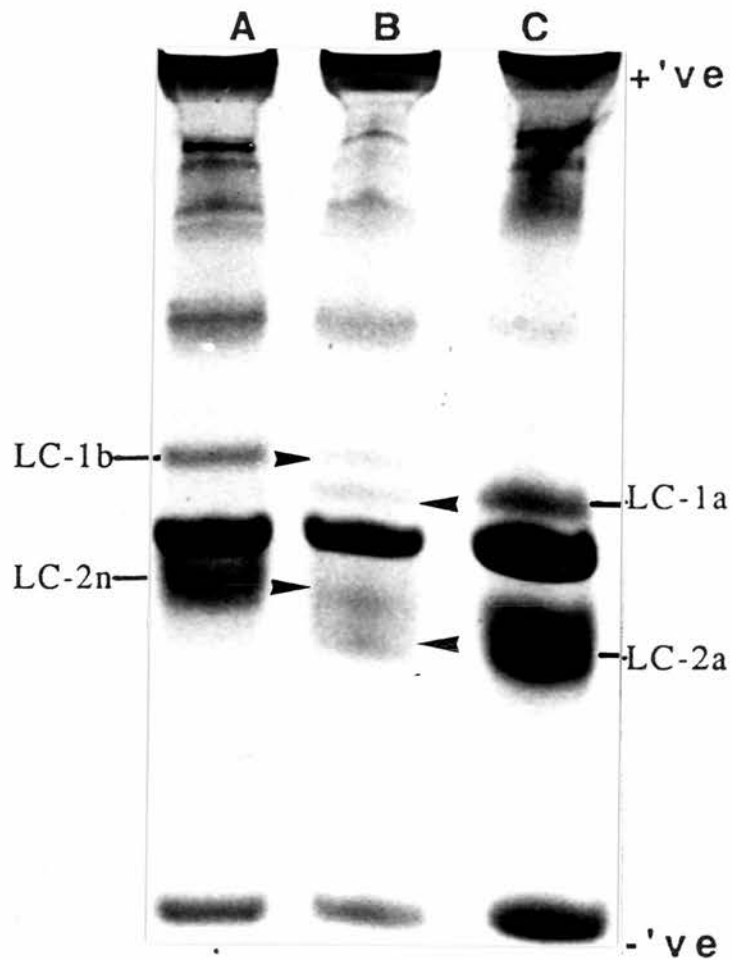
Figure 2.4 10% AU-PAGE pH10 of fast muscle myofibrils from
(A) *O. niloticus* (C) *O. andersoni* and (B) hybrid. All samples
contained 5mM Ca+2. Bands showing isoform differences
were identified as LC-1 and LC-2.

across the poorly polymerized interface. To eliminate the error induced by band spreading, actual positions are assumed to be at the center of the band. Actin and tropomyosin were identified by their abundance and the differential staining of Tm in silver stain and coomassie blue. Crockford (1989) noted that Tm stained well in coomassie blue; but, was nearly undetectable with silver stain. Figures 2.9a, 2.9b, 2.10a, and 2.10b were first silver stained and then stained in coomassie blue. In these two dimensional figures Tm appears as a strongly staining blue band in the center of the gel.

The positions of the lower molecular weight bands were taken relative to the actin and tropomyosin band. Rowlerson *et. al.* (1985) in comparing myosin light chains of nine fish species used actin and Tm bands as internal reference points on two dimensional electrophoretograms. In *O. niloticus* light chain 1 is slightly more basic than Tm while further down the gel LC-2 is slightly more acidic than Tm. Light chain 3, nearest the bottom of the gel, is the most acid of the light chains.

Among the Ca^{+2} regulatory proteins only troponin-I appears in the second dimension as a protein of intermediate pI on the basic end of tropomyosin directly below actin. Samples analysed in figures 2.5a, b, and c all contained 5mM Ca^{+2} to promote the binding of troponin I

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and troponin C. In the dissociated state (no Ca^{+2}) Tn-I and Tn-C migrate to opposite electrodes as the former has a basic pI and the latter an acidic pI. In the bound state Tn-I migrates as a single band of intermediate pI. Troponin-T was actually identified by its absence. Due to its strongly basic pI Tn-I does not enter the first dimension and is subsequently, absent on the second dimension.

Troponin C is also not resolved on the second dimension. This is probably due to low concentration in the myofibrillar samples and poor staining in coomassie blue. Similarly, troponin C was not resolved in SDS-PAGE; but, appeared with myosin light chain 2 as a large band filling the 18 kD to19 kD region of figure 2.2. The lower part of the band was hypothesized to be Tn-C according to its reported molecular weight of 18 kD (Ohtsuki *et. al.*, 1986). LC-2 in vertebrate striated muscle has reported values in the range of 18-19 kD (Swynghedauw, 1986). However, both Crockford (1989) and Huriaux & Focant (1978) have reported values for carp (*Cyprinus carpio*) LC-2 of 16.5 kD and 17.4 kD, respectively. To better discriminate Tn-C from LC-2 the staining method of Campbell *et. al.* (1983). was employed (fig. 2.6). The stain used was the cationic carbocyanine dye, "Stains all", which preferentially stains Ca^{+2} binding proteins blue. In
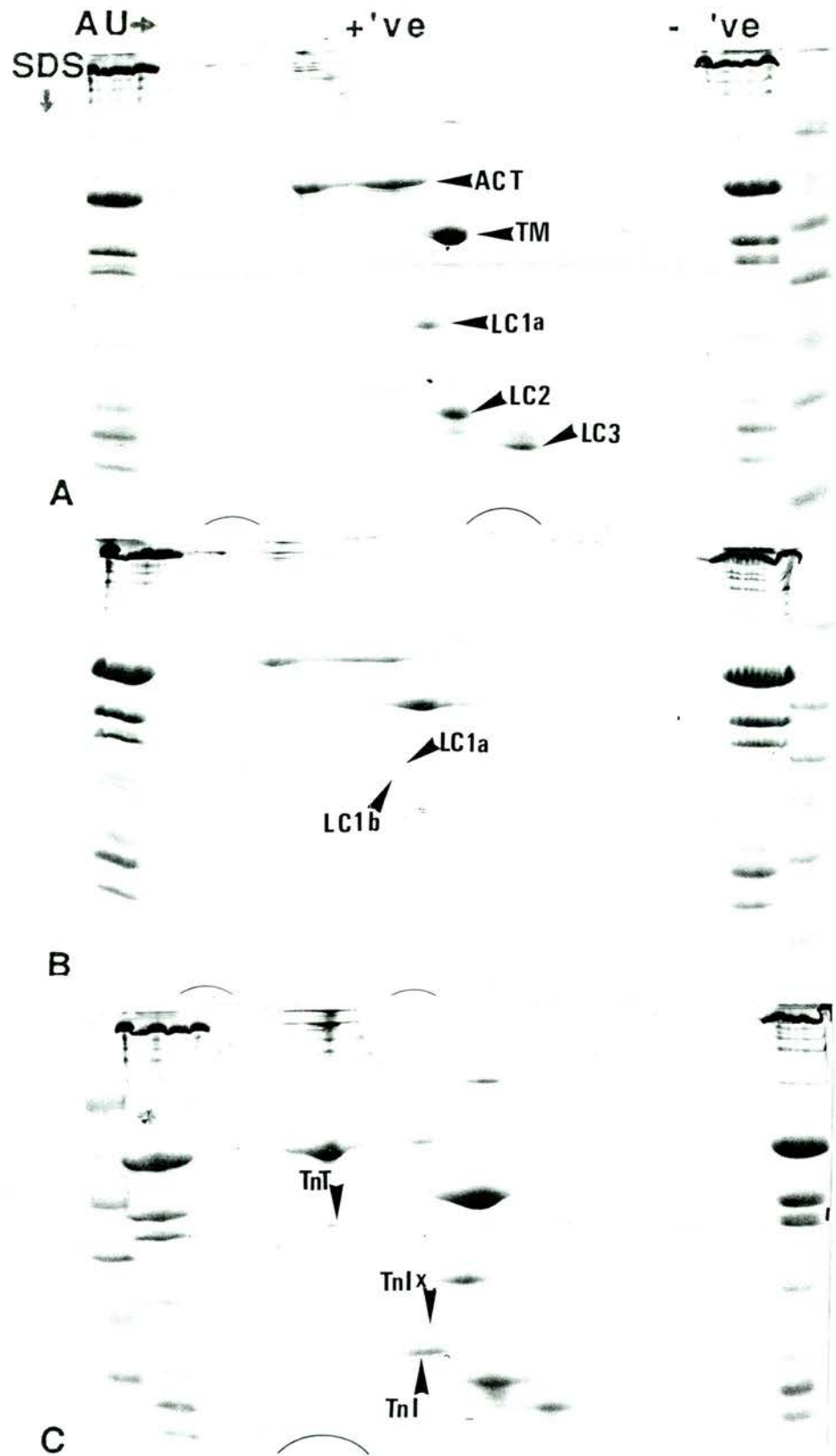
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Figure 2.5   Two dimensional electrophoretograms of (A) *O. niloticus* (B) hybrid (C) *O.andersoni*.
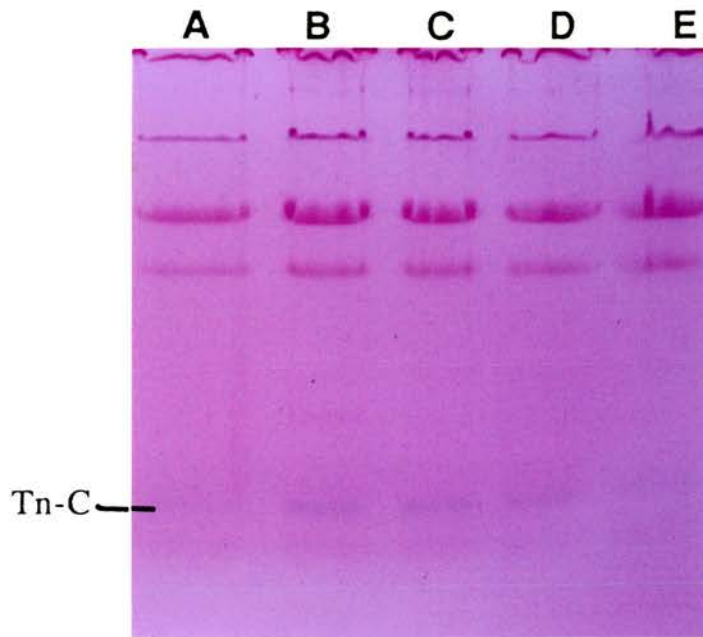
Figure 2.6   15%SDS-PAGE of fast myofibrils from *O. andersoni*, *O. niloticus*, and hybrids.   "Stains-all" staining was used to preferentially stain the Ca^{+2} binding protein Tn-C blue.   Key: (A) AN hybrid; (B) NA hybrid; (C) *O. niloticus*; (D) equal concentrations of *O. andersoni* and *O. niloticus* samples mixed; (E) *O. andersoni*.

O. niloticus the bottom half of the large 19-18 kD band
contains the blue staining Tn-C protein (figure 2.6 lane C).

O. andersoni

Fast muscle myofibrillar proteins of eighteen
individual *O. andersoni* were analysed on 15% SDS-PAGE.
Apparent molecular weights (Mr) of the protein bands were
identical to those observed in *O. niloticus* with the
exception of myosin light chain 1 and an extra troponin-I
isoform. The extra troponin-I isoform in *O. andersoni*,
designated troponin-Ix, has an apparent molecular weight
of 22.7kD (fig 2.2, lane I). *O. andersoni* myosin light chain
1, designated LC-1a, has a molecular weight of 23.5kD
(fig. 2.2, lane I). This is greater than the 22kD LC-1b
isoform in *O. niloticus.*

In subsequent SDS-polyacrylamide gels intraspecific
variation in LC-1 expression was observed in *O. andersoni.*
Figure 2.7 illustrates the two different SDS-PAGE banding
patterns of *O. andersoni.* Lane B, identical to figure 2.2,
lane I, shows the myosin light chain 1a homozygote. Lane
A illustrates the LC-1 heterozygote which contains both
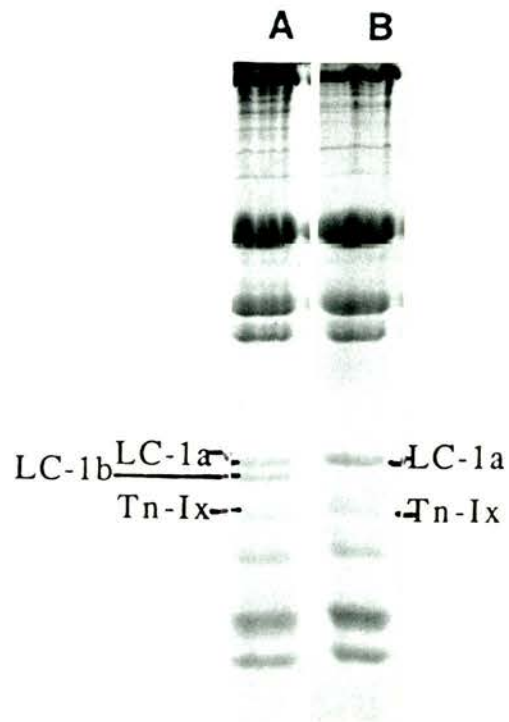LC-1a and LC-1b. Electrophoretic observation of

Figure 2.7   15%SDS-PAGE of *O. andersoni* fast muscle myofibrils. (A) *O. andersoni* heterozygous for LC-1.   (B) LC-1a homozygote.   Both contained the Tn-Ix isoform.

intraspecific variation in a myofibrillar protein has not been reported. To test the possibility that this novel finding was not a result of misidentification of the fish sampled, all *O. andersoni* fast muscle samples were tested at Stirling University using the electrophoretic stock identification technique of McAndrew and Majumdar (1983). All *O. andersoni* tested were properly identified (McAndrew, personal communication).

Of the eighteen *O. andersoni* individuals assayed using 15%SDS-polyacrylamide eight were heterozygous for LC-1 and ten were homozygous for LC-1a. Fifteen of the *O. andersoni* can be further subdivided into three different breeding groups each containing LC-1 hetero- and/or homozygotes. The identity of these breeding groups was:

Breeding group	Heterozygotes	LC-1a Homozygotes
Four	5	1
Five		6
Six	2	1

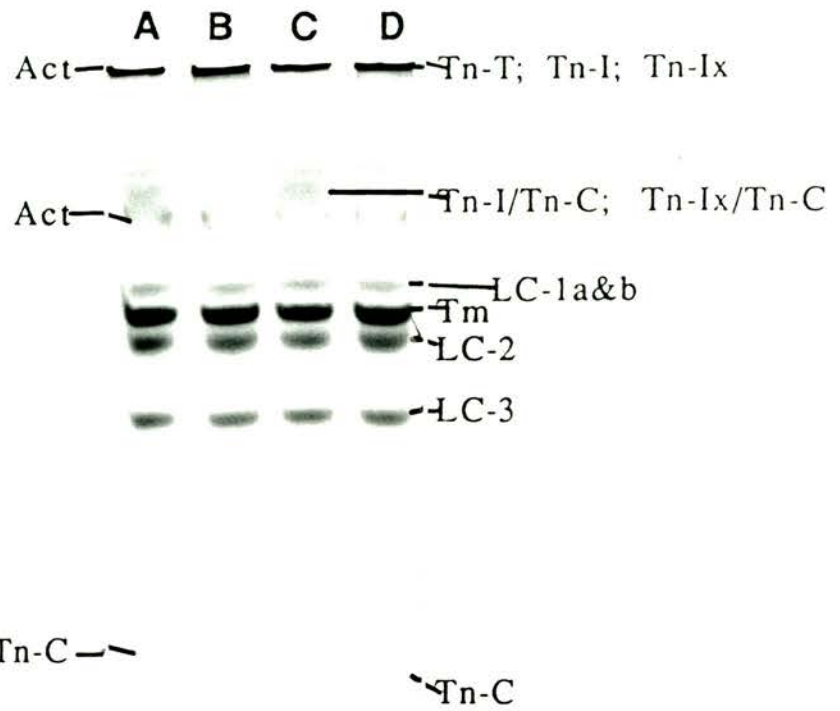determined by the female as the same *O. andersoni* male was used for each mating.

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Figure 2.8   10% AU-PAGE pH 10 of *O. andersoni* fast myofibrils with either 5mM Ca+2 (A & C) or 5mM Ca+2 and 10mM EGTA (B & D).   Key: lanes (A & B) LC-1 heterozygote;   lanes (C & D) LC-1a homozygote.

Both heterozygous and homozygous *O. andersoni* individuals were analysed with two dimensional electrophoresis. The relative positions of the myosin light chains in figures 2.9 and 2.10 are similar to those of *O. niloticus* in figure 2.5a. Unfortunately, in the heterozygous samples of figure 2.10 LC-1a and LC-1b were not separate enough to make any judgements on their relative pIs.

The first dimension AU-PAGE, shown in figure 2.8, illustrates the binding properties of troponin I and troponin C in the presence of Ca^{+2}. Heterozygous and homozygous samples appear identical under the same conditions. Obvious differences however, are apparent between samples containing Ca^{+2} or EGTA. In the Ca^{+2} samples (lanes A and C) bound Tn-I and Tn-C form a diffuse band immediately above the actin band. Small amounts of Tn-C, which are not bound to Tn-I or Tn-Ix, appear as a broad, lightly staining band at the bottom of the gel. In the absence of Ca^{+2} (EGTA samples; lanes B and D) the region above actin contains no band and the dissociated Tn-C forms a sharp, concentrated band of acidic pI. In these samples the basic Tn-I isoforms remain in the sample well at the top of the gel.
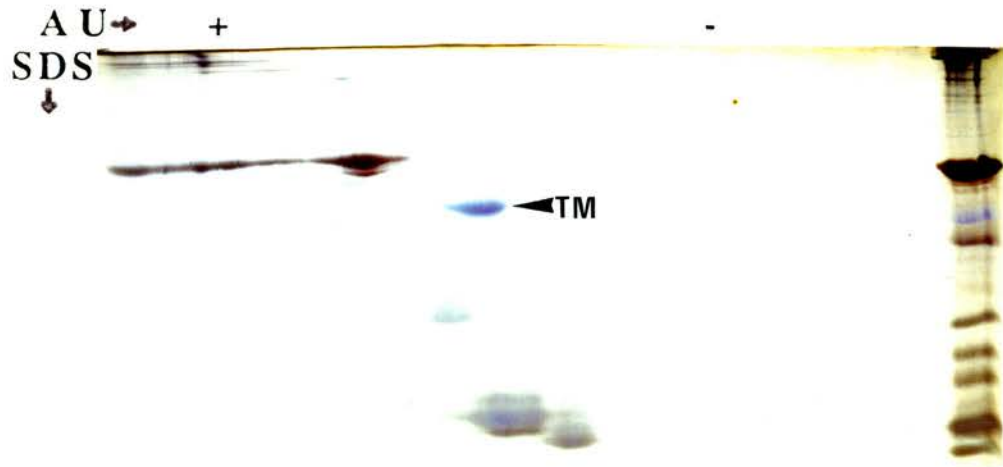
A (Ca+2/EGTA)
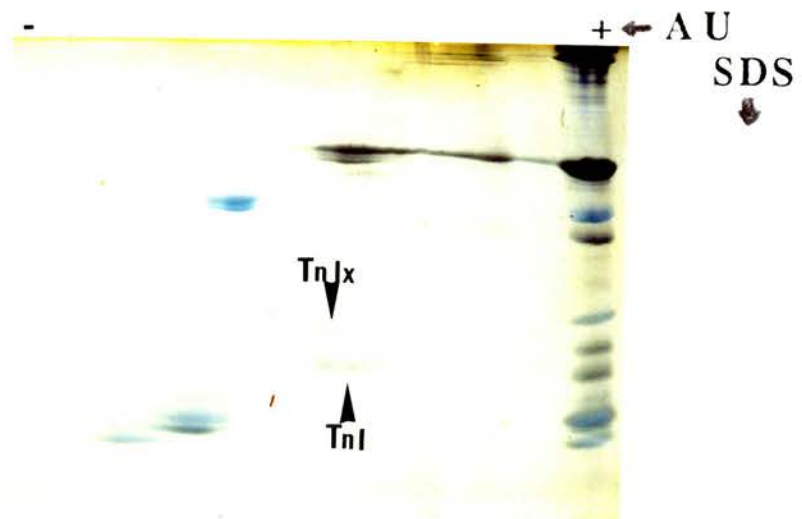
B (Ca+2)

Figure 2.9   Two dimensional electrophoresis of fast muscle myofibrils from *O. andersoni* homozygous for LC-1.   Both silver staining and coomassie blue staining were used in order to identify tropomyosin (Tm) which only stains with coomassie blue.   Artifactual band spreading is due to a poor interface between the sample gel piece and the stacking gel.

The Ca^{+2} induced change in mobility of Tn-I and Tn-Ix is shown in the two dimensional gels of figure 2.10a, & b. With the presence of Ca^{+2} in the first dimension the troponin-I's appear to have pI's slightly more basic than LC-1 (fig. 2.10b). Without Ca+2 in the first dimension their positions change dramatically to indicate strongly basic pI's (fig. 2.10a). When bound to Tn-C, Tn-Ix appears to have a more acidic pI than Tn-I (figs. 2.5c, 2.9b, & 2.10b). In the free state however, the two Tn-I isoforms appear to have equal pI's (figs. 2.9a, & 2.10a). The more acidic pI of bound Tn-Ix may represent a stronger binding affinity for Tn-C. Troponin C was not resolved in figures 2.9a, b and 2.10b; and, can only be seen as a negatively staining band in figure 2.10a. In "Stains-all" troponin C of *O. andersoni* (fig. 2.6, lane E) appeared as a blue band of apparent molecular weight identical to Tn-C of *O. niloticus* (lane C).

Troponin T (Tn-T), being a highly basic protein, remains mostly in the sample well only slightly entering the first dimension alkali-urea gel. A small trace of Tn-t is found at the far basic end of figure 2.5c . Two dimensional evidence of Tn-T can also be seen in figures 2.9a, 2.9b, and 2.10c. No change in the mobility of Tn-T due to Ca^{+2} is observed (figs. 2.9a, & 2.9b). The molecular weight of this basic protein on the second dimension

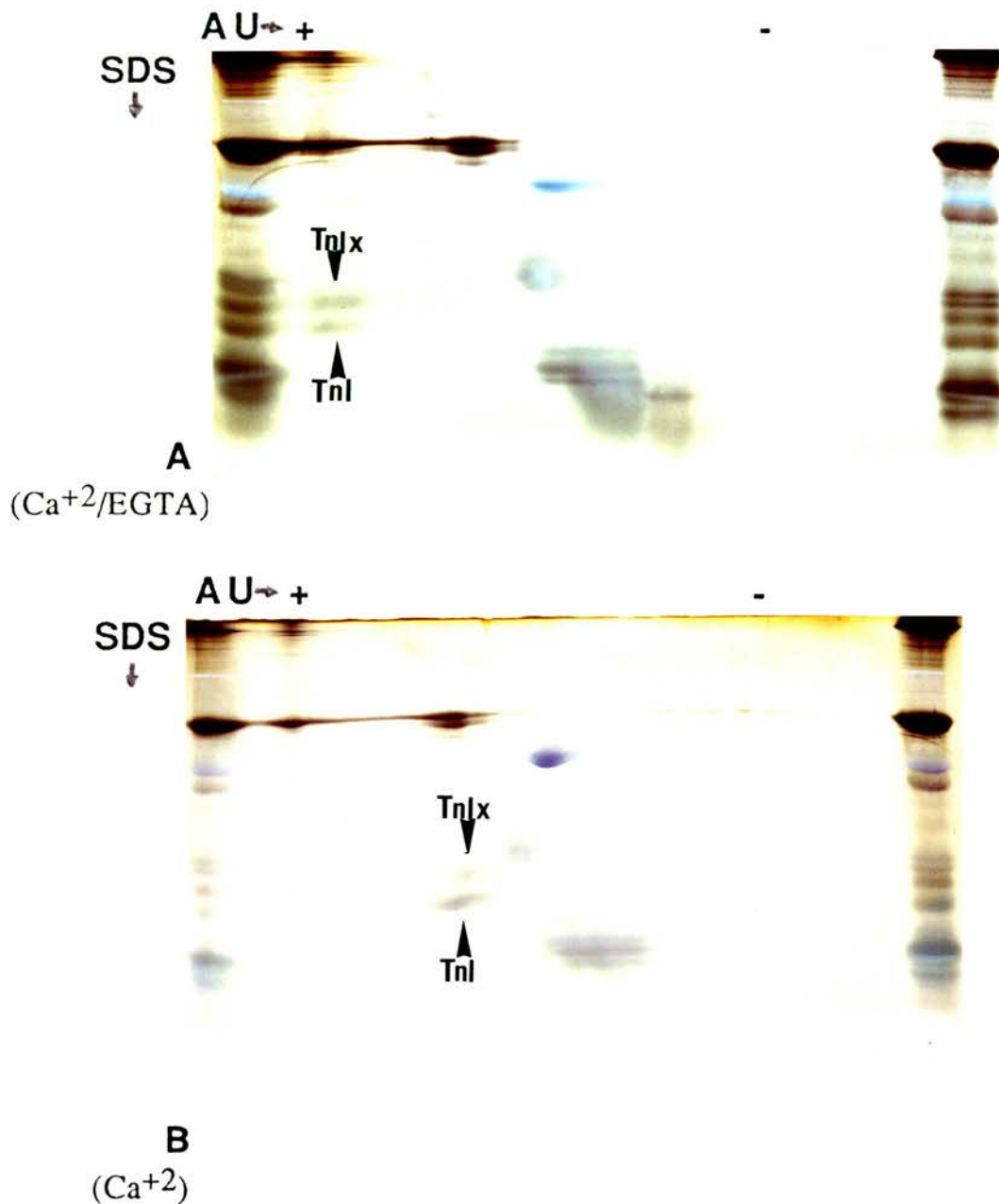Figure 2.10 Two dimensional electrophoresis of fast muscle myofibrils from *O. andersoni* heterozygous for LC-1. Note the change in apparent pI of the Tn-I isoforms with and without 10mM EGTA in the first dimension. In the presence of Ca^{+2} Tn-Ix seems to be slightly more acidic than Tn-I. This pI difference could be due to differential binding affinities for Tn-C as Tn-I and Tn-Ix have identical pI's in Ca^{+2}/EGTA.
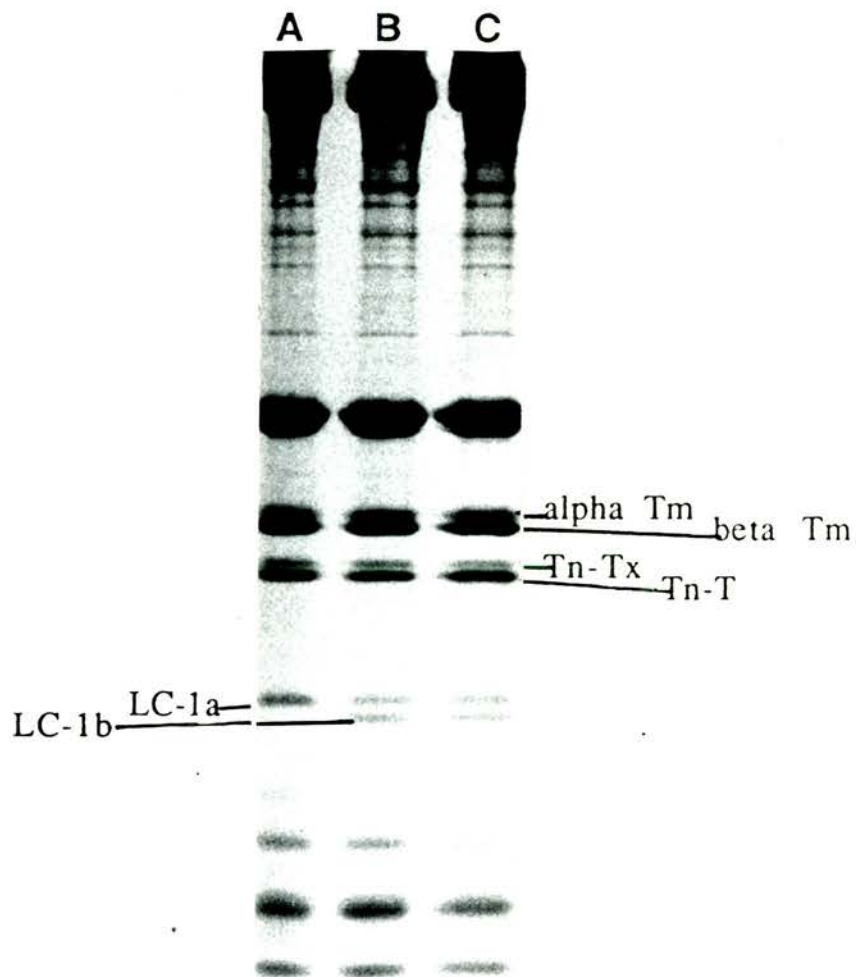
Figure 2.11 12.5% SDS-PAGE of *O. andersoni* fast muscle myofibrils. Note the isoforms of Tn-T and Tm present in both the hetero- and homozygous LC-1 individuals. Key: (A) LC-1a homozygote; (B & C) LC-1 heterozygote.

identifies it as Tn-T. Isoforms of Tn-T have been reported for rabbit (Schachat *et. al.*, 1985) and carp (Crockford and Johnston, 1989). Conclusive evidence of two Tn-T isoforms, Tn-T (Mr=32.6kD) and Tn-Tx (Mr=33kD) , in *O. andersoni* is shown in figure 2.11. Tn-Tx was not detected in two dimensional analysis. Furthermore, in *O. andersoni* the two known tropomyosin isoforms, alpha-Tm and beta-Tm, are apparent (fig. 2.11). Like Tn-T these tropomyosin isoforms were not resolved in two dimensional analysis.

Fertile F1 Hybrids

The sixteen hybrids surveyed were divided into two groups of progeny: seven NA hybrids [*O. niloticus* (female) X *O. andersoni* (male)]; and nine AN hybrids [*O. andersoni* (female) X *O. niloticus* (male)]. Fourteen of the hybrids were identical to both the *O. andersoni* LC-1 heterozygote and to a mixed sample of *O. andersoni* LC-1a homozygote and *O. niloticus* myofibrils in sample buffer (fig. 2.2). The only difference observed between the reciprocal hybrid groups occur in two AN samples which were homozygous for LC-1b (fig. 2.12). Therefore, the finding of intraspecific variation in LC-1 expression was extended to AN hybrids.
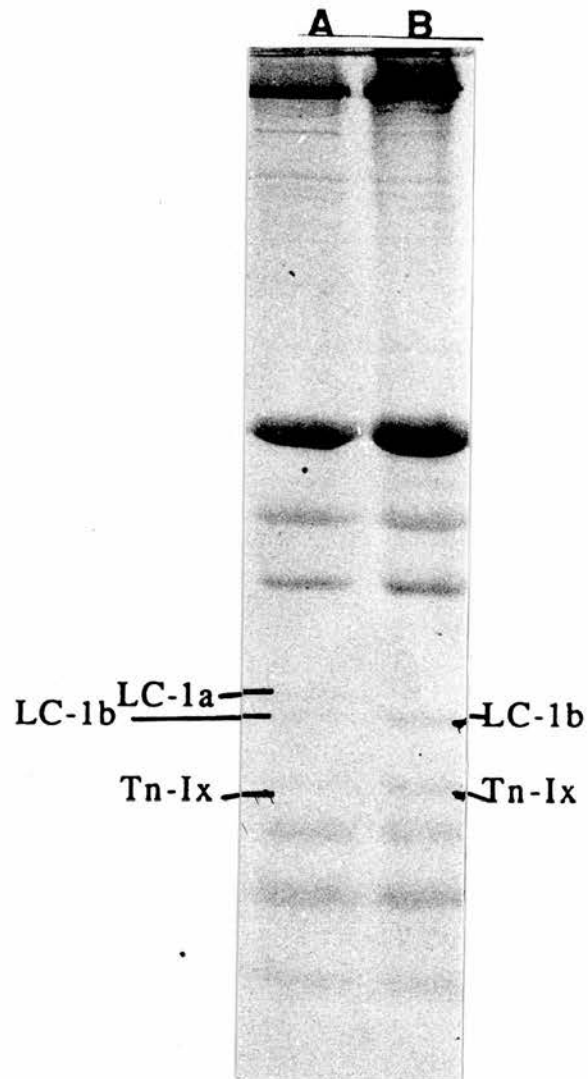
Figure 2.12   15%SDS-PAGE of AN hybrid fast muscle myofibrils.   (A) LC-1 heterozygote; (B) LC-1b homozygote.   All hybrids contained the Tn-Ix isoform.

Alkali-urea and two dimensional electrophoresis of the hybrid provided further evidence on the interspecific isoform differences between *O. andersoni* and *O. niloticus*. Figure 2.4, an alkali urea gel, illustrates that LC-1a from the *O. andersoni* homozygote and LC-1b of *O. niloticus* differ in pI. The hybrid (lane B) contains both the *O. andersoni* (lane C) acidic, lower band LC-1a, and the *O. niloticus* (lane A) basic, upper band LC-1b. The difference in pI's of LC-1a and LC-1b is also apparent in the second dimension SDS-PA gel of the hybrid (fig. 2.5b).

On SDS-PAGE (fig. 2.2) there appeared to be no interspecific isoform differences in myosin light chain 2. However, on examination of the AU-PAGE (fig. 2.4) there appears to be a slight difference in the charge of the band immediately below Tropomyosin. In the hybrid this band appears as a doublet containing both the acidic LC-2a of *O. andersoni* and the basic LC-2n of *O. niloticus*. This interspecific difference in LC-2 was not resolved in the second dimension.

The hybrids were identical to *O. andersoni* in expression of troponin I and troponin C isoforms (figs. 2.2; and 2.6). However, no solid evidence is available on the highly similar tropomyosin and troponin T isoforms for either *O. niloticus* or the hybrids. The ability of SDS-PAGE
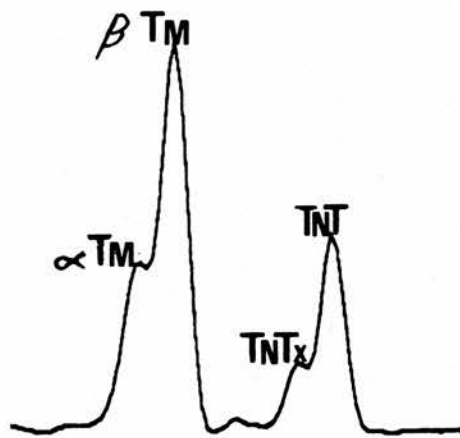
Figure 2.13 Densitometric scan of *O. andersoni* tropomyosin and troponin-T isoforms separated on 12.5%SDS-PAGE.
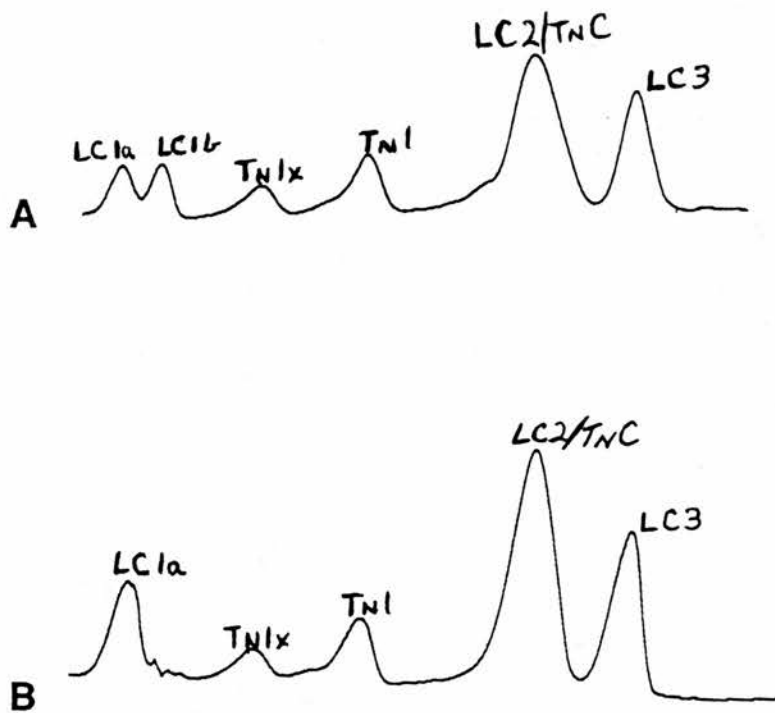
Figure 2.14 Typical densitometric scan of fast muscle myofibrils from *O. andersoni* separated on 15%SDS-PAGE. Key: (A) LC-1 heterozygote; (B) LC-1a homozygote. Data collected from these scans was used in statistical analysis.

to resolve relative molecular weight differences of less than 1kD depended on the distance of the lane from the edge of the gel. Maximal separation occurred only in the center three lanes. Moreover, the reproducibility of separation was poor from one gel to another. Densitometric scans of Tm and Tn-T isoforms illustrate that these proteins were too close to be detected as separate peaks (fig. 2.13).

Densitometric scanning enabled quantification of the relative amounts of each myofibrillar protein present in a particular sample. Typical scans of *O. andersoni* are presented in figure 2.14 Statistical analysis of alkali light chains, LC-1 and LC-3, is presented in table 1. Prior to hypothesis testing each distribution was assessed for normality using the Normal Scores test. If the sample distribution was normal, i.e. the Normal Scores test was not significant, then the parametric Student's TTest was used to test the null hypothesis. Non normal distributions were tested using the non-parametric Mann-Whitney test.

In *O. andersoni* and hybrid individuals LC-1a was significantly greater than LC-1b at ($P \leq 0.001$), and ($P \leq 0.05$), respectively. To test if the difference in LC-1a and LC-1b was constant among *O. andersoni* and the hybrids the LC-1a : LC-1b ratio was used. The ratios were not significantly different from one another ($P \leq 0.05$). The

Alkali Light Chains

Test	O. andersoni	Both	Hybrids
Normal Scores of LC-1a - LC-1b Distribution	Correlation = 0.991 N.S. at P ≤ 0.05 N=7; Mean=0.386 ± 0.1		Correlation 0.947 N.S. at P ≤ 0.05 N=10; Mean=0.2 ± 0.24
TTest of LC-1a - LC-1b $H_O = 0$	P = 0.0001 S. at P ≤ 0.0001		P = 0.025 S. at P ≤ 0.05
Normal Scores of LC-1a : LC-1b Distribution	Correlation = 0.986 N. S. at P ≤ 0.05 N=7; Mean=1.29 ± 0.08		Correlation=0.897 S. at P ≤ 0.05 N=10; Mean=1.40 ± 0.51
Mann-Whitney of LC-1a : LC-1b		P = 0.845 N. S. at P ≤ 0.05	
Normal Scores of LC-3 : LC-1 Distribution	Correlation = 0.977 N. S. at P ≤ 0.05 N = 17; Mean = 1.63 ± 0.26		Correlation = 0.98 N. S. at P ≤ 0.05 N = 9; Mean = 0.9 ± 0.47
TTest of LC-3 : LC-1 $H_O = 1$	P = 0.0000 S. at P ≤ 0.001		P = 0.55 N. S. at P ≤ 0.05
LC-1a + LC-1b	N=10; Mean=3.1 ± 0.29		
LC-1a only	N=7; Mean=3.04 ± 0.43		
Mann-Whitney (LC-1a + LC-1b) = LC-1a only		P = 0.92 N. S. at P ≤ 0.05	

Table 1 Statistical analysis of densitometric scanning data of alkali light chains in *O.andersoni* and F1 hybrid. Key: (N) population size; (S) significant; (NS) not significant; (H_O) null hypothesis.

Troponin-I

Test	O. andersoni	Both	Hybrids
(Tn-I - Tn-Ix)	N=17; Mean=3.15; S.D.=2.12		N=14; Mean=2.4; S.D.=0.
TTest of	P=0		P=0
(Tn-I - Tn-Ix)	N.S. at P < 0.0001		N.S. at P < 0.0001
$H_O = 0$			
Normal Scores	Correlation = 0.967		Correlation =0.887
of Tn-I : Tn-Ix	N.S. at P < 0.05		S. at P < 0.05
Distribution	N=17; Mean=2.6; S.D.=1.4		N=14;Mean=4.9;S.D.=4.4
Mann-Whitney		P = 0.17	
of Tn-I : Tn-Ix		N.S. at P < 0.05	
Correlation	r = -0.342		r = -0.175
Coefficient of	N.S. at P < 0.05		N.S. at P < 0.05
Tn-I to Tn-Ix			

Table 2 Statistical analysis of densitometric scanning data of troponin-I
isoforms in O. andersoni and F1 hybrid. Key (N) population size; (S.D.)
standard deviation; (S) significant; (NS) not significant; (H_O) null
hypothesis (r) Spearman's coefficient of rank correlation.

stoichiometry of the alkali light chains was tested using the LC-3/LC-1 ratio. This ratio was significantly different from the expected value of 1 in *O. andersoni* (P≤ 0.001). Finally, in *O. andersoni* the total amount of LC-1 in LC-1a/LC-1b individuals was not significantly different from the total amount of LC-1 in individuals expressing only LC-1a (P ≤ 0.05).

Possible relationships between the relative amounts of Tn-I and Tn-Ix expressed was also explored using scanning data. Statistical analysis, presented in table 2, found the amount of Tn-I was significantly greater than Tn-Ix in *O. andersoni* and the hybrids (P≤ 0.05). The difference between Tn-I isoform expression was constant as the Tn-I : Tn-Ix ratios of hybrids and *O. andersoni* were not significantly different from each other (P ≤ 0.05). An inverse relationship between the amount of Tn-I and Tn-Ix expressed was indicated by the negative correlation coefficients of *O. andersoni*, -0.342, and the hybrids, -0.175. However, further sampling is needed as these correlations were not significant at P ≤ 0.05.

Discussion

Characterization of myofibrillar proteins

O. niloticus

The protocol used to identify each component contractile protein was established using *O. niloticus* fast muscle myofibrils. Myosin heavy chain and actin were identified on basis of apparent molecular weight and dark staining intensity in coomassie blue. Identification of tropomyosin was based on apparent molecular weight, pI, and differential staining in silver stain and coomassie brilliant blue. Extremely basic pI and apparent molecular weight distinguished troponin T from tropomyosin and the other troponins. Besides characteristic molecular weight troponin I isoforms showed pI shifts on two dimensional electrophoretograms due to Ca^{+2} dependent binding to troponin C. Troponin C, which showed similar shifts in alkali urea electrophoresis, was not resolved on two dimensional gels. Instead, it was necessary to use "Stains-all" staining to distinguish Tn-C from myosin light chain 2. The myosin light chains were all identified from their apparent molecular weights and pIs. Using these methods the characteristics of *O. niloticus* myofibrillar proteins

were similar to those previously reported in fish and other vertebrates.

O. andersoni

The key difference between the fast muscle myofibrillar proteins of all the *O. andersoni* surveyed and those of other vertebrates studied to date is the expression of two troponin I isoforms: Tn-I and Tn-Ix. Troponin I isoforms correlated with slow or fast fibre type have previously been reported in skeletal muscle of rabbit (Dhoot & Perry, 1979; Salviati *et. al.*,1982) and bovine (Young & Davey, 1981). None of the fibre types examined contained two isoforms of Tn-I within the same fibre. Dhoot *et. al.*,(1978) found the localization of Tn-I fast and slow isoforms to be a feature of human, rat, mouse, hamster, rhesus monkey and babboon skeletal muscle. From this finding they suggest antibody staining for Tn-I as a reliable means of typing muscle cells.

The presence of two Tn-I isoforms in fast muscle myofibrils has only been reported for carp acclimated to 20°C (Crockford, 1989). Troponin Ix in warm acclimated carp appeared as a close satellite band of troponin I. Warm carp fast muscle troponin I isoforms did not exhibit the

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large apparent molecular weight differences shown in *O. andersoni* Tn-I and Tn-Ix isoforms. The 1.7kD difference between the *O. andersoni* troponin I's indicates that Tn-Ix is several amino acid residues larger than Tn-I. In rabbit fast and slow skeletal muscle the troponin I's were of similar size, while rabbit cardiac Tn-I was found to have an additional 26 amino acid residues (Grand *et. al.*, 1976). The additional residues of cardiac troponin I contained a siginificant phosphorylation site which was shown to modulate Ca^{+2} sensitivity of cardiac actomyosin (Perry, 1983; and England, 1983). The two troponin I isoforms showed identical pIs in the Ca^{+2} free state; however, Tn-Ix had a slightly more acidic pI than Tn-I in the presence of Ca^{+2}. This Ca^{+2} dependent pI difference may indicate differential binding affinities between the troponin I isoforms and Tn-C. In light of these previous findings in other vertebrates the existence of two Tn-I isoforms in fast muscle *O. andersoni* is an interesting discovery.

Fertile F1 hybrids

Fast muscle myofibrils of the fertile F1 hybrid of *O. andersoni* and *O. niloticus* matings contained all parental protein isoforms. In preliminary results the expression of both parental isoforms of LC-1 and LC-2 in the hybrid

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suggested a codominance relationship of parental alleles.
However, the discovery of intraspecific differences in *O.
andersoni* and hybrid myosin light chain 1 expression
challenged this hypothesis. In order to adequately test the
theory of codominance it would be necessary to identify the
banding pattern in each parent and their specific hybrid
offspring. Unfortunately, this test was not possible as
specific records linking parents to offspring were not
recorded.

Variation of contractile protein isoforms

Myosin light chains

The electrophoretic analysis of fast muscle myofibrils
revealed interspecific isoform differences in LC-1, LC-2
and Tn-I between *O. niloticus* and *O. andersoni*. Such a
large amount of interspecific variability between the
myofibrillar proteins of fast muscle has not been reported.
Other comparative studies have reported variability in the
alkali light chains among fish of different genus (Focant *et.
al.*, 1976). This variability is in marked contrast to the
more constant light chain expression seen in birds and

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mammals (Huriaux and Focant, 1985). However, in a definitive immunochemical/electrophoretic study of fast muscle from eight telosts, Rowlerson *et. al.* (1985) found no correlation of muscle ultrastructure, lifestyle, or taxonomic classification to alkali light chain expression. She speculated that the longer evolutionary history of teleosts has allowed for greater divergence in the alkali light chains. These results reported for *O. andersoni* and *O. niloticus* illustrate that striking light chain isoform variation is possible between species of similar taxonomy and lifestyle. Indeed, the ability of *O. niloticus* and *O. andersoni* to produce a fertile F1 hybrid indicates their close genetic similarity.

It is an interesting finding that no isoform differences in LC-3 were observed between the parent species and the hybrid. In separate studies using chicken (Nabeshima *et. al.*, 1984) and rat (Periasamy *et. al.*, 1984) muscle it was discovered that mRNA's for LC-1 and LC-3 resulted from alternative transcription of the same gene. The amino acid sequence's of LC-1 and LC-3 can be divided into two ends: the homologous COO^- end (141 amino acids from the COO^- terminus); and the divergent NH^{+3} end (8 amino acids in LC-3 and 49 in LC-1) (Weeds, 1975). There are three possible explanations why no LC-3 isoforms were observed: first, the electrophoretic techniques employed

were not sensitive enough; second, in the study animals LC-1 and LC-3 are coded by separate genes; third, the differences between LC-1a and LC-1b occur in the divergent NH+3 end.

Using the Holt and Lowey (1977) model of myosin, it is possible to hypothesize the existence of six isomyosins in fast muscle of *Ω. andersoni* and 12 isomyosins in the hybrid. These combinations are:

Theoretical Isomyosin	*Ω. andersoni* Light Chains	Hybrids Light Chains
1)	$(LC-1a)_2(LC-2)_2$	$(LC-1a)_2(LC-2)_2$
2)	$(LC-1b)_2$ "	$(LC-1b)_2$ "
3)	$(LC-1a)(LC-1b)(LC-2)_2$	$(LC-1a)(LC-1b)(LC-2)_2$
4)	$(LC-3)_2$ "	$(LC-3)_2(LC-2)_2$
5)	$(LC-1a)(LC-3)(LC-2)_2$	$(LC-1a)(LC-3)(LC-2)_2$
6)	$(LC-1b)$ " $(LC-2)_2$	$(LC-1b)$ " $(LC-2)_2$
7)		$(LC-1a)_2(LC-2a)(LC-2b)$
8)		$(LC-1b)_2$ " "
9)		$(LC-1a)(LC-1b)(LC-2a)(LC2b)$
10)		$(LC-3)_2$ " "
11)		$(LC-1a)(LC3)(LC2a)(LC2b)$
12)		$(LC-1b)$ " " "

Using non-denaturing pyrophosphate gel electrophoresis, isomyosins have been shown to exist within fast muscle in frog (Lannergren and Hoh, 1984) and chicken (Hoh *et. al.*, 1976). Staron and Pette (1987a & b) propose 18 possible isomyosins in type IIA fast fibers of the rabbit. While these isomyosins are postulated for *Ω. andersoni* and

hybrid fast muscle there is no actual proof that all the combinations exist *in vivo.* Pette and Staron (1988) conclude that the total number of isomyosins may be restricted *in vivo*"...because of preferential combinatorial patterns."

The original finding of this work is that LC-1 isoform expression varies not between recognized fibre types but, intraspecifically within one fibre type. Theoretically, homozygous individuals expressing only LC-1a (*O. andersoni*) or LC-1b (AN hybrids) have only 3 or 6 possible isomyosins, respectively. As shown above, heterozygous, LC-1a/LC-1b, individuals have twice as many possible combinations. The natural question arises as to whether the increased number of isomyosins possible in *O. andersoni* and hybrid heterozygotes confers any physiological and thus adaptational advantage.

It is assumed that the fish stocks at Stirling are genetically representitave of the wild populations from which they were started. The possibility of founder effect establishing a rare LC-1 allele in the homozygous condition in the captive *O. andersoni* population is remote (McAndrew, personal communication). Therefore, if the homozygous LC-1 condition exists in wild populations it probably is

neutrally selective. Absolute proof, however, would require the testing of *O. andersoni* from the wild.

Through the use of densitometry the relative stoichiometric amounts of each light chain in the myofibrils can be studied. In LC-1 heterozygotes LC-1a was always greater than LC-1b (P≤ 0.05). This difference in expression is conserved as the ratio of LC-1a:LC-1b was identical between hybrid and *O. andersoni* heterozygotes (P≤ 0.05). The possibility exists that the difference in LC-1 expression is artifactual due to differential staining properties in the two isoforms. However, it is more likely that these two results indicate skewed, non-random genetic expression of LC-1 isoforms. If expression were random between two LC-1 alleles then equivalent amounts of each LC-1 isoform is expected. Possible explanations are that either physiological or environmental factors are favoring translation of the LC-1a allele or modifier genes are regulating LC-1 expression through control of transcription, post transcription, and/or translation events.

Huriaux and Focant (1985) found the ratio of LC-3: LC-1 was greater than 1 for fish; whereas, the ratio was less than 1 for rabbit, chicken, and pig. The LC-3: LC-1 ratio varied between the fish studied without phylogenetic correlation. *O. andersoni* and the hybrid exhibited LC-3:LC-

1 ratios of 1.6 and 0.9, respectively. Statistically, the ratio in *O. andersoni* was significantly different from the expected value of 1 (P≤ 0.001). Such marked differences in the LC-3:LC-1 ratio extend the findings of Huriaux and Focant (1985) to fish as closely related as parent and hybrid species.

Troponin I

Troponin I expression varied interspecifically between the parent species since in none of the *O. niloticus* sampled was the troponin Ix isoform observed. Within hybrid and *O. andersoni* fast muscle the amount of Tn-I is greater than Tn-Ix (P≤ 0.0001). Furthermore, this difference is physiologically conserved since the ratio Tn-I:Tn-Ix is constant in both parent and hybrid (P≤ 0.05). In warm acclimated carp the predominance of Tn-I over Tn-Ix was also noted (Crockford, 1989). Differential expression in carp is most likely due to environmental influences on gene expression as Tn-Ix was only observed in warm acclimated animals. In Tilapia the cause of differential Tn-I isoform expression can only be theorized as one or a combination of the mechanisms already discussed for LC-1. It is interesting, although not statistically significant, that Tn-I and Tn-Ix were inversely related in their expression. An

inverse relationship would suggest possible feedback inhibition of one gene product on another.

Contractile Properties

There is limited evidence that Tn-I and myosin light chain isoforms are involved in the acclimatory response of fish muscle. Crockford (1989), in an electrophoretic study of fast muscle myofibrillar proteins of carp acclimated to different temperatures, found that cold acclimated carp contained an extra LC isoform, while warm acclimated fish contained an extra Tn-I. Johnston (1979) found evidence implicating the regulatory proteins (tropomyosin and the troponin complex) in temperature acclimation. If Tn-Ix were partially responsible for the cold tolerance of *O. andersoni* then similar ph·· ological tolerance would be expected in the ·   _. ·ıııs was not observed. Similarily, the presence of *O. andersoni* LC-1 and LC-2 isoforms in the hybrid do not correlate to cold tolerant physiology. Because the hybrid contractile properties resemble those of the less cold tolerant species, *O. niloticus*, this suggests recessiveness of the cold tolerant trait.

Genetics of intraspecific myosin light chain 1 expression

Any theories concerning the inheritance mechanisms of LC-1 in *O. andersoni* rely on the assumption that the isoform differences observed using denaturing electrophoretic techniques are a true representation of individual genotypes. Denaturing gel electrophoretic techniques have been developed to enable the study of non-soluble proteins and larger proteins constructed from subunits. There is no direct reported evidence which suggests that denaturing methods are more or less reliable than native techniques at estimating isoform differences in proteins. This is especially true when two dimensional techniques are applied to protein identification. Mikawa (1981) was confident enough in two dimensional electrophoresis of contractile proteins to support the conclusion that in a specific fibre type genetic expression of troponin subunits was linked to that of the myosin light chains. McAndrew and Majumdar (1983) made the assumption that average heterozygosity values obtained from native electrophoretic techniques probably reflected the amount of variation at other gene loci. This assumption remains to be proven for contractile proteins which until

this study have not appeared to vary intraspecifically according to genome.

From the two dimensional techniques applied to *O. andersoni* heterozygotes and homozygotes it is apparent that LC-1a and LC-1b differ in apparent molecular weight but have equivalent pI's. This is different from the situation in the hybrid, where LC-1a and LC-1b differed in both categories. The obvious reason for this discrepancy is that the LC-1b isoform in *O. andersoni* is not equivalent in pI to the LC-1b isoform in *O. niloticus*. In order to fully test this theory it would be necessary to examine an AN hybrid LC-1b homozygote for pI isoforms in LC-1b. Unfortunately, this was not possible. While the homozygous LC-1b geneotype was not observed among the *O. andersoni* sampled it is assumed to exist because of the LC-1b homozygous hybrid.

From the data presented on LC-1 in *O. andersoni* a Mendellian inheritance pattern with a nearly codominant relationship of LC-1a and LC-1b alleles is theorized. The scenarios represented in each of the three breeding groups are a homozygous LC-1a male mated with either a heterozygous LC-1 female (groups four and six) or a homozygous LC-1a female (group five). Examining group four by this theory the expected progeny of each genotype is three. Actual observed progeny were five heterozygotes and

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one homozygote. The chi-squared test, with the Yates correction for small sample size, found no significant difference at the P≤ 0.05 level between the observed and theoretically expected progeny genotypes. Testing this hypothesis with the hybrids is not possible as they were not separated into discrete breeding groups.

In order to fully substantiate the conclusion that intraspecific variation in LC-1 genotype is the result of Mendellian inheritance of codominant alleles more electrophoretic evidence from known mating pairs and offspring of *O. andersoni* is necessary. Furthermore, backcrossing of hybrid and parental strains could provide insight on possible linkage relationships in contractile protein genes. Such linkage relationships between metabolic enzyme genes have been proven using hybrid backcrossing in sunfish (Pasdar *et. al.*, 1984). Linkage relationships between the genes of myosin heavy chain, myosin light chain and actin have already been investigated using hybrid back- crossing in two mouse species. Robert *et. al.* (1985) examined genomic segregation patterns in mouse backcross progeny using cloned DNA probes for restriction fragment length polymorphisms. He found no evidence for linkage between any of the chosen contractile protein genes. If linkage exists between MHC and LC-1 genes in *O. andersoni* then it is possible that similar

intraspecific variation in MHC isoforms could be correlated to myosin light chain 1 isoform expression. In the next chapter this possibility is investigated using the technique of peptide mapping.

Chapter 3
Peptide Mapping of Electrophoretically Purified
Myosin Heavy Chain from *O. andersoni*
Introduction

It is well established that there is a close, positive, correlation between muscle fibre contraction velocity and myosin ATPase activity (Baranay, 1967). This fact has focused attention on the structure of myosin as a determinant of muscle contractile properties. Myosin is composed of four primary subunits (light chains 1,2,&3; and myosin heavy chain). Isoform differences in one or all of these subunits could contribute to enzymatic and physiological differentiation. In rabbit, MHC composition has been correlated to histochemically determined single fiber ATPase activity (Staron and Pette, 1986), and shortening velocity (Reiser *et. al.*, 1985; Sweeny *et. al.* 1986) Similar results were also reported for frog (Edman *et. al.*, 1988; and Lannergren, 1987) and carp (Scapolo and Rowlerson, 1987). The specific function of the myosin light chains is unclear (Syrovy, 1987). There is no correlation between ATPase activity (Wagner and Ginger, 1981; Staron and Pette, 1987a) or shortening velocity (Reiser *et.al.*, 1985) and light chain isoform composition.

Therefore, these findings have narrowed research on isomyosins to differences in the largest constituent subunit, the myosin heavy chain.

Several methods have been used to identify myosin isoforms present in muscle fibers: traditional histochemical staining for variation in ATPase activity (review by Spurway, 1980), immunohistochemical staining using monoclonal antibodies to specific myosin isoforms (Rowlerson *et. al.*, 1985; Scapolo and Rowlerson, 1987, Edman *et. al* 1988), immunoblotting of isolated myosins with monoclonal antibodies (Lied and Von Der Decken, 1985; Betto, Zerbato and, Betto, 1986; Sartore *et. al.*, 1987; and Rowlerson *et. al.*, 1985), pyrophosphate electrophoresis of native myosin (Hoh, McGrath, and White, 1976; and Lannergren and Hoh, 1984), and electrophoretic peptide mapping of enzymatically digested MHC (Rushbrook and Stracher, 1979; Bandman, Matsuda, and Strohman, 1982; Libera, 1981; and Carraro, and Catani, 1983). Of these methods the immunological and peptide mapping techniques have the greatest analytical value (Whalen, 1985). Peptide mapping was chosen for this study, due to the lack of suitable, specific antibodies for immunostaining.

Due to the large size and highly conserved structure of MHC the more traditional techniques of electrophoretic

analysis already described are inappropriate for the analysis of MHC isoforms (Rowlerson *et. al.*,1985). These properties can make the detection of apparent molecular weight differences between MHC isoforms impossible by SDS-PAGE. Peptide mapping by limited proteolysis, as first described by Cleveland *et. al.* (1977), provides a definitive technique for identifying the primary structure of the MHC. In digestion the protease cleaves the peptide bond between specific amino acid residues; thereby, breaking down the larger protein into smaller constituent peptides. The smaller peptide digests can be analyzed for differences in apparent molecular weight using standard SDS-PAGE. Differences in the digestion patterns produced from isolated MHCs indicates variation in the amino acid sequence.

Peptide mapping of has proven invaluble in assessing the changing expression of MHC isoforms during mammalian and avian development. In three separate studies, Whalen *et. al.*, (1979); Rushbrook and Stracher, (1979); and Hoh and Yeoh (1979), all demonstrated unique isoforms of embryonic MHC in rat, chicken, and rabbit. Bandman *et. al.*, (1981) used an *in vitro* ribosomal system (the reticulocyte lysate) which is incapable of post translational modification, to produce embryonic and adult MHC. The MHC isoforms obtained from the *in vitro* system were identical to corresponding MHC isoforms in muscle samples. This result confirms that

differences in embryonic and adult MHCs result from different mRNAs. Peptide mapping provided Rubinstein, Lyons, and Kelly (1989) with evidence for the involvement of neuronal and hormonal stimulation in the transition from embryonic to adult MHC. They conclude that this transition is endogenously controlled; but, "...closely orchestrated by the changing neuronal and hormonal status of the animal.".

Traditionally, histochemical techniques have identified muscle fibres according to their enzymatic or metabolic properties. More recently peptide mapping has been used to assess MHC isoform content within fibre types. These studies have established the existence of two fast MHC isoforms and one slow MHC isoform in rabbit skeletal muscle (Salviati, Betto, and Betto, (1982); and Staron and Pette, (1987a&b). Bandman *et. al.*,(1982) provided evidence that in adult chicken the two fast MHC isoforms result from post translational modification. Other fast MHCs related to specific muscles such as extraocular muscles (Sartore *et. al.*, 1987) have been discovered.

In the preceeding chapter electrophoretic evidence established the presence of intraspecific varation in the LC-1 isoform content between *O. andersoni* individuals. This study will employ peptide mapping to investigate if similar variation exists in MHC expression.

Materials and Methods

Preparation and electrophoretic concentration of MHC:

To analyze the enzymatic breakdown peptides of myosin heavy chain large concentrated amounts (>300ul) of MHC in SDS were needed. Purification of MHC was performed by first running 500uls of SDS myofibrillar protein sample on to a 3mm thick 7.5% acrylamide resolving gel with a 4% acrylamide stacking gel. Only two well formers were placed on each gel. These large wells were capable of holding 500ul of sample . Samples were run-in for 1/2h at 20mA/gel and 50v, then turned up to 60mA/gel and 200v for 10h.. After running the preparation gel was quick stained (5min) in the coomassie brillant blue solution and then placed into ultrapure water. The gel was washed in 5 changes of ultrapure water to rinse out excess stain and neutralize the pH of the gel. The MHC band was cut out with a sharp scalpel and chopped into fine pieces. Gel pieces were equilibrated for one hour at room temperature in 2X sample buffer containing only1% SDS.

Electrophoretic elution of MHC was performed in the apparatus developed by Crockford (1989) shown in figure 4.0. All elution equipment was cleaned by soaking in dilute Decon detergent and rinsing thoroughly with ultrapure
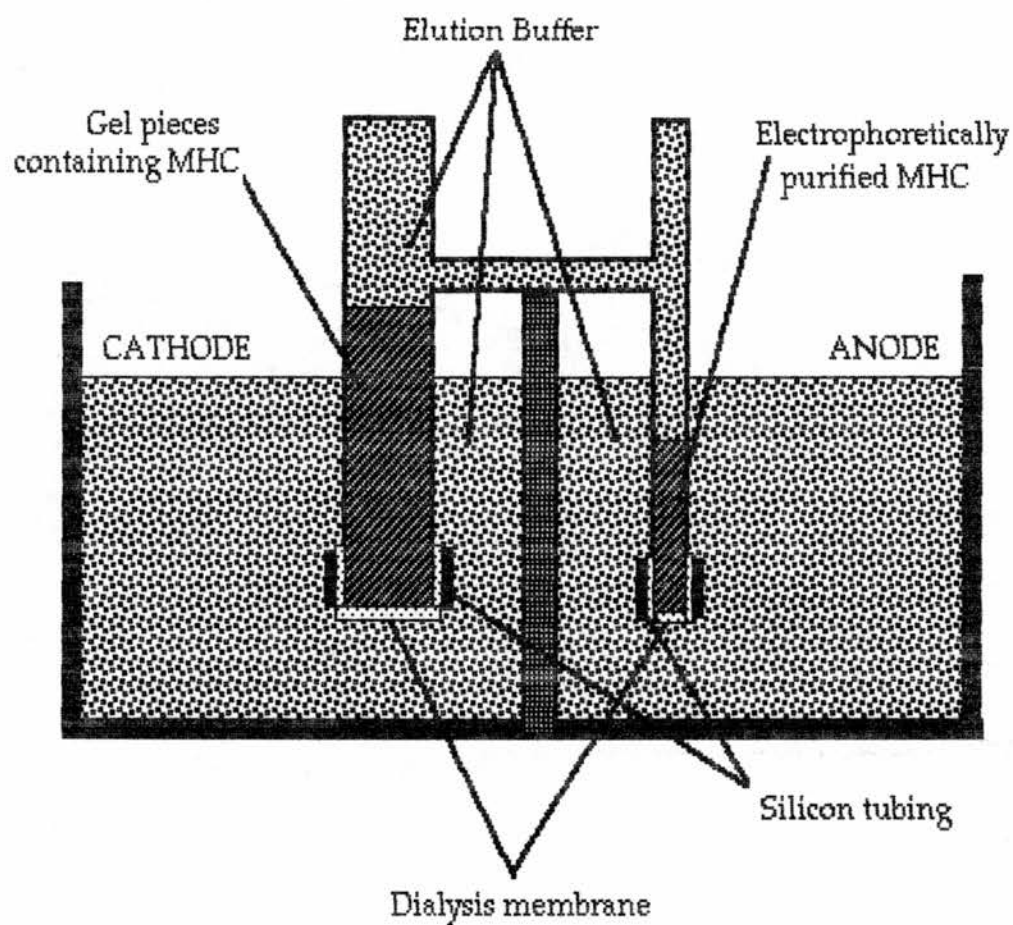
Figure 4.0 Electroelution chamber used for concentrating myosin heavy chain (MHC) for peptide mapping. Conditions of elution and buffer composition are described in text.

water. Gel pieces were loaded into the large chamber up to the level of the spanning tube. The pieces were overlayered with their equilibration buffer taking care that no buffer entered the spanning tube and that no air bubbles were trapped between the gel pieces. In order to recover the maximum amount of MHC it was necessary to elute the equilibration buffer as well as the gel pieces. The small chamber, the spanning tube, and the upper part of the large chamber were filled with elution buffer (10mM Tris-HCl, 0.1% SDS pH 7.4 at 20°C).

The chambers were aligned so that the current pulled the anionic SDS bound MHC across the spanning tube, towards the anode. MHC was collected above the dialysis tubing covering the bottom of the small chamber. Elution was performed at 100v and no more than 4mA for 2h. By that time all of the coomassie blue migrated to the small chamber. The collected protein was carefully removed by pippette and stored at -20°C. In order to check the purity of the concentrated MHC the collected samples were run onto a 15% acrylamide gel and visualized with coomassie blue staining.

Proteolytic digestion of MHC and peptide mapping:

Chymotrypsin (type 1-S from bovine pancreas Sigma Chemical Co.) and V8 protease (from *Staphylococcus aureus*

Sigma Chemical Co.) were dissolved in 2% SDS, 62mM Tris-HCl (pH 6.75),0.025% Bromophenol Blue to final concentrations of 62.5 activity units/ml and 100 activity units/ml, respectively. Proteolysis was initiated by vigorously mixing 1 part enzyme stock solution to 5 parts MHC and incubating at 25^0C. Digested samples were removed at 20, 45, and 90 minutes. Digestion was stopped by heating samples for 3min at 100^0C, then adding 2-mercaptoethanol to 2%. Samples were then stored at -20^0C. To insure that the digestion patterns were from proteolysis and not from random degradation of the MHC or the enzyme, it was necessary to incubate small control samples (20ul) of the enzyme stock solution and each MHC tested.

Peptide maps were produced by running sample digests and controls onto a15% SDS-polyacrylamide gel. To maximize band sharpness initial run-in time was extended until all of the sample entered the stacking gel (approx. 1 hour). After electrophoresis gels were fixed, stained with coomassie brilliant blue, and fully destained. The clear destained gel was then silver stained for maximum detection of peptide bands.

Results

The four *O. andersoni* individuals studied were selected for differing LC-1 expression, and breeding groups. Individuals A and C, heterozygous for LC-1, were from breeding groups 6 and 4, respectively. Individuals B and D, homozygous for LC-1a, were from breeding groups 5 and 4, respectively. Peptide mapping, of the electrophoretically purified MHC from these individuals, was used to investigate if differences in breeding group or LC-1 expression correlated to specific MHC isoform expression.

Chymotrypsin cleaves peptide bonds on the -COOH side of tyrosine, phenylalanine, and tryptophan. V8 protease cleaves on the -COOH side of aspartic acid and glutamic acid. The use of two enzymes of different cleavage sites increased the chances of detecting primary structure differences in MHC. Peptide maps produced from short incubation times (< 45min) were used to illustrate any differences in intermediate digestion. After 90min digestion was assumed to be complete.

Intermediate V8 digestion, figure 4.1, shows identical digestion banding patterns across all four lanes. The only apparent differences are in the staining intensity of the bands relative to one another. This is due to slight differences in the original concentration of the purified
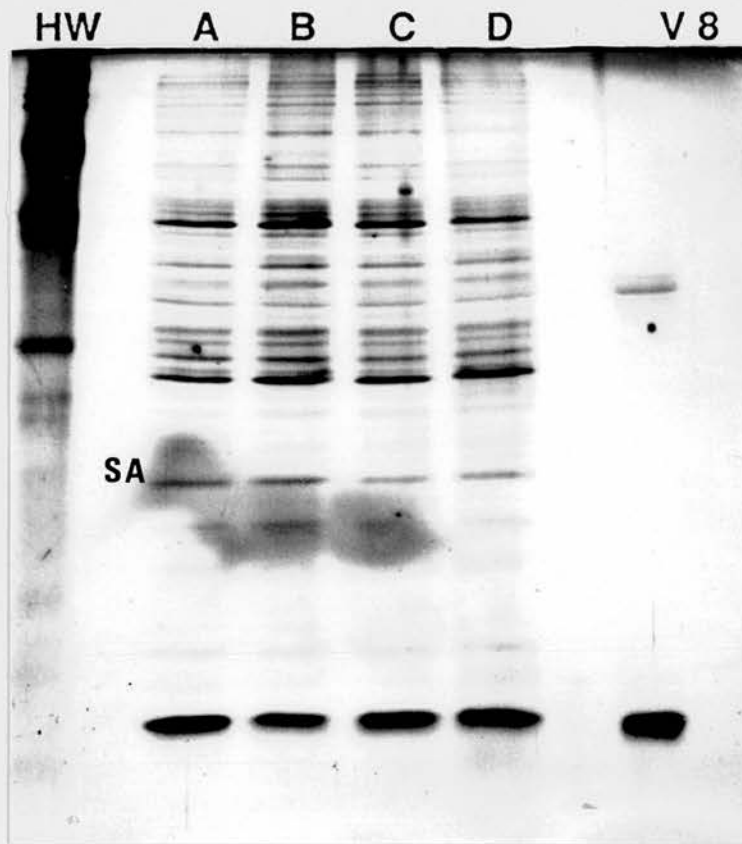
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Figure 4.1   15% SDS polyacrylamide peptide map of *O.*
andersoni MHC digested for 30min at 25ºC with V8 protease
from *Staphylococcus aureus* .   Peptides were stained with
the Sigma silver stain kit.   Key: (HW) high molecular weight
marker; (A) LC-1 heterozygote, breeding group 6; (B) LC-1
homozygote, breeding group 5; (C) LC-1 heterozygote,
breeding group 4; (D) LC-1 homozygote, breeding group 4;
(SA) staining artifact; (V8) V8 protease control incubated for
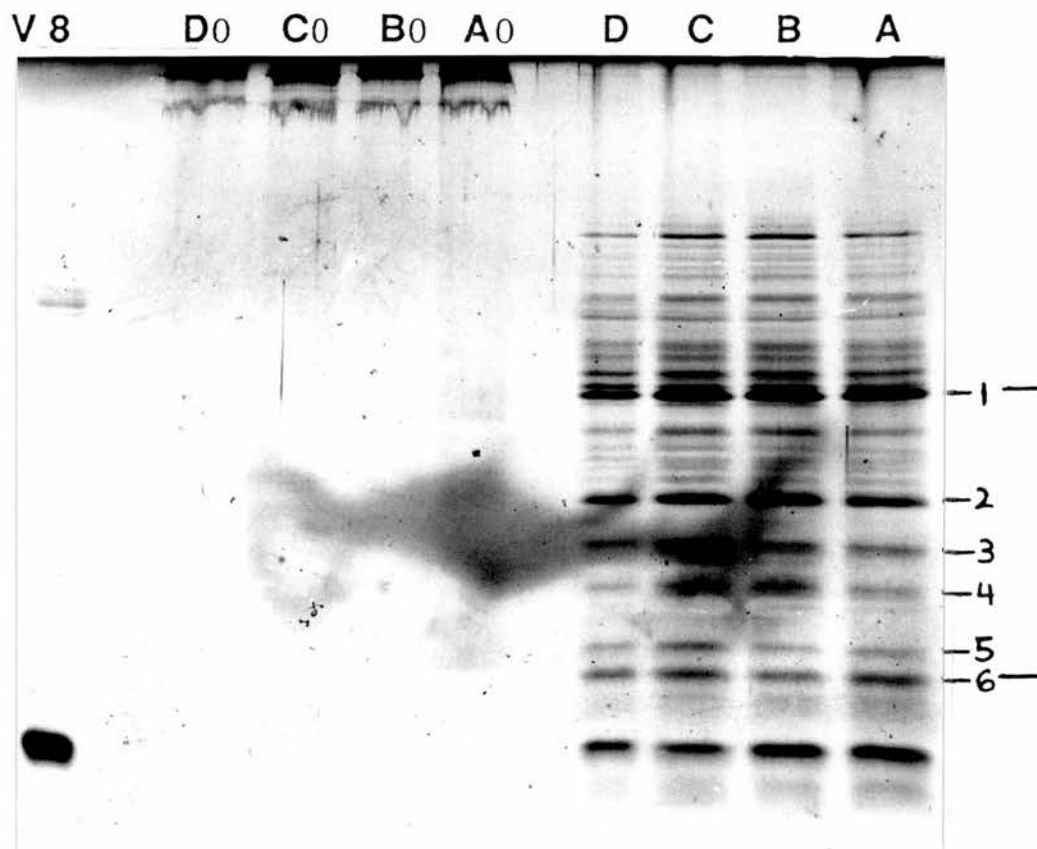30min at 25ºC.

Figure 4.2   15% SDS polyacrylamide, silver stained, peptide maps of *O. andersoni* MHC digested for 90min with V8 protease. Key as described in figure 4.1. Lanes Ao-Do on the left are undigested MHC controls incubated for 90min at 25°C. No appreciable non-enzymatic breakdown is apparent.   The disappearance of the upper bands and the increase in the lower bands, as compared to the 30min digestion maps, indicates more complete digestion.   No differences were observed between any of the digests.

MHC. Figure 4.2 shows the final V8 digestion patterns along with incubated but undigested MHC controls. It was important to run the controls alongside the final digestion patterns to eliminate any possible banding differences due to non-specific degradation. It was equally important to run an incubated protease control along with each experiment to show which bands resulted from MHC digestion.

A comparison of figures 4.1 and 4.2 shows a disappearance of the uppermost bands in the 90min digestion. This indicates more a complete and final digestion pattern. No differences are apparent in either the intermediate or final digestion patterns between the four MHCs studied.

Intermediate chymotrypsin peptide maps (figure 4.3) of 20min and 45min incubations all show similarity, indicating the reproducibility of the gels. The final 90min maps in figure 4.4 strongly resemble the earlier maps in figure 4.3 only with greater band definition. This suggests that chymotrypsin cleaved nearly all the available substrate bonds of MHC before the 20min incubation sampling. Peptide maps B-D in figures 4.3 and 4.4 were identical at each incubation time. Lane A, however, lacked bands 1, 2, and 3 in figure 4.3. Band 4 of lane A in each case is of stronger intensity than the corresponding bands in B-D. Band 2
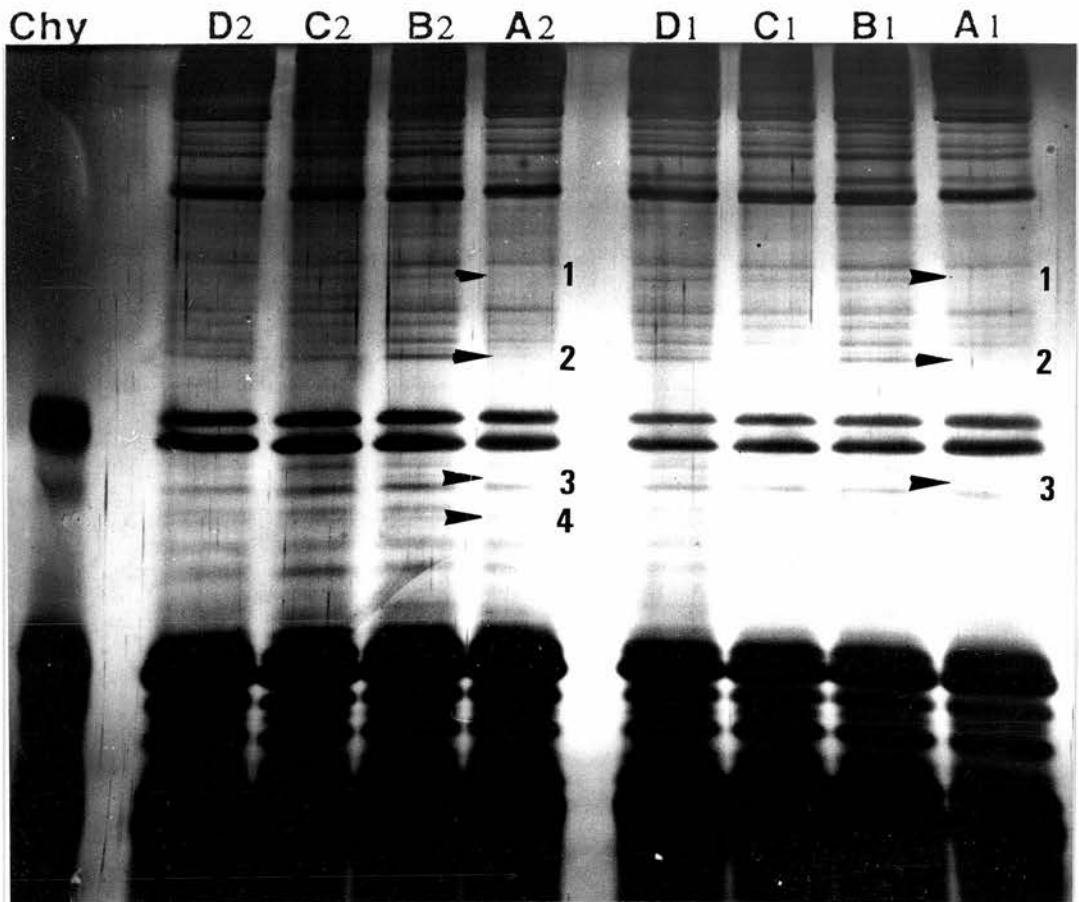
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Figure 4.3   15% SDS polyacrylamide, silver stained, peptide maps of *O. andersoni* MHC digested for 20min and 45min with chymotrypsin.   Identical digestion patterns were produced at 20min and 45min.   Banding differences in lanes A1 and A2 are highlighted by arrows. Key (A, B, C, & D) as described in figure 4.1; (1) 20min digestion; (2) 45min digestion; (Chy) chymotrypsin incubated for 45min at 25°C.
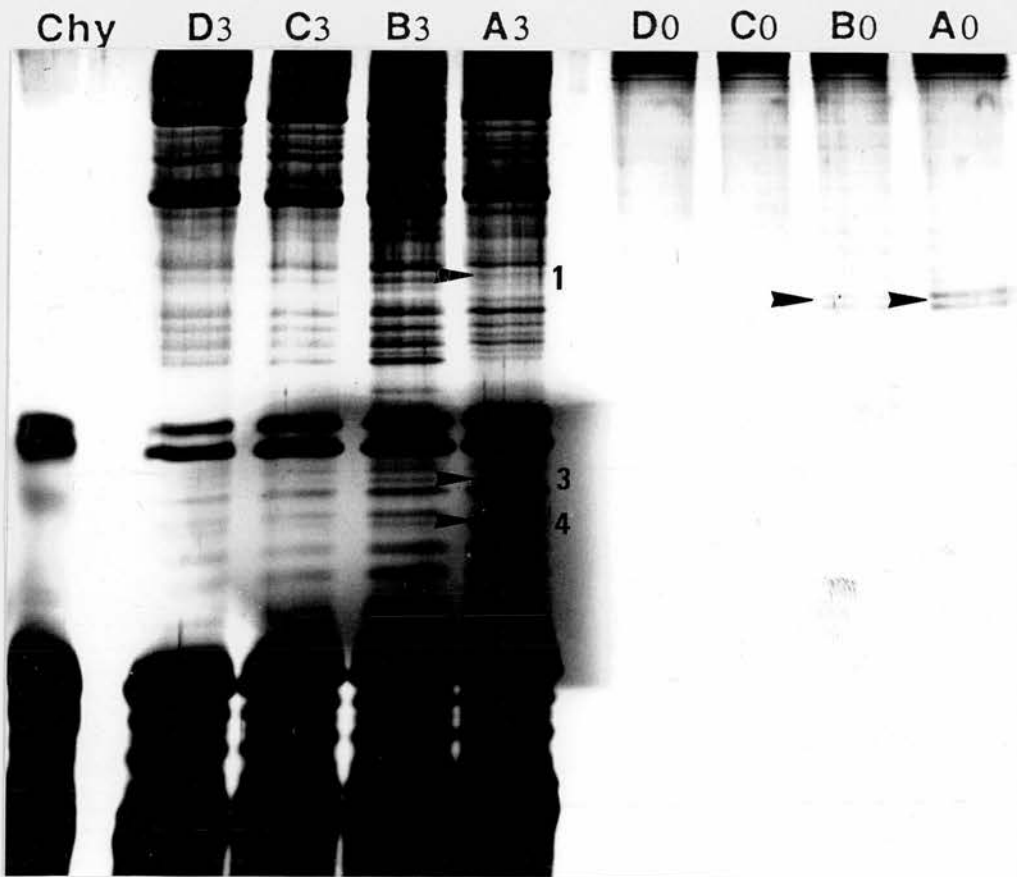
Figure 4.4   15% SDS polyacrylamide, silver stained, peptide
maps of *O. andersoni* MHC digested for 90min with
chymotrypsin.   Lanes Ao-Do are undigested MHC controls
incubated for 90min at 25°C.   Note the slight breakdown
products (pointed out by arrows) in the undigested samples.
Differences in lane A3 highlighted by arrows. The dark
background of lanes A3, B3, C3 is a silver staining artifact.
Key: (A, B, C, & D) as described in figure 4.1; (3) 90min
digestion; (Chy) chymotrypsin incubated for 90min.
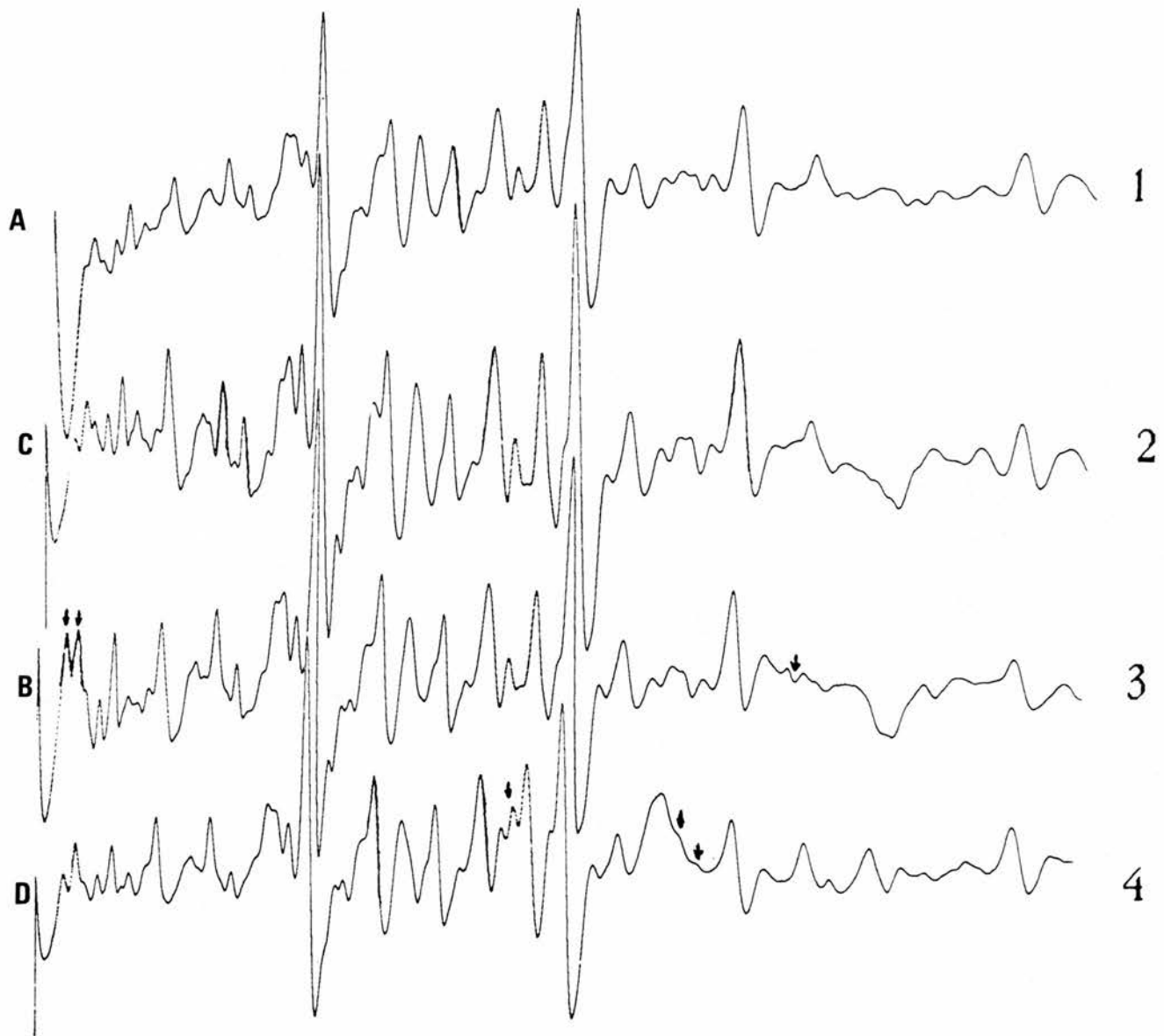
Figure 4.5 Densitometric scans of MHC peptide maps obtained after intermediate 30 min digestion with V8 protease. Scans A and C are of *O. andersoni* individuals heterozygous for LC-1. *O. andersoni* individuals B and D expressed only LC-1a. Individuals C and D are siblings from breeding group 4. A and B are from breeding groups 6 and 5, respectively Each peptide map scanned appeared identical. Slight differences in the scans (highlighted by arrows) do not correlate to the isoform differences in LC-1 or breeding.

Figure 4.6 Densitometric scans of MHC peptide maps obtained after final 90min digestion with V8 protease. Scans are lettered as previously described. Differences are observed but do not correlate to LC-1 expression or breeding. Each peptide map scanned appeared identical. The peak marked A is artifactual. The disappearance of peaks in the bracketed region indicates more complete digestion.

Figure 4.7 Densitometric scans of MHC peptide maps obtained after intermediate 45 min digestion with 1-Chymotrypsin. Scans are lettered as previously described. Arrows 3 and 4 correlate to bands 3 and4 in lane A2 of figure 4.3. Other differences are also noted in the scans; but, are not observed in figure 4.3.
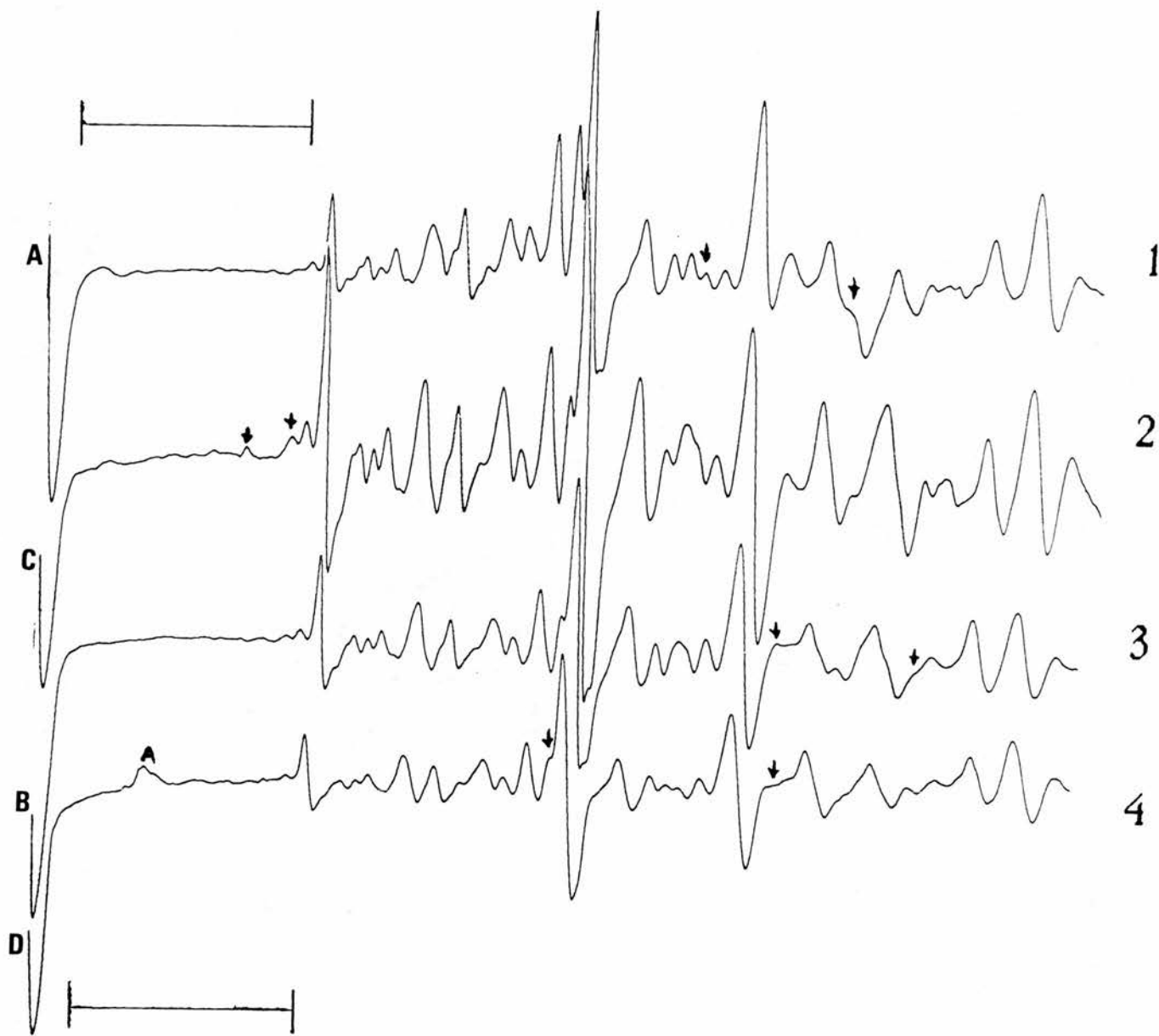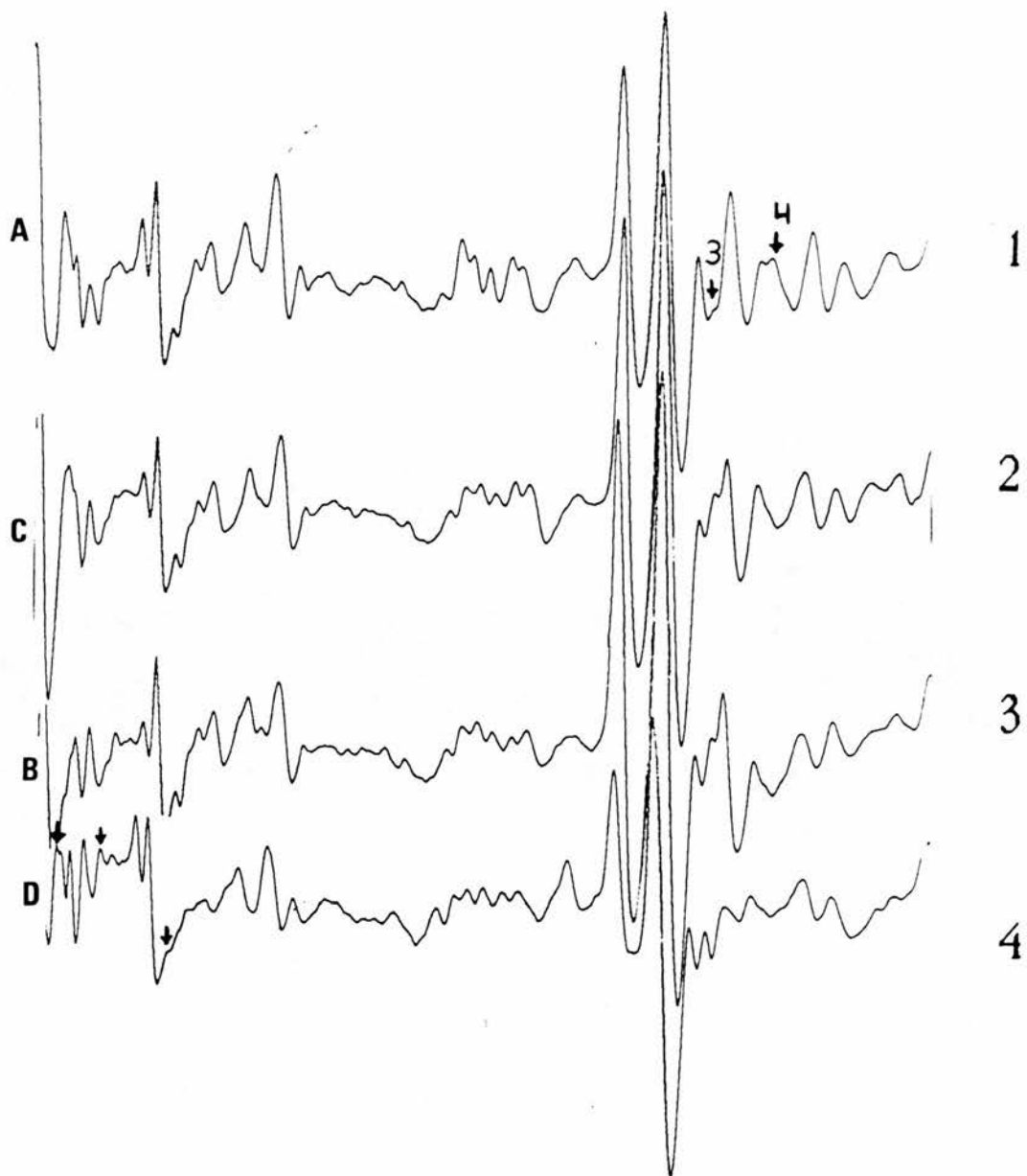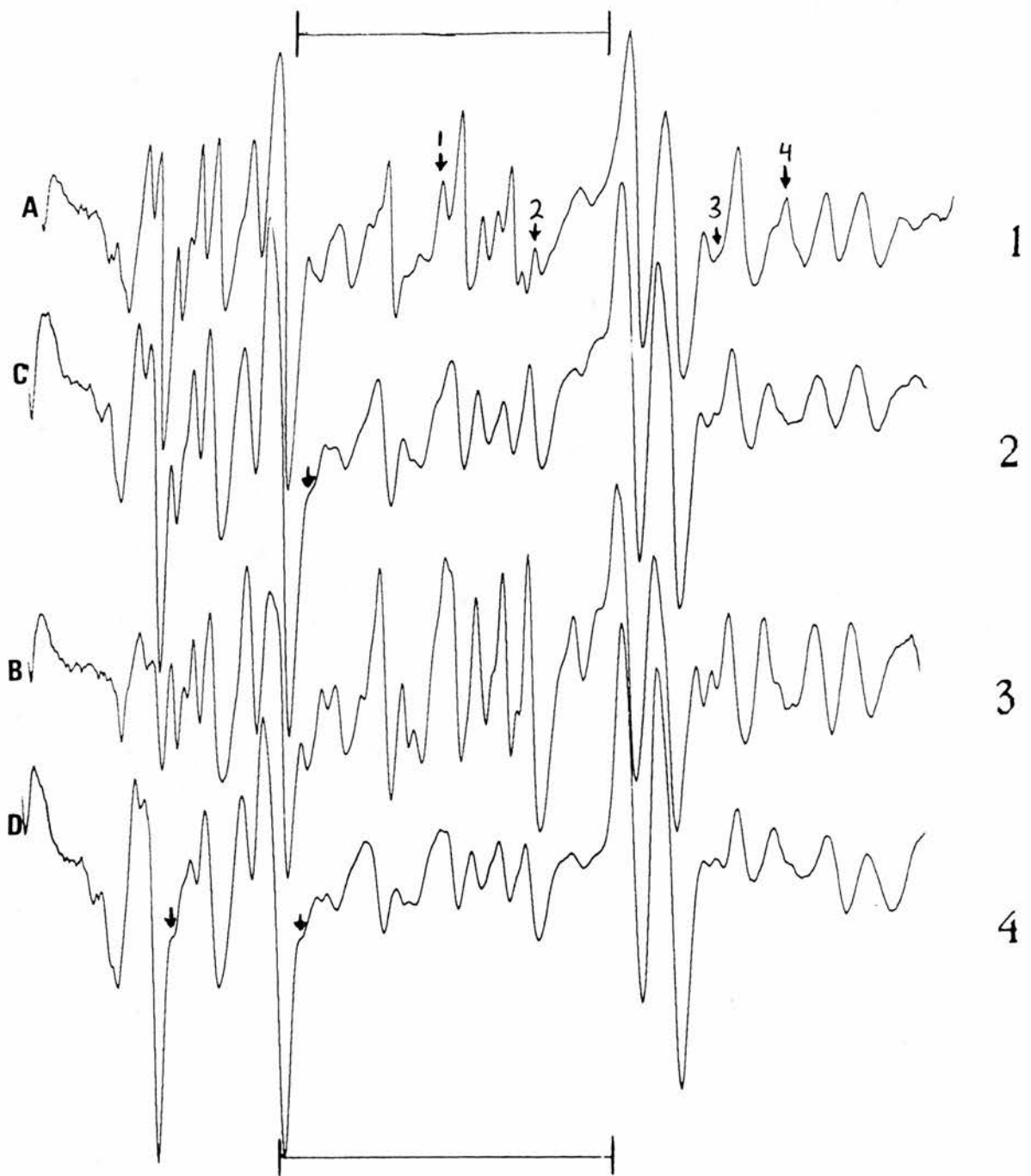
Figure 4.8 Densitometric scans of MHC peptide maps obtained after final 90min digestion with Chymotrypsin. Scans are lettered as previously described. Arrows 1, 2, 3, & 4 correlate to differences noted in lane A3 of figure 4.4. Other differences are apparent; but, do not correlate to observed differences in the gel.

appears in lane A3 (figure 4.4); but, at a lesser intensity than lanes B3-D3. Because chymotryptic digestion appeared nearly complete at 20min it is unlikely that the banding differences noted in lane A up to 90min were due to differences in digestion.

Each of the gels in figures 4.1-4.4 were scanned at 420nm using a Shimadzu CS-9000 gel scanner. Differences are noted between the 30min (figure 4.5) and 90min (figure 4.6) scans of the V8 peptide maps; however, these differences are not constant, and are probably due to slight differences in band resolution. The scans of the 45min (figure 4.7) and 90min (figure 4.8) chymotrypsin digestions reinforce the differences in individual A already noted in figures 4.3 and 4.4.

Discussion

The definitive method of peptide mapping using gel electrophoresis was first described by Cleveland *et. al.* (1977) who compared two methods of peptide mapping. Either, digestion of protein still contained within gel pieces, or digestion of electrophoretically eluted proteins. In the former method gel pieces containing the protein to be analyzed are loaded and run onto a second gel along with protease. When the bromophenol blue dye front nears the end of the stacking gel the current is switched off for 30min to allow for proteolysis. In the latter, chosen for the present study, digestion is carried out under controlled conditions and stopped at selected time intervals. The digested samples are then loaded and run on a standard SDS-polyacrylamide gel.

Cleveland *et. al.* (1977) reported no differences in the peptide maps obtained from either method. Others (Libera, 1981; Rushbrook and Stracher, 1979; and Bandman *et. al.* 1981 & 1982) have all reported on the reproducibility of the Cleveland method using gel pieces. It is the finding of this study and that of Crockford (1989) that the peptide maps produced from eluted MHC were superior to maps from gel pieces in band resolution and reproducibility.

Figure 4.9, lane 3, is a peptide map of *O. andersoni*
MHC produced from gel pieces digestion (courtesy of T.
Crockford). Comparison of this banding pattern with the
maps of figure 4.2 shows the increased resolution of the
fine bands using the elution method. The corresponding
bands in figures 4.2 and 4.9 are numbered for easy
comparison. There are three probable contributing factors
in the elution method which allow for band sharpness (ie.
resolution) and reproducibility. Firstly, digestion of the
eluted protein is carried out under precisely controlled
conditions of temperature and time. This limits any
uncontrollable effects of the stacking gel on proteolysis.
Secondly, volume measurement of eluted MHC, rather than
equal sizing of gel pieces, is more accurate and easily
adjusted to any differences in protein concentration.
Finally, blemishes in the interface of the gel sample piece
with the stacking gel can affect band shape in the resolving
gel. This is not a problem with the eluted sample as it is a
liquid. Perhaps the greatest advantage of the elution
technique is the ability to produce several peptide maps
from a single sample. This further increases the
reproducibility between experiments.

Close inspection of the intermediate and final peptide
maps from V8 proteolysis reveals no isoforms of MHC
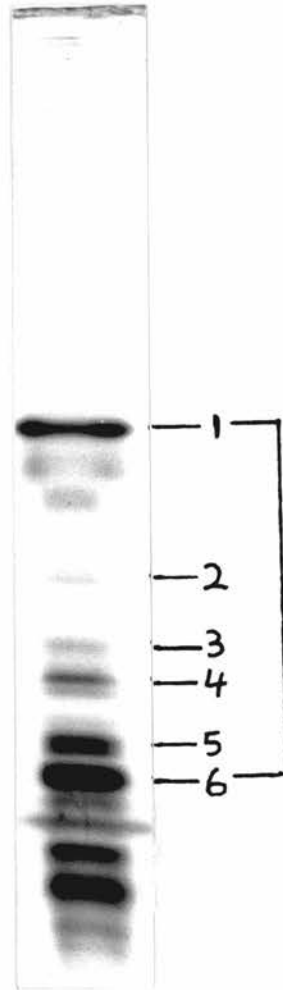between the four individual *O. andersoni*. Densitometric

Figure 4.9 V8 peptide map of *O. andersoni* produced from gel pieces digestion (courtesy of T. Crockford). The bracked region corresponds to the bracked region in figure 4.2. Certain bands are numbered for easier comparison between figure 4.9 and 4.2. Note the lack of band sharpness in this gel compared to the elution technique presented in figure 4.2.

scanning showed slight differences between the V8 maps; however, these differences were not maintained from intermediate to final digestion and did not correlate to LC-1 expression. Comparison of the chymotryptic peptide maps did reveal slight differences between the MHC of individual A and that of individuals B-D. These banding differences were maintained from intermediate to final digestion and were supported by densitometric scanning data. The isoform of MHC expressed in A did not correlate with LC-1 expression as individual C was also heterozygous for LC-1. The only other possible known difference between A and B, C, & D was breeding group.

The general conclusion of this peptide mapping study is that intraspecific variation in MHC expression exists among the four *O. andersoni* sampled. In previous sampling, differences in MHC peptide maps were observed between two *O. andersoni* (T. Crockford personal communication) This intraspecific variation in MHC does not correlate with either the hetero- or homozygous LC-1 genotype. It is possible that parental lineage is a determinant of MHC isoform expression; however, further investigation of siblings and parents would be necessary to establish this relationship.

Previous studies using peptide mapping have linked differences in MHC isoform expression to development,

disease, or muscle fibre type. These correlations depend on the basic assumption that MHC is not subject to individual allelic variation; but, is constant among similar phenotypes. The preliminary findings of this study challenge this basic assumption.

Chapter 4

General Discussion

The qualities of tilapiine fishes which make them ideal for aquaculture ie. fast growth, general hardiness across a range of environmental conditions, short regeneration time and ability to produce interspecific and intergenic fertile F1 hybrids also make them suitable for genetic studies (McAndrew, 1989; and Kornfield *et. al.* 1979). Mutungi (1987) used tilapiine fishes to study the thermal dependence of muscle contraction in two closely related species. He found that fast fibres from *O. andersoni* generated higher maximum isometric tensions at temperatures below 12-15⁰C than homologous fibres from *O. niloticus.* This finding correlated with the greater cold tolerance of *O. andersoni.* In the present study the fast muscle contractile proteins of *O. andersoni, O. niloticus* and their F1 hybrid have been analysed to provide an insight into the genetic mechanism underlying the inheritence of cold tolerance. In the course of characterizing the contractile proteins this project has uncovered unusual intra- and interspecific variation in the expression contractile protein isoforms.

In general, the characteristics of the fast muscle contractile proteins in the three study fish agreed with the well established characteristics for fish and higher

vertebrates. Previous studies have correlated the existence of particular isoforms with muscle fibre type (Staron and Pette, 1987a; 1987b; and Hoh *et. al.* 1976), developmental stage (Whalen, 1985; and Hoh and Yeoh, 1979) and disease state (Rushbrook and Stracher, 1979; Rushbrook *et. al.* 1987) The protein isoforms expressed within vertebrate skeletal muscle have not been shown to vary intraspecifically as long as the conditions of the animal and muscle sampling are identical.

This study found that, despite controlled conditions, the expression of myosin light chain 1 isoforms varied intraspecifically in *O. andersoni* and the F1 hybrid progeny of *O. andersoni* (female), *O. niloticus* (male) crosses. Intraspecific variation in *O. andersoni* consisted of two possible genotypes either LC-1a homozygote or LC-1 heterozygote. Available data suggested that LC-1 genotypes were expressed in Mendellian ratios among progeny. Furthermore, preliminary peptide mapping results indicated that *O. andersoni* myosin heavy chain isoforms also varied intraspecifically. Myosin heavy chain variation however, was not correlated to differences in myosin light chain 1 expression. In general, the finding of intraspecific variation complicates interpretation of inheritance patterns in the hybrid; however, as such variation has never before been observed *O. andersoni* provides a unique opportunity to study this phenomena.

The possible physiological ramifications of myosin light chain 1 and myosin heavy chain isoform composition could be investigated by first measuring the contractile properties of isometric force (Po) and maximum velocity of shortening (Vmax) of a single skinned muscle fibres; then, analysing the contractile proteins of each fibre using electrophoretic techniques. Using such a protocol Reiser *et. al.* (1985) found a significant correlation between MHC isoform composition and Vmax in single fast fibres of the rabbit. He also found that variations in alkali light chain composition occurred independently of MHC isoform variation. Similar correlation was also reported in frog (*Xenopus*) single muscle fibres (Lannergren, 1987). While the specific alkali light chain isoform composition was not correlated to Vmax, Greaser *et al.* (1988) found that in rabbit single fibres the stochiometric LC-3 : LC-1 ratio was positively related to differences in Vmax.

A potentially interesting genetic study utilizing the interspecific F1 hybrid of *O. andersoni* and *O. niloticus* would be to repeat the investigation of Robert *et. al.* (1985). In this study he used the method of gene cloning to produce DNA restriction fragment length polymorphisms (RFLPs) as probes for actin, myosin heavy chain, and myosin alkali light chain genes (reviewed by Solomon and Goodfellow, 1983). These RFLP probes enabled Robert *et. al.* (1985) to follow the segregation of these genes in the progeny of an interspecific

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mouse F1 hybrid crossed with its parent species (backcross progeny). Linkage in expression of the contractile protein genes was not observed. To establish the chromosomal location of the actin, MHC and alkali LC genes Robert *et. al.* (1985) analysed other polymorphic genes of known location for similar segregation. He found that indeed each of the contractile genes studied was located on a different chromosome. With these results Robert *et. al.* (1985) was able to reject the hypothesis of Buckingham (1985) that coexpression of contractile protein isoforms resulted from common regulation of isogenes in close proximity to one another on the DNA. Backcrossing of the *O. niloticus, O. andersoni* F1 hybrid would provide a means of investigating the structural relationships of contractile protein genes in fish. *O. niloticus* and *O. andersoni* present a good model for such genetic studies as they are genetically distinct enough to exhibit polymorphism at several loci (McAndrew and Majumdar, 1984); but, are similar enough to produce fertile hybrid offspring.

A good example of interspecific contractile protein polymorphism between the two parental species occurred in troponin I expression. *Oreochromis niloticus* fast muscle contained a typical single Tn-I isoform; whereas, *O. andersoni* contained both the *O. niloticus* Tn-I and another isoform, Tn-Ix, of higher apparent molecular weight. This is the first known report of two such dissimilar Tn-I isoforms in fast

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muscle. This difference could provide a useful model in the study of Ca^{+2} regulation of contractility. The possible significance of two troponin-I isoforms to ATPase activity could be investigated by comparing the Ca^{+2} sensitivity of natural actomyosin ATPase preparations from *O. andersoni* and *O. niloticus*. If interspecific differences in Ca^{+2} sensitivity were found then the regulatory proteins from *O. andersoni* could be cross hybridized onto desensitised *O. niloticus* actomyosins and visa versa. Cross hybridization acts as a check to see if the Ca^{+2} sensitivity differences in ATPase activity observed were due to elements of the regulatory complex. Johnston (1979) successfully used this protocol to identify alterations in the actomyosin Mg^{+2} Ca^{+2} ATPase of goldfish acclimated to different temperatures. Another possibility for investigating the physiological significance of Tn-Ix would be to measure the Ca^{+2} sensitivity of tension development in skinned single fibres from *O. niloticus* and *O. andersoni*. Greaser *et. al.* (1988) performed this experiment using rabbit single muscle fibres and found that different isoforms of troponin T correlated to differences in Ca^{+2} sensitivity.

In conjunction with physiological findings, detailed biochemical analysis of the two troponin I isoforms could reveal differences in phosphorylation, and binding to troponin C. Two dimensional analysis of samples containing 5mM Ca^{+2} suggested a greater binding affinity of Tn-Ix for Tn-C than Tn

I for Tn C. More in depth analysis is necessary however, to establish this point. Finally, it would be informative to analyse cardiac and slow muscle of *O. andersoni* for expression of the Tn-Ix isoform.

The pattern of isoform expression obtained from the fast muscle myofibrillar samples was taken to be indicative of the pattern within single fast muscle fibres. It is possible to make this assumption when studying fish muscle as the myotomal muscle of fish is anatomically segregated into homogeneous fibre type regions (see Johnston, 1981 for review). Crockford, (1989) tested this assumption in carp by comparing the electrophoretic patterns of single fast muscle fibres and fast muscle myofibril samples. He found no differences in the patterns obtained. Despite this it is possible that the intraspecific and interspecific differences observed in this study were due to heterogeneous fibre types within fast muscle. Comparison of the contractile protein isoforms in single fast fibres with the myofibrillar samples of this study could be accomplished in conjunction with analysis of the contractile properties of single fibres already proposed.

The findings of this project indicate that *Oreochromis niloticus* and *O. andersoni*, in particular, and tilapiine fish in general may provide a useful model for the study of comparative muscle physiology. It is possible that the polymorphism illustrated in the study species may in future

studies elucidate the connection between genotypic protein expression and phenotypic physiological traits.

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