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FUNCTIONALLY SIGNIFICANT ALLELIC VARIATION IN MYOSIN LIGHT CHAIN COMPOSITION IN A TROPICAL CICHLID

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Summary

Single fast muscle fibres in the tropical fish *Oreochromis andersonii* were found to contain two myosin light chains (LC1s; $LC1_{f1}$* or $LC1_{f2}$*). Breeding experiments confirmed that the different LC1s were of allelic origin and their inheritance patterns conformed to Mendelian expectations (1:2:1). The LC1s differed in apparent relative molecular mass by 800–900. No other differences in myosin subunits were found between the LC1 genotypes. The molar ratios of LC3:$LC1_{(total)}$ in the fast muscle of *O. andersonii* homozygous for $LC1_{f1}$* or $LC1_{f2}$* and heterozygous for both alleles were 2.0:1, 2.1:1 and 2.2:1, respectively, as determined by capillary electrophoresis. The maximum contraction velocity (V_{max}) of single skinned muscle fibres was determined at 20 °C by the slack-test method. V_{max} values (fibre lengths s^{-1}) for fast muscle fibres from *O. andersonii* which were homozygous for either $LC1_{f2}$* or $LC1_{f1}$* were 5.3 and 3.3, respectively, compared with 3.8 when both alleles were present. Crosses between *Oreochromis niloticus* and *O. andersonii* produced F1 hybrids which were heterozygous for either $LC1_n/LC1_{f1}$* or $LC1_n/LC1_{f2}$*, where $LC1_n$ is the myosin light chain for *O. niloticus*. The distribution of myosin light chain genotypes in hybrid offspring was not significantly different from the expected Mendelian 1:1 ratio (47 %: 53 %). The V_{max} (fibre lengths s^{-1}) of muscle fibres containing $LC1_{f2}$* from hybrid *Oreochromis* was 4.3 compared with 3.1 for the $LC1_{f1}$* genotype. The results are consistent with a functionally significant allelic variation in myosin LC1 in fast muscle fibres from *O. andersonii* which is also expressed in hybrid genotypes.

Key words: muscle contraction, myosin, fish, allelic variation, tilapia, *Oreochromis niloticus*, *Oreochromis andersonii*.

Introduction

The three-dimensional structure of the myosin head region of the molecular motor has been described. Each head contains two light chains wrapped around a long (approximately 8.5 nm) α-helix extending from the thick part of the myosin head (subfragment-1) to the carboxy terminus (Rayment *et al.* 1993). The removal of light chains from myosin has been shown to reduce shortening velocity in an *in vitro* motility assay by a factor of 10 without significantly decreasing ATPase activity (Lowey *et al.* 1993). In rat hindlimb muscles, the unloaded shortening velocity of fast fibres was found to be positively correlated with their myosin light chain 3 content, whereas ATPase activity was not (Bottinelli *et al.* 1994). These results indicate that light-chain/heavy-chain interactions are important for the conversion of chemical energy into movement.

In a previous study, we found that single fast myotomal muscle fibres from the tropical freshwater fish *Oreochromis andersonii* had multiple myosin light chain 1 bands on SDS–PAGE gels (Crockford *et al.* 1991). In birds and mammals, different alkali light chains of myosin arise from a single gene by an alternative RNA transcription and splicing mechanism (Periasamy *et al.* 1984; Nabeshima *et al.* 1984). Thus, the alkali light chains (LC1 and LC3) have identical C-terminal sequences of 141 amino acids, but differ in the lengths and sequences of their N-terminal segments. In contrast, in the mullet (*Mugil cephalus*), there are 29 amino acid differences between the LC1 and LC3 sequences spread throughout all regions of the polypeptide chains, suggesting that the alkali light chains in fish are the products of two separate genes (Dalla Libera *et al.* 1991). There is a report on allelic variation in myosin light chain 1 in chicken fast muscle, although the physiological characteristics of the putative genotypes were not investigated (Rushbrook and Somes, 1985). In order to determine whether the multiple fast muscle LC1 bands observed in *O. andersonii* were due to allelic variation, we have assessed the Mendelian inheritance of myosin subunits in

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two families and in hybrid crosses with *O. niloticus*. The maximum unloaded shortening speeds of skinned fibres from individuals with the different LC1 genotypes were also measured in order to determine whether the differences in myosin light chain composition had any functional significance.

Materials and methods

The African cichlid species *Oreochromis andersonii* (Castelnau; Zambesi, Zimbabwe) and *Oreochromis niloticus* (Linnaeus; Lake Manzala, Egypt) were studied. The fish came from a wild collection (Institute of Aquaculture, Stirling, Scotland) that has been electrophoretically tested for species purity (McAndrew and Majumdar, 1983). Potential broodstock of both species were anaesthetised in 1:10 000 benzocaine, biopsied and tagged with passive integrated transponders (PIT; Avid, Norco, CA, USA) (McAndrew, 1981). Biopsied tissue was frozen and stored in liquid nitrogen until the genotype was determined using the techniques of Crockford *et al.* (1991). The breeding and maintenance of all experimental fish were as described by McAndrew and Majumdar (1989). Fish weighing 5 g were transferred to aquaria at the Gatty Marine Laboratory, St Andrews, Scotland. They were maintained in a recirculating freshwater system at 27–29 °C and with a 16 h:8 h light:dark cycle. All fish were fed *ad libitum* on commercial trout food (Ewos, Bathgate) until they weighed 180–265 g (18–24 cm total length).

Breeding experiments

Both male and female *O. andersonii* putatively identified as $LC1_{f1}$*/$LC1_{f2}$* heterozygotes (two males and two females) were crossed to produce two full sib-families. Initial screening of *O. niloticus* showed that all fish were homozygous for $LC1_n$. A series of hybrid crosses was carried out between male and female *O. andersonii* which were heterozygous for LC1 (four fish) and *O. niloticus* (11 fish), to produce a total of 11 hybrid families.

Experimental studies

Fish were killed by stunning and pithing at the age of 6–8 months, when they had reached a sufficient size for the isolation of single muscle fibres (18–24 cm total length). Fast muscle samples were taken for physiological experiments and frozen in liquid nitrogen for subsequent confirmation of the myosin genotype.

Measurement of contractile properties

Maximum (unloaded) contraction velocity (V_{max}) was determined for single skinned fast myotomal muscle fibres at 20 °C by the slack-test method as described previously (Edman, 1979; Johnston and Gleeson, 1987). Briefly, single fast muscle fibres were isolated under silicone oil (MS 550, BDH/Merck Ltd, Lutterworth, UK) at 0–4 °C and mounted between two stainless-steel hooks connected to a force transducer (AME 801, Horten, Norway) and a servo system. Resting sarcomere length was measured by laser diffraction and set at 2.3 μm. The fibres were lowered into the first of three baths (temperature controlled to ±0.1 °C) and chemically skinned for 10–15 min in a relaxing solution containing 1 % Brij 58 (polyoxyethylene 20 cetyl ether). The ionic composition of the relaxing solution was (mmol l^{-1}) 1,4-piperazine-bis(ethanesulphonic acid) (Pipes), 25; ethyleneglycol-bis(β-aminoethylether)-*N*,*N*-tetraacetic acid (EGTA), 15; $MgCl_2$, 6.8; ATP, 6; phosphocreatine, 27.5; and 25–50 i.u. ml^{-1} creatine phosphokinase. Free ion concentrations were calculated using an iterative computer program (Johnston and Gleeson, 1987). The main ionic species in the activating solution were at the following concentrations pMg^{2+}=2.99, pMgATP=2.27, pCa^{2+}=4.23, pH 7.2 at 20 °C, and ionic strength was 0.185 mol l^{-1}. Once steady tension had been reached, 1 ms releases were given (sufficient to abolish tension) and the time required to take up the slack was recorded. The fibres were re-extended to their original length after each release. The slope (unloaded contraction velocity) was determined using least-squares regression from a plot of the applied length change *versus* the time taken to redevelop force (Johnston and Gleeson, 1987).

Sodium dodecyl sulphate gel electrophoresis (SDS–PAGE)

Fast fibres were dissected from the deep white muscle below the dorsal fin. One-dimensional and two-dimensional SDS–PAGE and the preparation of myofibrils were carried out as described previously (Crockford *et al.* 1991). Gels, 13 % acrylamide, were fixed for 1.5 h in 20 vols of 12.5 % trichloroacetic acid, 3 % sulphosalicylic acid, 0.017 % formaldehyde and stained with either colloidal Coomassie Blue G-250 (Neuhoff *et al.* 1988) or silver (Bloom *et al.* 1987).

The myosin subunits of individuals with different LC1 genotypes were analysed by two-dimensional electrophoresis (LC2 and LC3). Myosin heavy chain composition was investigated by peptide mapping with *Staphalococcus aureus* V8 protease, chymotrypsin, papain and trypsin (Crockford *et al.* 1991). To estimate the molar ratios of light chains, Coomassie-Blue-stained gels were scanned at 550 nm using a Shimadzu (CS-9000) densitometer and integration software.

SDS capillary electrophoresis (SDS–CE)

Crude myosin was prepared from a 4 g sample of fast muscle (Crockford and Johnston, 1995). Mixed myosin light chains for analysis by SDS–CE were purified by a modification of the procedure of Wagner (1982), as described by Crockford and Johnston (1995).

Mixed myosin light chain samples (0.5 mg ml^{-1}) were dialysed against 10 mmol l^{-1} Tris–HC1 (pH 6.75 at 20 °C), 0.1 % SDS, 1 mmol l^{-1} DL-dithiothreitol (DTT), and analysed on a Biofocus 3000 capillary electrophoresis apparatus (BioRad Laboratories Ltd, UK). Uncoated fused-silica capillary tubes 47 cm×50 μm (Sigma, Poole, UK) and

SDS–CE running buffer (pH 9.0) with sieving polymers (BioRad Laboratories Ltd, UK) were used. Before runs, the capillary was purged (at 690 kPa) for 140 s with 0.1 mmol l^{-1} NaOH, for 100 s with 0.1 mmol l^{-1} HCl and then filled for 200 s with SDS–CE run buffer. Prior to sample loading, the capillary tip was dipped three times into 20 % SDS–CE run buffer. Samples were loaded electrophoretically at 5 kV for 5 s and the runs monitored at 215 nm for 30 min. An internal standard of benzoic acid (0.1 mg ml^{-1}) was included in samples and used to calculate relative migration times. The following standard proteins (1 mg ml^{-1} in 10 mmol l^{-1} Tris–HCl, pH 6.76 at 20 °C, 0.1 % SDS, 1 mmol l^{-1} DTT: α-lactalbumin, soyabean trypsin inhibitor, trypsinogen, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase and egg albumin; Sigma-Aldrich Co. Ltd, Poole, UK) were used to calculate apparent relative molecular mass (M_r). The molar ratios of the light chains were calculated from integration of the peak areas using the integrator package supplied with the Biofocus 3000 (Version 3.01, BioRad Laboratories Ltd, UK).

Statistical analysis

The V_{max} of 15–19 muscle fibres from five individuals of each genotype was measured. Normality of the data was tested using the N-score procedure (Minitab Inc. USA) and the effects of LC1 genotype on V_{max} were analysed using a one-way analysis of variance (ANOVA) and Tukey's test. The significance of F1 ratios in breeding experiments were calculated by comparing the numbers observed with those expected using the χ^2-test.

Results

Breeding experiments

The myosin light chain genotypes of parental fish and their offspring were compared using SDS–PAGE (Fig. 1). The genotypes from two families of *O. andersonii* were not significantly different from the Mendelian 1:2:1 distribution ($LC1_{f1}$*/$LC1_{f1}$*:$LC1_{f1}$*/$LC1_{f2}$*:$LC1_{f2}$*/$LC1_{f2}$*) expected from segregating alleles at an autosomal locus. The distribution of light chains in family 1 was 0.9:2.0:1.1 (N=22; P>0.90) and in family 2 was 0.9:2.2:0.9 (N=22; P>0.75). The genotypes derived from two families of hybrid *O. andersonii* and *O. niloticus* also conformed to the expected Mendelian distribution (Fig. 2B). The $LC1_n$/$LC1_{f1}$*:$LC1_n$/$LC1_{f2}$* ratio in family 1 was 1.0:1.0 (N=18; P>0.99) and in family 2 was 0.9:1.1 (N=20; P>0.50). Similar results were obtained when light chains were analysed using SDS–CE (Fig. 3).

Analysis of proteins

SDS–CE separated the myosin light chains from *O. andersonii* into well-defined peaks and was able to distinguish between $LC1_{f1}$* and $LC1_{f2}$* homozygotes, and heterozygotes (Fig. 4). Analysis by SDS–PAGE showed that single fibres from heterozygotes contained both $LC1_{f1}$* and $LC1_{f2}$*. On the basis of their separation by SDS–CE, the apparent relative molecular masses (M_r) of $LC1_{f1}$* and $LC1_{f2}$* were estimated

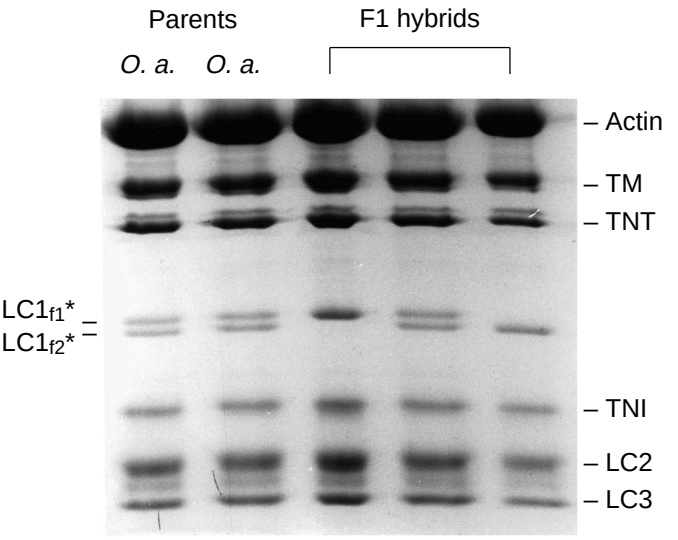

Fig. 1. SDS–PAGE analysis of the expression of myosin light chain 1 in *Oreochromis andersonii* (*O. a.*). Parental fish expressing both $LC1_{f1}$* and $LC1_{f2}$* were selected, crossed and compared with their F1 hybrid offspring. Representative examples from the three groups of offspring are shown. $LC1_{f1}$*, upper myosin light chain 1; $LC1_{f2}$*, lower myosin light chain 1; LC2, light chain 2; LC3, light chain 3; TM, tropomyosin; TNT, troponin T; TN1, troponin I.

as 27 100 and 26 200 respectively. These are similar to the values of M_r previously reported for these LC1 isoforms using SDS–PAGE (27 500 and 26 900 respectively) (Crockford *et al.* 1991). Densitometric analysis of SDS–PAGE and SDS–CE gels showed equal contents of $LC1_{f1}$* and $LC1_{f2}$* in heterozygous individuals (Figs 1, 4).

In the fast muscle of individual *O. andersonii* homozygous for $LC1_{f1}$* or $LC1_{f2}$*, and heterozygous for both alleles, the LC3:$LC1_{(total)}$ ratios were 2.2:1±0.13, 2.3:1±0.11 and 2.1:1±0.17, respectively, from densitometry of SDS–PAGE and 2.0:1±0.19, 2.1:1±0.21 and 2.2:1±0.27, respectively, from SDS–CE (mean ± S.E.M., N=10 per genotype). There were no significant differences in the LC3:LC1 ratio between the genotypes.

No other differences were found in either myosin subunits LC2 and LC3 or other myofibrillar proteins by SDS–PAGE and SDS–CE between individual fish with different myosin LC1 genotypes (Figs 2B, 3). Peptide mapping with four different proteolytic enzymes also revealed no evidence for differences in myosin heavy chain composition between individuals (Figs 5, 6).

SDS–CE was able to distinguish between the two hybrid genotypes (Fig. 3). All offspring contained an equal amount of $LC1_n$ and either $LC1_{f1}$* or $LC1_{f2}$*, representing the two LC1 genotypes in *O. andersonii*. Analysis of the myosin subunits of individuals with different LC1 genotypes, by SDS–PAGE and SDS–CE (LC2 and LC3; Figs 2B, 3) and peptide mapping (myosin heavy chain; Fig. 6), revealed no other variations between hybrid fish. All hybrid individuals appeared to inherit their myosin isoforms equally from both parents.

Fig. 2. (A) Breeding experiments were performed between *O. niloticus* (*O. n.*) and *O. andersonii* (*O. a.*) heterozygous for the LC1 alleles in order to produce hybrid genotypes. A range of morphological and pigment characteristics in the hybrids were intermediate between those of the parental fish. For example, note the striped caudal fin in *O. niloticus* (arrowhead) but not in *O. andersonii* and the intermediate pigmentation pattern of the hybrid. (B) SDS–PAGE of myofibrillar proteins in the F1 hybrid offspring. Representative examples from the parents and both F1 genotypes are shown. $LC1_{f1}$*, upper myosin light chain 1; $LC1_{f2}$*, lower myosin light chain 1; LC_n, myosin light chain 1 from *O. niloticus*; other abbreviations as in Fig. 1.

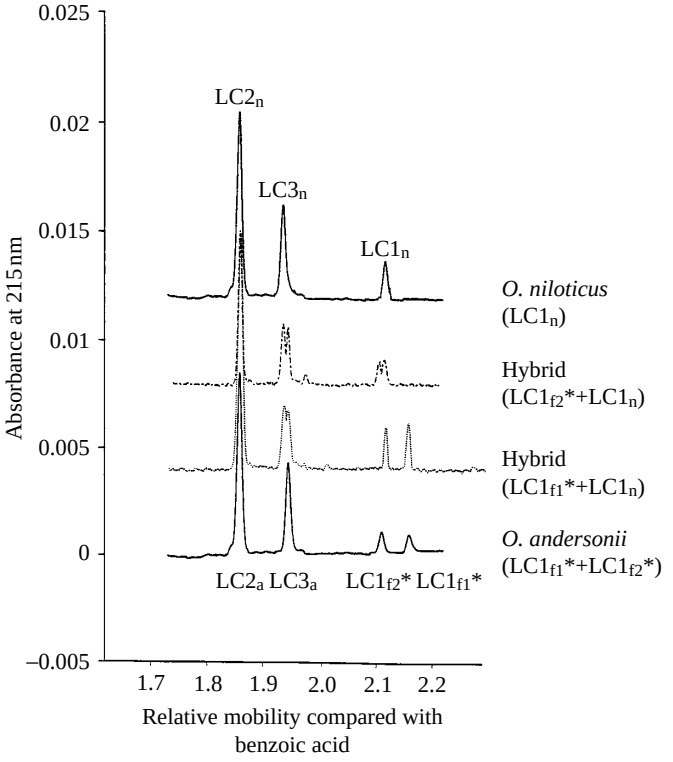

Fig. 3. Representative SDS capillary electrophoresis traces of the light chains of *Oreochromis niloticus* and *Oreochromis andersonii* heterozygous for myosin light chain 1 and for hybrids between the two species. $LC1_n$, myosin light chain 1 from *O. niloticus*; $LC2_a$ and $LC2_n$, myosin light chain 2 from *O. andersonii* and *O. niloticus* respectively; $LC3_a$ and $LC3_n$, myosin light chain 3 from *O. andersonii* and *O. niloticus* respectively; other abbreviations as in Fig. 1. The baseline of the traces was offset to allow the peaks to be seen.

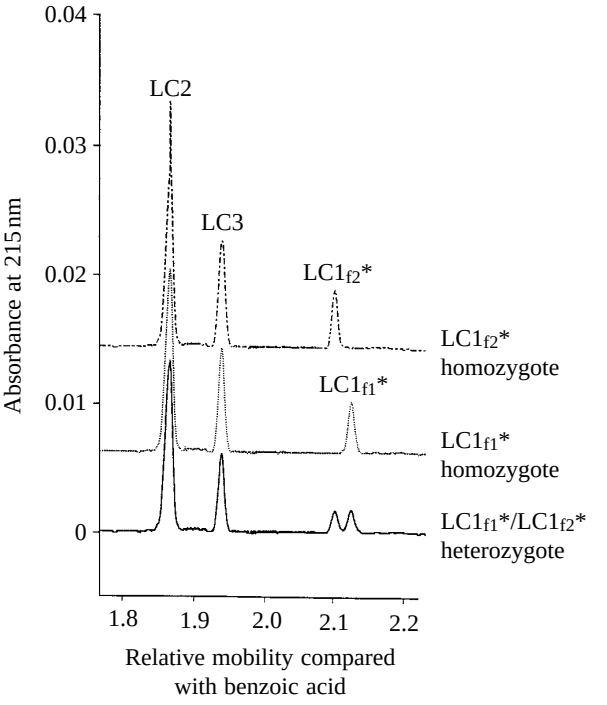

Fig. 4. Representative SDS capillary electrophoresis traces of the light chain genotypes of *O. andersonii*. Labels as in Fig. 1. The baseline for the upper two traces was offset to allow the peaks to be seen.

Muscle contractile properties

The maximum tensions generated by fast muscle fibres from the different *O. andersonii* genotypes were not significantly different at around $300\,kN\,m^{-2}$, whereas the maximum (unloaded) contraction velocity (V_{max}) was found to be dependent on LC1 composition (Fig. 7). Fibres from fish homozygous for $LC1_{f2}$* or $LC1_{f1}$*, and heterozygous for these light chains, had V_{max} values of 5.2±0.35, 3.3±0.23 and 3.8±0.30 fibre lengths s^{-1} respectively (mean ± S.E.M., N=16–19; Fig. 7). Thus, fibres from fish homozygous for $LC1_{f2}$* were significantly faster than those from the $LC1_{f1}$* homozygotes (P<0.01) or $LC1_{f2}$*/$LC1_{f1}$* heterozygotes (P<0.01), which were not significantly different from each other.

The maximum tension generated by the hybrid genotypes was also around $300\,kN\,m^{-2}$. The V_{max} values of fast muscle fibres from the parental *O. andersonii* heterozygote and *O. niloticus* were not significantly different (3.9±0.46 muscle lengths s^{-1} for *O. andersonii* and 3.6±0.51 muscle lengths s^{-1} for *O. niloticus*). The V_{max} of the hybrid offspring was found to be dependent on the *O. andersonii* LC1 allele inherited (Fig. 8). The V_{max} (fibre lengths s^{-1}) of muscle fibres isolated from offspring of the genotype $LC1_n$/$LC1_{f1}$* was significantly lower than that for the genotype $LC1_n$/$LC1_{f2}$* (P<0.03).

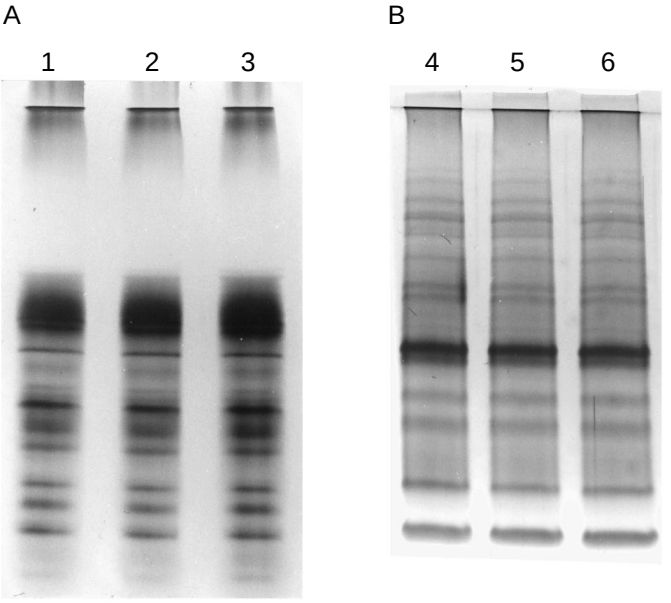

Fig. 5. Silver-stained 15% SDS–PAGE peptide maps of the fast muscle myosin heavy chain of *O. andersonii* comparing individuals of different myosin light chain 1 composition. Lanes 1 and 4, $LC1_{f1}$* homozygotes; lanes 2 and 5, $LC1_{f1}$*/$LC1_{f2}$* heterozygotes; lanes 3 and 6, $LC1_{f2}$* homozygotes. The proteolytic enzymes used were (A) *Staphylococcus aurus* V8 protease and (B) type 1-S chymotrypsin from bovine pancreas.

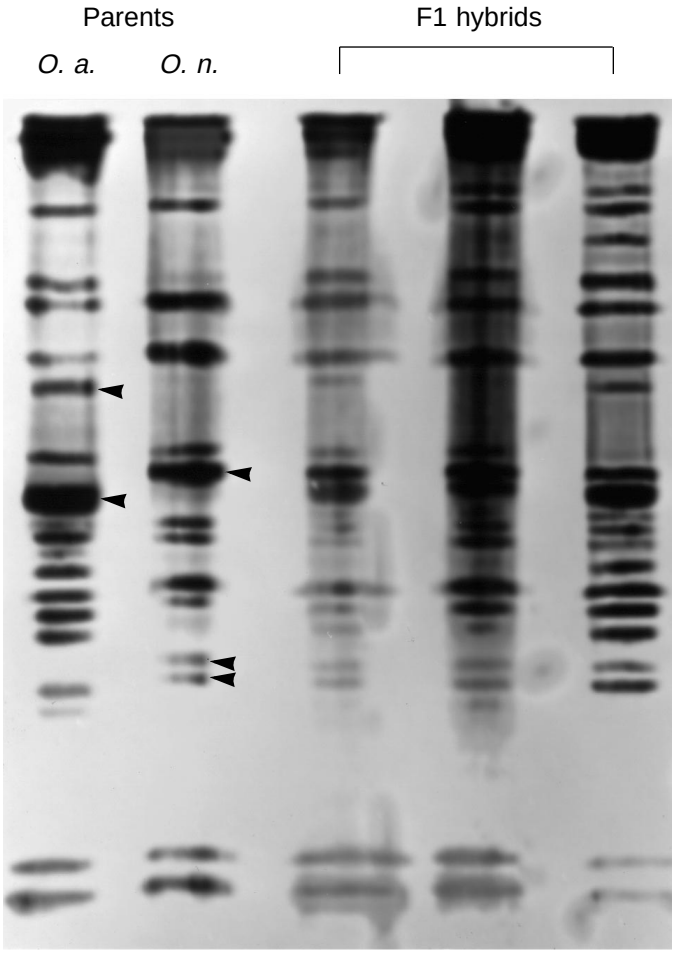

Fig. 6. Silver-stained 15 % SDS–PAGE peptide maps of the fast muscle myosin heavy chain digested with papain. Maps for parental *O. niloticus* (*O. n.*) and *O. andersonii* (*O. a.*) and representative F1 hybrids are shown. Arrowheads indicate bands specific to the parental species.

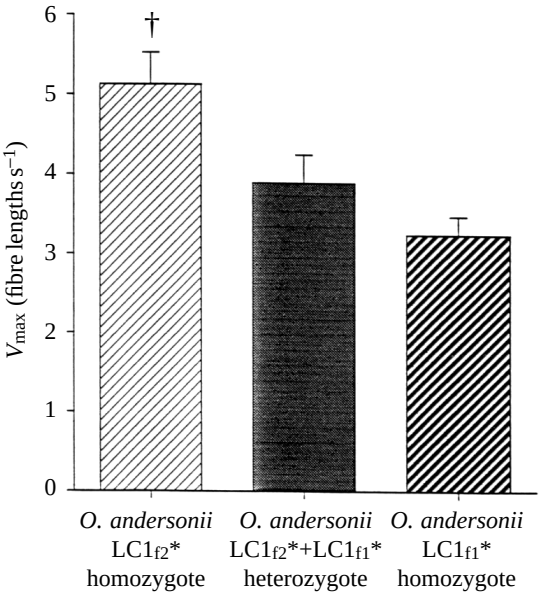

Fig. 7. Maximum (unloaded) contraction velocity (V_{max}) of muscle fibres from *Oreochromis andersonii* with different myosin light chain 1 compositions. Values are mean + S.E.M., N=16–19 muscle fibres from five individuals per genotype. †Significantly different from the $LC1_{f1}$ homozygote and the $LC1_{f2}$*+$LC1_{f1}$* heterozygote at the P<0.01 level.

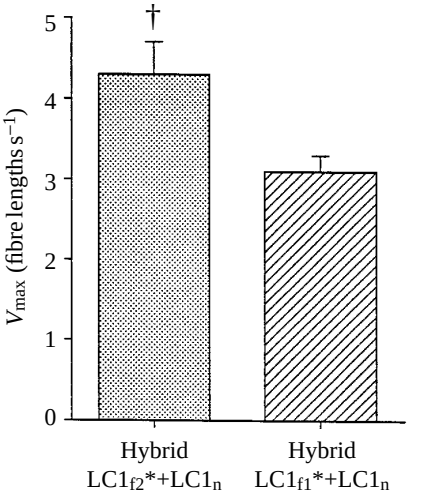

Fig. 8. Maximum (unloaded) contraction velocity (V_{max}) of muscle fibres from *O. niloticus* × *O. andersonii* hybrids. Results are mean + S.E.M., N=15 muscle fibres from five individuals per genotype. †Significantly different at the P<0.03 level.

Discussion

The present study is consistent with a functionally significant allelic variation in myosin light chain 1. It has previously been suggested that the presence of multiple LC1s in fast muscle may be due to the presence of other muscle fibre types (Rowlerson *et al.* 1985) or to the expression of one fibre-type-specific light chain in a different fibre type (Staron and Pette, 1987). Changes in myosin light chains have also been observed in fish during temperature acclimation (Crockford and Johnston, 1990) and at different developmental stages (Crockford and Johnston, 1993). The presence of *O. andersonii* homozygous for $LC1_{f1}$* and for $LC1_{f2}$* shows that the double banding pattern found in heterozygotes for this allele was not due to contamination of fast muscle with another fibre type (Figs 1, 4). The possibility of acclimation or developmental stage being responsible for the observed results was excluded by studying sibling groups maintained in the same tanks under identical conditions. Instead, a different protein was being expressed throughout the tissue in a pattern consistent with allelic variation. Different races of chickens have also been reported to contain allelic variants of myosin LC1 in the pectoralis major muscle, although no functional significance was described in that case (Rushbrook and Somes, 1985).

The V_{max} of single fast muscle fibres from the rabbit (Greaser *et al.* 1988) and the rat (Bottinelli *et al.* 1994) has been shown to increase in parallel with their LC3:LC1 ratio. To ensure that the difference in V_{max} observed with allelic

variation in LC1 was not due to a change in the relative content of LC1, densitometry of myofibrillar proteins separated by both one-dimensional SDS–PAGE and capillary electrophoresis (Figs 1, 4) was used to calculate the LC3:LC1 ratio. The LC3:LC1 ratio was the same in the three genotypes and could not, therefore, account for the observed differences in contraction velocity. On the basis of peptide mapping with four different proteolytic enzymes, no differences in myosin heavy chain composition could be detected between the LC1 genotypes. Similarly, there was no evidence for polymorphism in any other myofibrillar protein using two-dimensional gels and SDS–CE. Therefore, our results strongly suggest that the differences in the contraction velocity are due to the type of LC1 present, with the presence of the larger LC1 ($LC1_{f1}$*) resulting in a significantly reduced speed of contraction. Thus, in the heterozygotes, there was co-dominance at the protein expression level, with equimolar proportions of $LC1_{f1}$* and $LC1_{f2}$*. In contrast, at the phenotypic level, as measured by V_{max}, there was over-dominance of the $LC1_{f1}$* light chain (Fig. 7). Furthermore, the physiological effect of the $LC1_{f1}$* allele was still apparent in the hybrid fish (Fig. 8).

The atomic structure of the myosin heads has been determined by X-ray diffraction (Rayment *et al.* 1993). The actin and ATP binding sites are located in the globular head region of subfragment-1, whilst the light chains are wrapped around a long single α-helix of approximately 73 amino acids at the carboxy terminus. It has been suggested that the light chains keep the heavy chain α-helix in an extended configuration and provide it with sufficient rigidity to act as a lever (Rayment *et al.* 1993; Spudich, 1994). Lowey *et al.* (1993) found that, although the light chains of rabbit myosin were not essential for ATPase activity, they increased the speed of contraction of actin in an *in vitro* motility assay by up to 10 times, possibly by amplifying the working stroke of the crossbridge. Interestingly, it was found that a myosin with a shortened lever arm from a genetically engineered slime mould moved actin at about half the velocity of the native myosin in an *in vitro* motility assay (Uyeda and Spudich, 1993). However, both movement and ATPase activity are subject to mechanical constraints in muscle which are absent in the *in vitro* assays. The finding that variations in alkali light chain structure alters V_{max} in intact muscle fibres, in which the three-dimensional lattice structure arrangement of the proteins is preserved, is therefore of fundamental interest. Structural studies of myosin in *O. andersonii* could provide valuable insights into the function of molecular motors, particularly the lever arm concept (Spudich, 1994).

Rome and co-workers have suggested that *in vivo* muscle fibres are recruited over a narrow range of contraction speeds (V) at which power output and efficiency are near optimal, corresponding to V/V_{max} values of 0.17–0.36 (Rome *et al.* 1988, 1992). The identification of allelic variations in myosin light chain genes which result in altered V_{max} therefore provides an ideal test of the idea that V/V_{max} represents a fundamental constraint in muscle design. Future studies will investigate the influence of different LC1 genotypes on both muscle contraction velocity *in vivo* and swimming speed.

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