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SCALING OF INTRINSIC CONTRACTILE PROPERTIES AND MYOFIBRILLAR PROTEIN COMPOSITION OF FAST MUSCLE IN THE FISH *MYOXOCEPHALUS SCORPIUS* L.

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Summary

Contractile properties were determined for fast muscle fibres from short-horn sculpin (*Myoxocephalus scorpius* L.) ranging from 5 to 35 cm total body length (L) and from 2.0 to 758 g body mass. Maximum unloaded shortening velocity (V_0) decreased with increasing body size, scaling as $19.5L^{-0.34}$, and isometric twitch activation and tetanus relaxation times became longer, scaling as $12.0L^{0.31}$ and $19.5L^{0.42}$ respectively. Myofibrillar Mg^{2+}/Ca^{2+}-ATPase activity scaled as $2.51L^{-0.28}$. In order to investigate the mechanisms underlying the scaling of contractile properties, myofibrillar protein composition and I filament lengths were determined. One-dimensional SDS–PAGE and two-dimensional isoelectric focusing/non-equilibrium isoelectric focusing–PAGE revealed no differences in the myofibrillar protein isoforms of myosin light chains, actin, tropomyosin, troponin-T and troponin-C in fish of differing body size. Peptide maps of purified myosin heavy chains digested with eight different proteolytic enzymes were also similar in all fish examined. Three isoforms of troponin-I were present in fish less than 20 cm in total length with relative molecular masses of 17 (TnI_{f3}), 22 (TnI_{f2}) and 23 (TnI_{f1}). The ratio of TnI isoforms varied with body length, and only TnI_{f3} was present in fish greater than 28 cm total length. The length of I filaments was independent of body length. Thus, although the expression of TnI isoforms changes during growth, the underlying mechanism responsible for increased shortening velocity with decreased body size remains unknown.

Key words: body length, fish, mechanics, muscle, myofibrillar proteins, *Myoxocephalus scorpius*, scaling, short-horn sculpin.

Introduction

Fish axial muscles are composed of a series of metamerically arranged myomeres. There is considerable diversity in body form and swimming style within the fishes (Lindsey, 1978), particularly with respect to the wavelength of body curvature. Many species, including cod (*Gadus morhua*) and trout (*Salmo trutta*), use a subcarangiform swimming style in which the head is held rather rigid and the wavelength of body curvature increases towards the tail. Muscle contractile properties have been shown to vary from anterior to posterior myotomes in species using continuous subcarangiform or carangiform swimming styles. For example, in cod (Davies *et al.* 1995), saithe (*Pollachius virens*; Altringham *et al.* 1993) and scup (*Stenotomus chrysops*; Rome *et al.* 1993), times for isometric muscle twitches increased from anterior to posterior myotomes. In contrast, in the short-horn sculpin (*Myoxocephalus scorpius*), a specialist ambush predator, twitch and tetanus activation and relaxation times were not significantly different between rostral (0.32 total body lengths; L) and caudal ($0.77L$) myotomes, although they were consistently greater towards the tail (Johnston *et al.* 1995).

To produce work during swimming, muscle fibres need to undergo cycles of shortening and lengthening which require the muscle to be activated to produce force during shortening and to be relaxed during lengthening. However, Altringham and Johnston (1990) found that the time needed to activate or relax muscle increased with fish body length. Videler and Wardle (1991) calculated the expected decreases in maximum tailbeat frequency from *in vitro* isotonic shortening times, finding a good match with observed values in the literature. Work with isolated muscle fibres has demonstrated that the duration of the length-change (strain) cycle required to produce maximum power output increases with increased body size in line with the rise in muscle activation times and decrease in tailbeat frequencies used during swimming (Altringham and Johnston, 1990; Anderson and Johnston, 1992). Myofibrillar ATPase activity has been shown to decrease with increased body length in gadoids (Witthames and Greer-Walker, 1982), suggesting that there are changes in myofibrillar protein expression with growth.

Previous studies on single muscle fibres isolated from toad

(*Xenopus laevis*; Lännergren, 1987), rat (Bottinelli *et al.* 1994*a*; Bottinelli and Reggiani, 1995) and human (Larsson and Moss, 1993; Bottinelli *et al.* 1996) have shown correlations between contractile properties and myofibrillar protein isoform composition. The maximum shortening velocity, the maximum power output and the shortening velocity and force at which muscles produce maximum power were all dependent on myosin heavy chain composition. Myosin heavy chain composition is also a major factor in determining the myofibrillar ATPase activity of muscle fibres during isometric studies (Bottinelli *et al.* 1994*b*; Steinen *et al.* 1996). Variation in maximum shortening velocity in fast muscle fibres with the same heavy chain composition has been found to correlate with the ratio of myosin light chain isoforms in rat (Bottinelli and Reggiani, 1995) and carp (*Cyprinus carpio*; Crockford and Johnston, 1990). The existence of multiple contractile protein isoforms provides a mechanism for producing a continuous range of muscle phenotypes (reviewed in Pette and Staron, 1988). For instance, there are more than ten different combinations of light and heavy chain isoforms expressed in mammalian muscle fibres without consideration of the expression of different troponin or tropomyosin subunits. Variations in troponin-T isoforms have been shown to result in changes in the Ca^{2+} sensitivity of force development in both rabbit (Greaser *et al.* 1988) and chicken fast muscle fibres (Reiser *et al.* 1996).

The aim of the present study was to determine the effects of body size on the contractile properties and myofibrillar ATPase activity of fast muscle in the short-horn sculpin. This fish can grow up to a standard length of 60 cm, but adults longer than 30 cm are rarely caught (Whitehead *et al.* 1986). Myofibrillar protein composition and I filament length were also investigated to determine what factors could be contributing to the observed general slowing in contractile properties with increased body length. The force–velocity relationships of muscle fibres isolated from anterior abdominal and caudal myotomes were also determined. The information gained in the present study was required for a complete description of the scaling of muscle performance *in vivo* reported in the accompanying paper (James and Johnston, 1998).

Materials and methods

Short-horn sculpin (*Myoxocephalus scorpius* L.) were caught by local fishermen in lobster creels or by trawling in the Firth of Forth, Scotland, from October to November in 1993, 1994 and 1996. Fish (N=49) were acclimated at 10–12 °C for 5–7 weeks in recirculating seawater tanks (photoperiod 12 h:12 h light:dark). Fish were fed twice a week on live shrimps (*Crangon crangon*) and chopped squid.

Preparation of muscle fibre bundles

For studies of contractile properties, fish (N=33: N=27 for scaling studies and N=6 for rostral:caudal comparison) were stunned using a blow to the head and pithed to destroy the central nervous system. Total body length (L) and mass ranged from 6.2 to 32.8 cm and from 2.6 to 594 g, respectively (Fig. 1). An incision was made from the anus to the pectoral gill arch, and the anterior abdominal myotomes (0.35L from the snout) were removed. Small bundles of 6–20 fibres (30–100 fibres per bundle in the four smallest fish) were dissected from anterior abdominal myotomes in Ringer's solution maintained at 4 °C. The Ringer's solution contained (in mmol l^{-1}): NaCl, 143; sodium pyruvate, 10; KCl, 2.6; $MgCl_2$, 1.0; $NaHCO_3$, 6.18; NaH_2PO_4, 3.2; $CaCl_2$, 2.6; Hepes sodium salt, 3.2; Hepes, 0.97. The ionic composition of the Ringer matched that of serum in the short-horn sculpin (Hudson, 1968). Sodium pyruvate provided a readily absorbed energy source (Altringham and Johnston, 1988) and the buffer Hepes was used to maintain pH at 7.40 at 12 °C. In six of the fish studied (L=19.0±0.21 cm; mean ± S.E.M.), muscle fibre bundles were removed from both anterior abdominal myotomes (0.30L) and caudal myotomes (0.75L). An aluminium foil T-shaped clip was folded over the myoseptum at each end of the muscle fibre bundle. The preparation was then transferred to a flow-through chamber of Ringer maintained at 12 °C. The foil clips were used to attach the preparation to a force transducer at one end (AME 801, SensoNor, Norway) and a servo arm at the other. The remaining anterior abdominal myotomes were fast-frozen in liquid nitrogen and stored at −20 °C for subsequent analysis of myofibrillar protein isoform composition.

Isometric contractile properties

Muscle fibre preparations were held at constant length. Stimulus amplitude (8–15 V), pulse width (0.8–1.5 ms) and fibre length were adjusted to maximise twitch force. The fibre length for maximal twitch force corresponded to sarcomere lengths between 2.20 and 2.25 μm, as measured by laser diffraction. Stimulation frequency was adjusted to maximise tetanus height (80–140 Hz). Time to peak twitch force, time from peak twitch to 50 % relaxation, time from stimulus to 90 % twitch relaxation, time to 50 % peak tetanic force, time to peak tetanic force and time from last stimulus to 50 % tetanic relaxation were all measured. Preparations were then used to determine maximum unloaded shortening velocity or the force–velocity relationship.

Unloaded shortening velocity

Maximum unloaded shortening velocity (V_0) was determined from 17 fish (6.2–32.8 cm total length) using the slack-test method (Edman, 1979). Preparations were stimulated to produce maximal tetanic force and subjected to a rapid shortening step of sufficient size to reduce the force generated to zero. This protocol was repeated 6–8 times using different magnitudes of length step with a 5 min recovery time allowed between each run. The time taken from the rapid shortening step to the beginning of force development was plotted against the magnitude of the length step. V_0 of the fibre bundle was then calculated as the slope of the first-order polynomial line fitted to the data by least-squares regression (Edman, 1979; Rome *et al.* 1990).

The force–velocity relationship

The force–velocity relationship was determined for muscle fibres in six fish of similar length (19.0±0.21 cm total length, 127±19.9 g body mass; mean ± S.E.M.). Muscle fibres were isolated from anterior abdominal (0.30L) and caudal (0.75L) myotomes. Each muscle preparation was stimulated under isometric conditions to produce a tetanus. When maximum force was achieved, the muscle preparation was subjected to an initial rapid shortening step to reduce force to a new level and then a shortening step of a lower constant velocity to maintain constant force for 8–15 ms (Altringham and Johnston, 1988). This protocol was repeated 16–20 times with each muscle fibre preparation, with 5 min between each repetition to allow the muscle to recover. For each muscle preparation, a best-fit hyperbolic–linear curve (Marsh and Bennett, 1986) was fitted to the force–velocity (P–V) data using the software package Regression (Blackwell Scientific-Software, Oxford, England). The curvature of the P–V relationship was estimated using the power ratio $\dot{W}_{max}/V_{max}P_{max}$, where $\dot{W}_{max}$ is the maximum power output produced during force–velocity studies, V_{max} is the maximum shortening velocity and P_{max} is the maximum isometric force (Marsh and Bennett, 1986). Muscle power output was calculated as the product of force and velocity. The highest calculated value of power output was assumed to represent the maximum muscle power output.

Determination of fibre bundle cross-sectional area and mass

On completion of the *in vitro* experiments, each muscle preparation was frozen rapidly in isopentane cooled to −159 °C in liquid nitrogen. Transverse sections, 10 µm thick, were cut and stained for myosin ATPase activity at pH 9.4 using the method of Johnston *et al.* (1974). A microscope drawing arm was used to draw the outline of each fibre in cross section. The cross-sectional area of the muscle fibre bundle was then determined using a digital planimeter interfaced to a microcomputer running Videoplan software (Kontron, Eching, Germany). The cross-sectional area was calculated for 17 of the preparations used for scaling studies and all of the preparations used for the rostral:caudal comparison. Muscle mass was calculated using the measurements of muscle length and cross-sectional area, assuming a density of 1060 kg m^{-3} (Méndez and Keys, 1960).

Determination of myofibrillar ATPase activity

Rostral fast muscle was dissected rapidly from 11 fish (Fig. 1) and macerated using an ice-cold scalpel blade. Macerated muscle was added to 10 vols of solution A containing (in mmol l^{-1}): imidazole, 20; KCl, 100; EDTA, 1 (pH 7.2 at 0 °C) and homogenised for 15 s using a Polytron blender (Kinetica GMBH, Switzerland). The homogenate was centrifuged at 3000 g for 5 min at 2 °C. The pellet was resuspended in 10 vols of solution B containing (in mmol l^{-1}): imidazole, 20; KCl, 100 (pH 7.2 at 0 °C). The suspension was centrifuged at 400 g for 2 min and the pellet was resuspended in 10 vols of solution B. The myofibrillar protein concentration was determined using the microbiuret method (Itzhaki and Gill, 1964) and adjusted to approximately 2 mg ml^{-1} using solution B. ATPase activity of the myofibrils was determined by incubation at 12 °C in an assay medium containing (in mmol l^{-1}): KCl, 50; imidazole, 40; $MgCl_2$, 7; and either $CaCl_2$, 5 (Mg^{2+}/Ca^{2+}-ATPase activity) or EGTA, 5 (Mg^{2+}/EGTA-ATPase activity). The ATPase reaction was started with the addition of ATP (final concentration 5 mmol l^{-1}) and was stopped after 12 min by the addition of 10 % (w/v) trichloroacetic acid. Precipitated protein was removed by centrifugation at 5000 g for 5 min. Inorganic phosphate concentration in the supernatant was measured as described in Bers (1979).

Preparation of myofibrils for electrophoresis

Myofibrils were prepared using rostral fast muscle from seven fish ranging from 6 to 33 cm total body length (Fig. 1). All of the steps in the sample preparation were carried out at 0–4 °C in order to minimise proteolytic breakdown. The tissue was homogenised using a chilled hand-held glass homogeniser in 20 vols of ice-cold preparation buffer containing (in mmol l^{-1}): Tris–HCl, 10; NaCl, 50; EDTA, 1; pH 7.4 at 20 °C; and the following proteolytic enzyme inhibitors, 50 µg ml^{-1} phenylmethonylsulphonyl fluoride, 0.5 µg ml^{-1} leupeptin, 1 µg ml^{-1} pepstatin A and 0.2 µg ml^{-1} aprotinin (Sigma Ltd, Poole, England). The homogenate was centrifuged at 15 000 g for 10 min and the supernatant discarded. The pellet was rehomogenised, washed and centrifuged a further four times in 20 vols of ice-cold preparation buffer. The final pellet contained washed myofibrils.

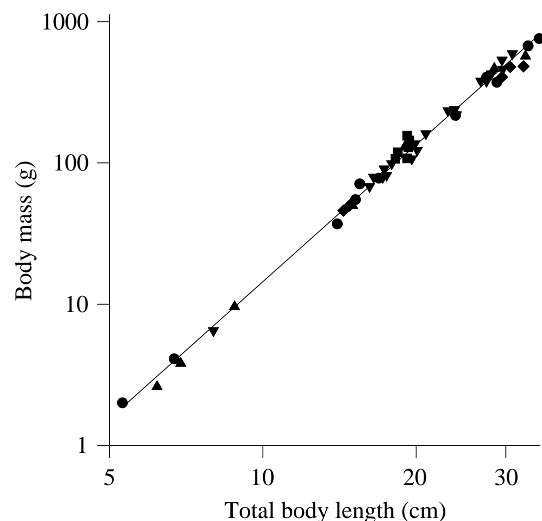


Fig. 1. Size range and experimental usage of fish: circles, myofibrillar ATPase studies; squares, force–velocity studies (anterior abdominal compared with caudal preparations); triangles, isometric, V_0 (maximum unloaded shortening velocity) and gel-electrophoresis studies; inverted triangles, isometric and V_0 studies; diamonds, electron microscopy studies. Body mass scaled as $1.02L^{3.22}$, r^2=0.99, P<0.001, where L is total body length. The line represents a first-order polynomial fitted to the log–log data using a least-squares regression.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was carried out as described by Laemmli (1970) with the inclusion of 10 mmol l^{-1} DL-dithiothreitol (DTT) in the sample buffer. Myofibrils were resuspended in a solution containing: 60 mmol l^{-1} Tris–HCl, pH 6.75 at 20 °C, 2 % (m/v) SDS, 10 % (v/v) glycerol, 10 mmol l^{-1} DTT and 0.002 % (m/v) Bromophenol Blue to give a final protein concentration of 2 mg ml^{-1}. The samples were heated to 80 °C for 3 min and centrifuged at 5000 ***g*** for 5 min prior to use.

Alkali urea polyacrylamide gel electrophoresis

Alkali urea polyacrylamide gel electrophoresis (AU–PAGE) was carried out by a modification of the method of Focant *et al.* (1976) as described in Crockford (1987). The electrode and gel buffers used were 50 mmol l^{-1} glycine, 5 mmol l^{-1} $CaCl_2$, pH 9.0 at 20 °C. Myofibrils were dissolved in 3 vols of 12 mol l^{-1} urea, 20 mmol l^{-1} glycine in 1 mmol l^{-1} Tris, pH 8.9 at 20 °C, 10 mmol l^{-1} DTT, 0.001 % (m/v) Bromophenol Blue and either 5 mmol l^{-1} $CaCl_2$ or 10 mmol l^{-1} EGTA to give a final protein concentration of 5 mg ml^{-1}. Gels were stained rapidly with Coomassie Brilliant Blue G-250 and washed with several changes of water. The bands of interest were cut out using a razor blade and equilibrated with SDS sample buffer for 1 h. The gel pieces were then placed in sample wells of one-dimensional SDS–PAGE gels and run as normal.

Isoelectric focusing/non-equilibrium isoelectric focusing polyacrylamide gel electrophoresis

Two-dimensional electrophoresis was carried out as described by O'Farrell (1975) using either isoelectric focusing (IEF–PAGE) or non-equilibrium isoelectric focusing polyacrylamide gel electrophoresis (NEIEF–PAGE) as the first dimension for acidic or basic proteins, respectively. Myofibrils were homogenised in 8 mol l^{-1} urea, 1 % (v/v) Ampholyte, pH 3–10, 1 % (v/v) Ampholyte, pH 5–7, 5 % (v/v) glycerol, 5 % (m/v) DTT, 2 % (v/v) Noniodet P-40 (NP40) and 0.001 % (m/v) Bromophenol Blue. Samples were warmed to 30 °C for 1 h and centrifuged at 5000 ***g*** for 5 min prior to use. IEF–PAGE and NEIEF–PAGE pH gradients were prepared in tube gels using the following combination of ampholines (v/v) (Pharmacia, Uppsala, Sweden): for basic proteins, 1.67 % Pharmalyte, pH 3–10, and 3.33 % Pharmalyte, pH 8.5–10.5; and for acidic proteins, 1.67 % Pharmalyte, pH 3–10, 1.67 % Pharmalyte, pH 4–6.5, and 1.67 % Pharmalyte, pH 2.5–5.0 (Crockford and Johnston, 1993). Gels were fixed for 2 h in 12 % (m/v) trichloroacetic acid, 3 % (v/v) sulphosalicylic acid. Gels were rinsed thoroughly prior to being stained with 0.1 % (m/v) Coomassie Brilliant Blue G-250 in 2 % (v/v) H_3PO_4, 10 % (m/v) ammonium persulphate plus 20 % (v/v) methanol.

Identification of myofibrillar protein isoforms

Myosin heavy chain and actin were identified by their relative molecular mass (M_r) and abundance on SDS–PAGE. Tropomyosin was identified by its anomalous migration on SDS gels in the presence and absence of 8 mol l^{-1} urea (Crockford and Johnston, 1993). Troponin-C was identified by its characteristic blue stain with 'Stains all' (Campbell *et al.* 1983). The myosin light chains were identified by their characteristic migration in the neutral-to-acidic range on two-dimensional IEF–PAGE gels (Rowlerson *et al.* 1985; Martinez *et al.* 1990) and by purification from adult white muscle using a Sepharose Q column (Pharmacia, Uppsala, Sweden) (Crockford and Johnston, 1995). Troponin-I and -T have basic isoelectric points and different relative molecular masses (Johnston and Ball, 1996). Troponin-I, -T and -C were purified using a DEAE Sephadex ion-exchange column in the presence of 8 mol l^{-1} urea (Greaser and Gergely, 1971). Purified troponin-I, -T and -C were run on a one-dimensional SDS–PAGE gel and used to identify the respective troponins on the two-dimensional gels. The apparent relative molecular mass (M_r) of each myofibrillar protein was estimated using standard proteins of known M_r (Sigma Ltd, Poole, England). Troponin-I and -T were also identified by western blotting using the Biorad mini trans-blot cell according to the method of Burnette (1981). During western blotting, monoclonal antibodies to rabbit skeletal muscle troponin-T (clone JLT-12, Sigma Ltd) and monoclonal antibodies to mouse skeletal muscle troponin-I (clone C5, Advanced Immunochemical Inc., CA, USA) were used.

Densitometric analysis

Densitometry was carried out on one-dimensional SDS–PAGE gels using a Shimadzu CS-9000 densitometer at 550 nm. Scans were then analysed quantitatively using NIH Image (National Institutes of Health, USA) on an Apple Macintosh.

Myosin heavy chain mapping

Myosin heavy chains were purified using one-dimensional SDS–PAGE on 8 % acrylamide gels. The gels were stained rapidly in Coomassie Brilliant Blue G-250, and the myosin heavy chain bands were cut out with a razor blade. Peptide maps were then run on 15 % SDS–PAGE gels, as described previously, using the proteases endoproteinase Glu-C from *Staphylococcus aureus* V8, papain from papaya latex, trypsin from bovine pancreas, ficin from fig tree latex, clostripain from *Clostridium histolyticum*, elastase from porcine pancreas, thermolysin from *Bacillus thermoproteolyticus rokko* and α-chymotrypsin from bovine pancreas (Sigma Ltd, Poole, England) (Crockford and Johnston, 1993).

Measurement of filament lengths

An anterior abdominal muscle fibre bundle was dissected from five fish (Fig. 1). An aluminium foil T-shaped clip was folded over the myoseptum at each end of the muscle fibre bundle. The preparation was then pinned out on a strip of Sylgard and immersed for 30 min in fixative, which contained: 2.5 % (v/v) glutaraldehyde, 2.5 % (m/v) paraformaldehyde, 1 % (m/v) sucrose, 0.2 mol l^{-1} $CaCl_2$, 0.1 mol l^{-1} NaCl and 0.1 mol l^{-1} sodium cacodylate; pH 7.4 at 20 °C. Fibre bundles were washed in the same solution without fixative and post-fixed for 60 min

in 2 % (m/v) osmium tetroxide in 0.1 mol l^{-1} sodium cacodylate. Each fibre bundle was washed in buffer, dehydrated through an ethanol series, stained *en bloc* with uranyl acetate in 70 % (v/v) alcohol, then embedded in Araldite resin. Semi-thin sections were cut and stained with Toluidine Blue. The orientation of the block was adjusted to cut ultra-thin (90 nm) longitudinal sections. Sections were mounted on copper 300 mesh grids and stained with lead citrate and uranyl acetate for 8 min each. For each fish, 2–3 A and I filament lengths and Z line widths were measured from each of 4–5 negatives taken at a magnification of 19 600×. I filament length was measured as the Z line width plus the thin filament length on each side of the Z line. Shrinkage was determined by assuming that A filament length was 1.50 µm and that I and A filaments underwent a similar amount of shrinkage (Sosnicki *et al.* 1991).

Statistics

Unpaired, two-sided *t*-tests were used for statistical comparisons between sets of data. Scaling relationships were calculated by fitting a first-order polynomial to the log–log data using a least-squares regression. The scaling relationship was represented by a power equation of the form $y=ax^b$, where a is the y-axis intercept, b is the slope of the regression line and x is either total body length (cm) or body mass (g). The slope of each regression line was found to be significantly different from zero ($P<0.001$), except for the time to peak tetanic force and maximum isometric stress. No set of data showed a significant deviation from linearity ($P>0.3$). The standard error of the estimate of the hyperbolic–linear curve-fitting procedure (SEE) was calculated as: $\mathrm{SEE}=\sqrt{\mathrm{RSS}/(N-2)}$, where RSS is the residual sum of squares and N is the number of data points.

The term ‘significant’ has been used to signify a P value of less than 0.05.

Results

Isometric contractile properties

The times to peak twitch force (Fig. 2A), from peak twitch force to 50 % relaxation (Fig. 2B) and from the stimulus to 90 % twitch relaxation all increased significantly with body length: scaling as $12.0L^{0.31}$, $2.88L^{0.67}$ and $17.0L^{0.49}$ respectively. The time from the last stimulus to 50 % tetanus relaxation also increased significantly with fish length (Fig. 2C), scaling as $19.5L^{0.42}$. In contrast, the time to peak tetanic force and the mean maximum isometric stress were 60.6±2.6 ms and 195±7.6 kN m^{-2} respectively (mean ± S.E.M.) and were independent of body length ($r^2<0.03$, $P>0.5$).

There was a tendency for the time to 50 % peak tetanic force and the time from the last stimulus to 50 % tetanus relaxation to be consistently greater for caudal than for anterior abdominal fibre bundles in each fish (Table 1). These differences, although not significant, are indicative of a change in contractile properties from relatively fast to slow from the anterior to posterior of the fish.

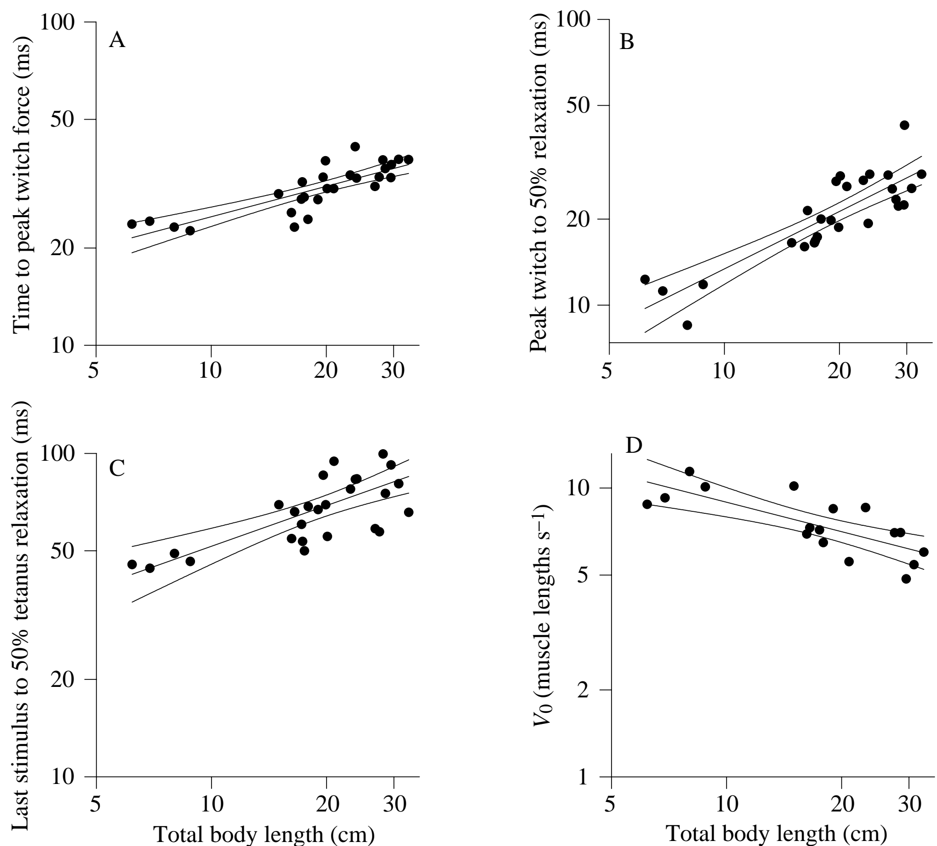


Fig. 2. Scaling of isometric properties and maximum unloaded shortening velocity. (A) Time to peak twitch force ($r^2=0.65$, $P<0.001$). (B) Time from peak twitch force to 50 % relaxation ($r^2=0.72$, $P<0.001$). (C) Time from last stimulus to 50 % tetanus force relaxation ($r^2=0.51$, $P<0.001$). (D) Maximum unloaded shortening velocity (V_0) ($r^2=0.56$, $P<0.001$). The lines represent a first-order polynomial fitted to the log–log data using a least-squares regression and the 95 % confidence limits of this line. A, B, C, $N=27$; D, $N=17$.

Unloaded shortening velocity and the force–velocity relationship

Maximum unloaded shortening velocity (V_0) decreased significantly with increasing fish length scaling as $19.5L^{-0.34}$ (Fig. 2D). Maximum shortening velocity (V_{max}) was 38 % higher in anterior abdominal (0.30*L*) than in caudal (0.75*L*) muscle fibres ($P<0.05$; Table 1). A very good fit to the force–velocity data was achieved using the hyperbolic–linear equation, yielding r^2 values of 0.99 for muscle fibres isolated from both anterior abdominal and caudal myotomes (Table 1). The mean maximum power output was 23 % higher in anterior abdominal than in caudal myotomes, although this difference was not statistically significantly (Fig. 3; Table 1). The curvature of the force–velocity relationship, calculated using the power ratio, was not significantly greater in caudal than abdominal myotomes, 0.166±0.01 (mean ± S.E.M.) compared with 0.138±0.01, respectively ($P>0.05$; Table 1).

Muscle filament lengths

I filament lengths showed no significant change with increased body size, being 1.77±0.01 µm (mean ± S.E.M.) and 1.81±0.03 µm in fish of 14.6±0.20 cm (N=2) and 30.9±0.88 cm (N=3) total body length, respectively. Z line thickness was also similar in both size classes; 0.045±0.005 µm and 0.046±0.002 µm in fish of 14.6 cm and 30.9 cm, respectively.

Myofibrillar ATPase activity

Mg^{2+}/Ca^{2+}-myofibrillar ATPase activity (µmoles phosphate released mg^{-1} myofibrillar protein min^{-1}) decreased with increasing fish body length scaling as $2.51L^{-0.28}$ (Fig. 4). In contrast, both myofibrillar ATPase activity in the presence of EGTA (0.70±0.05 µmol mg^{-1} min^{-1}; mean ± S.E.M.) and Ca^{2+} sensitivity (69.8±3.9 %) were independent of body length.

Myofibrillar protein isoforms

One-dimensional SDS–PAGE and two-dimensional IEF/NEIEF–PAGE gels showed very few differences in the migration patterns of myofibrillar proteins between fish of different length (Figs 5–7). The mobility of actin was identical in all sizes of fish with an M_r of 45 (Fig. 5). Alkali light chains (LC1 and LC3) had identical M_r values and isoelectric points in all sizes of fish analysed (Figs 5, 6). The M_r of LC1 and LC3 were 26 and 16, respectively. Myosin light chain 2 (LC2; M_r 21) was also identical in all fish analysed, with major and minor isoforms separated on the basis of their isoelectric points (Figs 5, 6). The M_r of myosin heavy chain (MHC) was 200. Peptide maps of electrophoretically purified MHC produced by digestion with eight different proteases did not reveal any consistent differences between different sizes of fish (results not shown).

Tropomyosin was present as a single spot on two-dimensional gels, with an M_r of 38, in all fish examined. 'Stains-all' demonstrated that troponin-C was expressed as a single isoform, in each size of fish studied, with an M_r of 20. Three isoforms of troponin-I were present in the one-dimensional–PAGE and two-dimensional-NEIEF gels of fish less than 20 cm total body length (Figs 5, 7) with relative molecular masses of 17 (TnI_{f3}), 22 (TnI_{f2}) and 23 (TnI_{f1}).

Table 1. *Comparison of the contractile properties of muscle fibre bundles from anterior abdominal (0.30 total body lengths,* L*, from snout) and caudal (0.75*L*) myotomes*

	Units	Anterior abdominal myotomes	Caudal myotomes
Maximum isometric stress	$kN\,m^{-2}$	159±8.2	161±5.1
Time to 50 % peak tetanus	ms	27.5±2.8	33.0±2.7
Time from last stimulus to 50 % tetanus relaxation	ms	73.2±10.3	84.8±6.5
Maximum unloaded shortening velocity	muscle lengths s^{-1}	7.6±0.7*	5.5±0.5
A	–	0.15±0.07	0.15±0.06
B	muscle lengths s^{-1}	0.79±0.27	0.32±0.13
C	muscle lengths s^{-1}	3.5±0.5	3.0±0.3
r^2	–	0.99	0.99
SEE	–	0.11±0.02	0.10±0.01
Maximum power output	$W\,kg^{-1}$	180±18.9	146±16.9
Power ratio ($\dot{W}_{max}/V_{max}P_{max}$)	–	0.138±0.01	0.166±0.01

A, *B* and *C* are the constants for the hyperbolic–linear equation, r^2 and SEE are the correlation coefficient and the standard error of the estimate, which indicate the goodness of fit of the hyperbolic–linear curve to the force–velocity data.

$\dot{W}_{max}$ is the maximum power output produced during force–velocity studies; V_{max} is the maximum shortening velocity and P_{max} is the maximum isometric force (Marsh and Bennett, 1986).

Values are mean ± S.E.M.

The total body length of the fish used was 19.0±0.21 cm and body mass was 127±19.9 g (N=6).

*Signifies a probability of less than 0.05 that the differences between the anterior abdominal and caudal myotomes are due to chance alone.

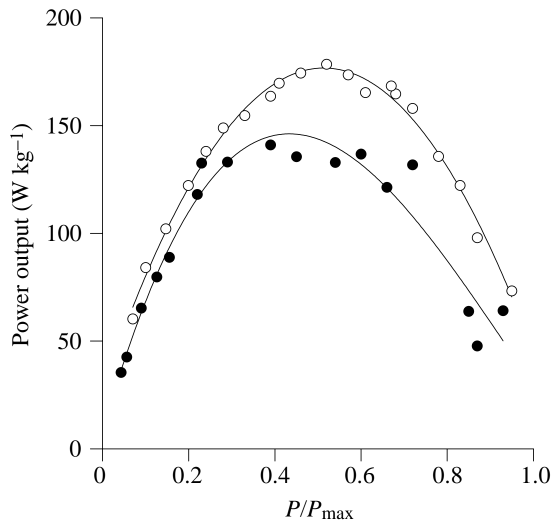


Fig. 3. The effects of body position on the muscle power output calculated from force–velocity data. Open and filled symbols represent typical data for anterior abdominal and caudal fibre preparations, respectively. Each line represents a third-order polynomial fitted to the data using a least-squares regression. P/P_{max} is the force produced during force–velocity studies normalised to the maximum isometric force.

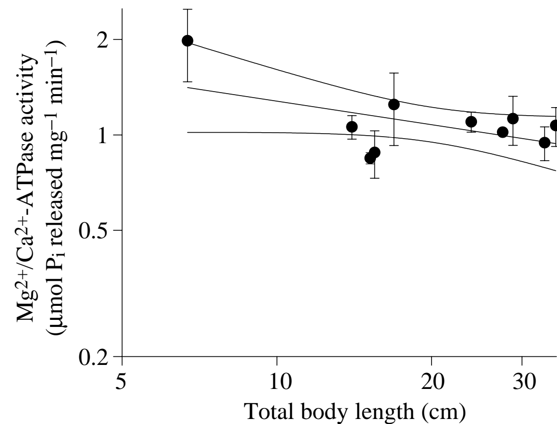


Fig. 4. The effects of total body length on Mg^{2+}/Ca^{2+}-myofibrillar ATPase activity (µmol phosphate released mg^{-1} min^{-1}). The lines represent a first-order polynomial fitted to the log–log data using a least-squares regression and the 95 % confidence limits (r^2=0.22, P<0.05). The log–log data represent mean ± S.E.M. from each fish, 1–4 fish were used in each case.

However, only TnI_{f3} was present in fish greater than 28 cm total body length. The ratios TnI_{f1}:TnI_{f2}:TnI_{f3} increased from 2:1:4 in fish less than 9 cm to 1:1:10 in 15–19 cm fish, whereas TnI_{f3} was the only isoform present in fish greater than 28 cm total length.

Myofibrillar proteins run in the presence of Ca^{2+} possessed an additional band to proteins run in the presence of EGTA. When this additional band was cut out from the one-dimensional AU–PAGE gel and run on an SDS–PAGE gel, it was identified as the 23 kDa isoform of TnI. Western blots of myofibrillar proteins developed against antibodies for actin and troponin-T confirmed the identification of actin and troponin-T. Troponin-T was present as a single spot on two-dimensional NEIEF–PAGE gels, with an M_r of 32, in all fish examined (Fig. 7). The anti-troponin-I antibody confirmed the identity of the troponin-I bands, although there was cross reactivity with troponin-T and an unknown protein with an approximate molecular mass of 100 kDa.

Discussion

Changes in the contractile properties of muscle along the length of the fish

Time-dependent contractile properties of muscle have been found to become slower along the length of the trunk in a number of pelagic species including cod (*Gadus morhua*; Davies *et al.* 1995), saithe (*Pollachius virens*; Altringham *et al.* 1993) and scup (*Stenotomus chrysops*; Rome *et al.* 1993). In the present study on short-horn sculpin, a sedentary benthic fish, isometric tetanus activation and relaxation times tended to be shorter (although not significantly so) in anterior abdominal myotomes than in caudal myotomes, as reported in the study by Johnston *et al.* (1995). However, maximum shortening velocity was 38 % greater and the curvature of the force–velocity relationship, as indicated by the power ratio, was lower (although not significantly so) in anterior abdominal than in caudal myotomes (Table 1). These new findings suggest that anterior abdominal myotomes (0.30L) have at least some contractile properties indicative of a faster muscle type than caudal myotomes (0.75L) and this may be true generally for fish that swim using their trunk musculature.

The effects of body size on muscle contractile properties

Increases in body size are matched by a general slowing of the contractile properties of muscle, demonstrated in the present study by twitch and tetanus kinetics and maximum shortening velocity. In mammals, scaling relationships are usually expressed in relation to body mass (M). The scaling exponents for time to peak twitch force and time from peak force to 50 % twitch relaxation in the present study were $M^{0.08}$ and $M^{0.20}$ respectively, virtually identical to the exponents found by Altringham *et al.* (1996) in *Xenopus laevis* fast muscle of $M^{0.07}$ and $M^{0.19}$. Both *Xenopus laevis* and short-horn sculpin use their fast muscles for burst locomotion. However, this relationship does not hold true for all species since time to peak force for the lizard *Dipsosaurus dorsalis* fast muscle fibres scaled as $M^{0.21}$ (Johnson *et al.* 1993). Curtin and Woledge (1988) reported that the maximum shortening velocity of fast muscle fibres (V_{max}) was scale-independent in dogfish. However, we found that V_0 of fast muscle fibres scaled as $M^{-0.10}$, similar to the values determined using mammalian fast muscle fibres of $M^{-0.07}$ for V_0 by Rome *et al.* (1990) and $M^{-0.13}$ for V_{max} by Seow and Ford (1991). Our results are consistent with previous studies on mammals (Rome *et al.* 1990) and amphibians (Altringham

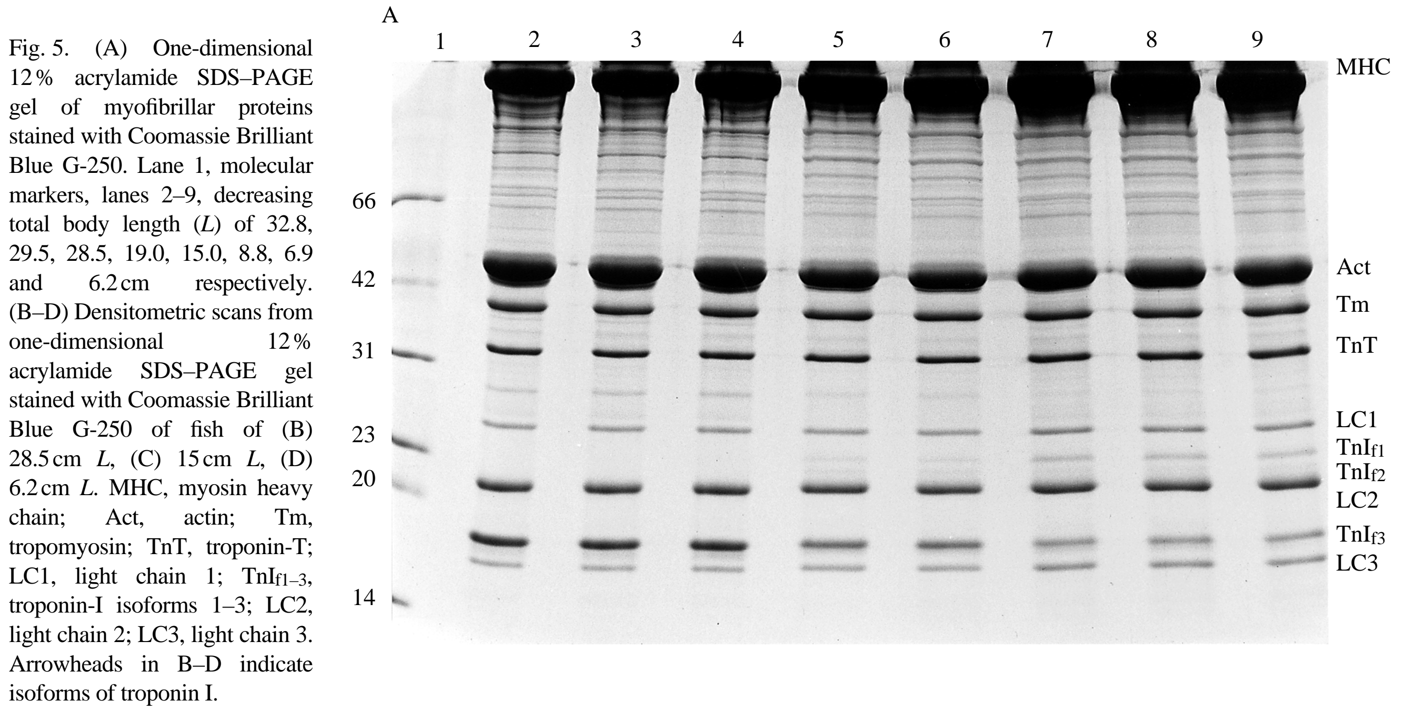


Fig. 5. (A) One-dimensional 12 % acrylamide SDS–PAGE gel of myofibrillar proteins stained with Coomassie Brilliant Blue G-250. Lane 1, molecular markers, lanes 2–9, decreasing total body length (*L*) of 32.8, 29.5, 28.5, 19.0, 15.0, 8.8, 6.9 and 6.2 cm respectively. (B–D) Densitometric scans from one-dimensional 12 % acrylamide SDS–PAGE gel stained with Coomassie Brilliant Blue G-250 of fish of (B) 28.5 cm *L*, (C) 15 cm *L*, (D) 6.2 cm *L*. MHC, myosin heavy chain; Act, actin; Tm, tropomyosin; TnT, troponin-T; LC1, light chain 1; $TnI_{f1–3}$, troponin-I isoforms 1–3; LC2, light chain 2; LC3, light chain 3. Arrowheads in B–D indicate isoforms of troponin I.

et al. 1996) that found with increased body size there was a more marked alteration in relaxation times than in either activation times or maximum unloaded shortening velocity (V_0). The small changes in V_{max}, V_0 and muscle activation times with body size in fast muscle presumably enable rapid movements across the whole range of body sizes (Rome *et al.* 1990; Altringham *et al.* 1996).

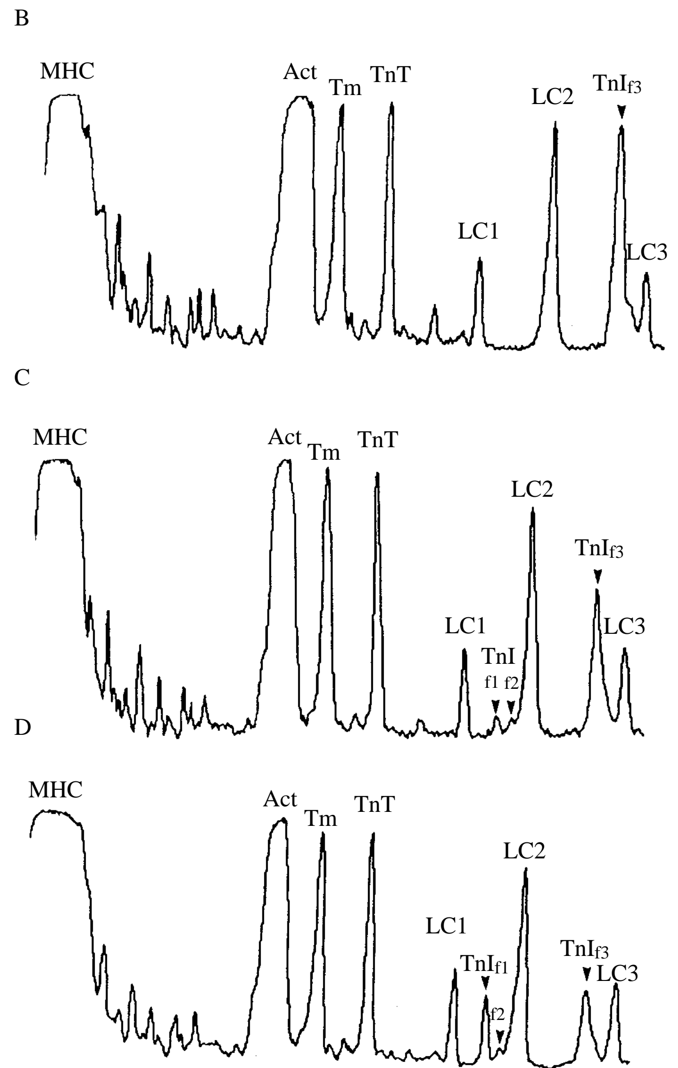
Filament lengths

I filament lengths were found to increase from 2.12 to 2.18 μm in rat gastrocnemius (Heslinga and Huijing, 1993) and from 2.28 to 2.48 μm in rat soleus (Heslinga *et al.* 1995) during development from 14 to 16 weeks after birth. In contrast, we found no significant differences in I filament lengths between fish of 15 cm (1.77±0.01 μm) and 31 cm (1.81±0.03 μm) total body length. The I filament lengths measured in this study are comparable with those obtained for carp red and white muscles (1.83–2.02 μm) (Akster, 1985; Sosnicki *et al.* 1991; van Leeuwen *et al.* 1990).

Our measurements of I filament length are subject to error as we corrected for shrinkage by assuming that the A filament length was 1.5 μm (Sosnicki *et al.* 1991) and that I filaments would undergo the same magnitude of shrinkage as A filaments. However, it would seem unlikely that shrinkage of I filaments would vary with body size; hence, I filament length could not have accounted for the changes in maximal shortening velocity or maximal unloaded shortening velocity measured in the present study.

Changes in the expression of contractile proteins with growth

Myofibrillar protein composition was determined to investigate alternative mechanisms for the changes in



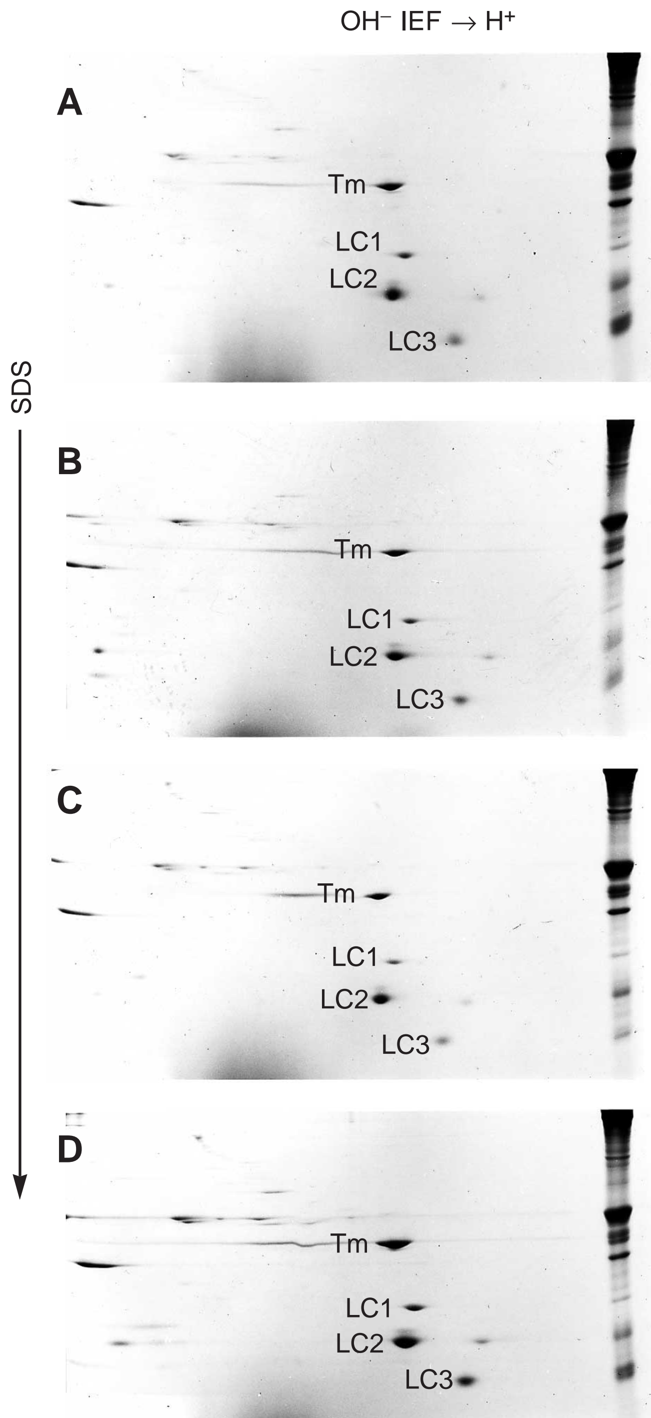


Fig. 6. Two-dimensional IEF–PAGE gels of neutral-to-acidic myofibrillar proteins resolved on 13 % acrylamide SDS–PAGE gels and stained with Coomassie Brilliant Blue G-250. (A–C) Fish of total lengths 32.8, 15.0 and 6.2 cm, respectively. (D) A mixture of proteins from 32.8 and 6.2 cm fish run on the same gel. Tm, tropomyosin; LC1, light chain 1; LC2, light chain 2; LC3, light chain 3.

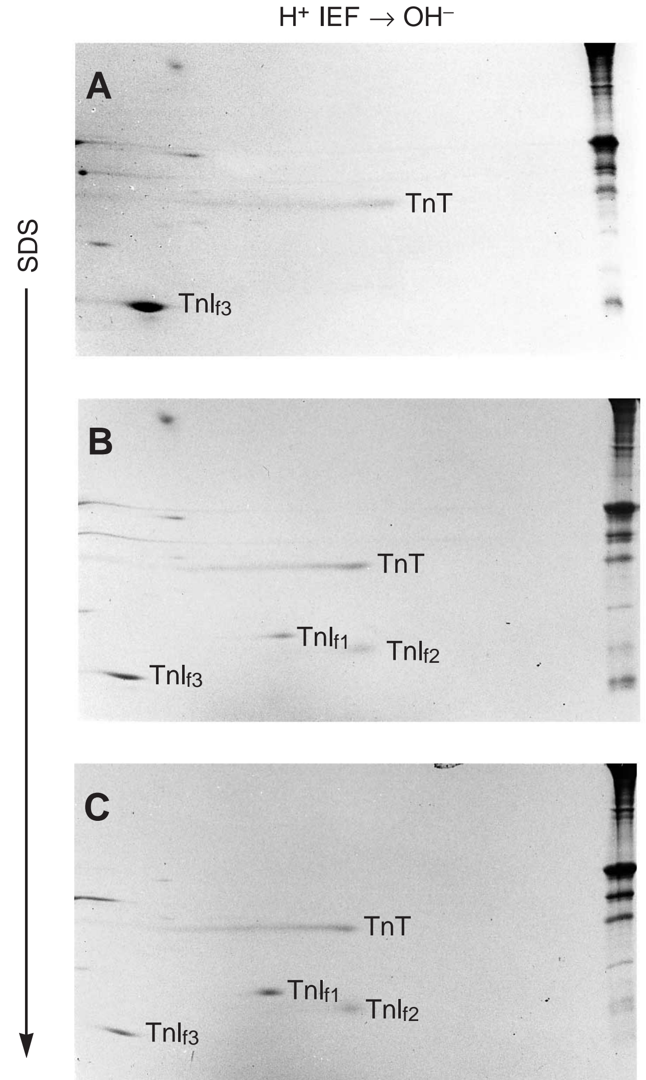


Fig. 7. Two-dimensional NEIEF–PAGE gels of neutral-to-basic myofibrillar proteins stained with Coomassie Brilliant Blue G-250. (A–C) Fish of total lengths of 32.8, 15.0 and 6.2 cm, respectively. TnT, troponin-T; TnI_{f1-3} troponin-I isoforms 1–3.

contractile properties and myofibrillar ATPase activity with body size. The changes in myosin heavy and light chain isoform composition that occur during mammalian development result in a slowing of the mechanical properties of muscle and changes in myofibrillar ATPase activity (for a review, see Moss *et al.* 1995). Alterations in myosin heavy or light chain composition can cause changes in V_{max}, but removal of myosin light chains has little effect on myosin ATPase activity (for a review, see Moss *et al.* 1995). In the present study, no evidence was found for changes in myosin composition. However, it is possible that very subtle differences in myosin heavy chain primary structure went undetected even though eight proteolytic enzymes with different specificities for peptide bonds were used. In a previous study, myofibrillar ATPase activity increased with cold acclimation in killifish (*Fundulus heteroclitus*) without detectable changes in myosin heavy chain composition (Johnson and Bennett, 1995).

Changes in troponin-T isoforms described during development in the dragonfly *Libellula pulchella* (Fitzburgh and Marden, 1997) and chicken (Reiser *et al.* 1992) have been

associated with decreases in isometric muscle activation and relaxation times due to an increase in Ca^{2+} sensitivity. Differences in troponin-T isoforms between fibre types in rabbit muscle have also been shown to affect Ca^{2+} sensitivity (Greaser *et al.* 1988); however, no changes in troponin-T isoform composition were found in the present study.

In the present study, the only differences in myofibrillar protein composition detected in white muscle of the short-horn sculpin with growth were the changes in the relative abundance of troponin-I isoforms. Changes in troponin-I isoforms with growth have been found previously in herring larvae (*Clupea harengus*; Johnston *et al.* 1997). It is possible that changes in troponin-I isoforms could alter the interaction of troponin-C with Ca^{2+}, contributing to the observed alterations in twitch activation times; however, they would be unlikely to affect unloaded shortening velocity (V_0).

In previous studies, an increase in the expression of the α-isoform of the ryanodine receptor has been associated with faster contractile properties (for a review, see Coronado *et al.* 1994). Therefore, modifications in muscle activation rates might also be due to differential expression of ryanodine receptor isoforms throughout growth, resulting in altered Ca^{2+} release from the sarcoplasmic reticulum (SR). The mechanisms underlying increases in relaxation times with growth were not investigated in the present study, but probably involve decreases in the rate and capacity of Ca^{2+} uptake by the sarcoplasmic reticulum. For example, it has been demonstrated that the timing and regulation of SR Ca^{2+}-ATPase isoform shifts during muscle development are independent of the timing and regulation of MHC isoform shifts (for a review, see Gunning and Hardeman, 1991). Muscles with shorter twitch relaxation times have higher SR Ca^{2+}-ATPase activity, increased amounts of SR and higher levels of Ca^{2+}-binding proteins than slow-twitch muscles (for a review, see Dux, 1993). Relaxation rate in frog skeletal muscle is directly correlated with parvalbumin content (Hou *et al.* 1991), whereas twitch duration in cicada muscle is directly correlated with the volume ratio of muscle fibres to sarcoplasmic reticulum and T-tubules (Josephson and Young, 1987).

In conclusion, the *in vitro* contractile properties of fast fibres from short-horn sculpin undergo major changes during growth with increases in activation and relaxation times and more than twofold decreases in maximum unloaded shortening velocity (V_0). These changes in contractile properties with growth may, in part, result from changes in the expression of troponin-I isoforms, but no evidence was found for altered expression of myosin heavy chain, myosin light chain, troponin-C or troponin-T isoforms or changes in I filament lengths. In contrast, major differences in myosin heavy chain composition (Rowlerson *et al.* 1985; Crockford and Johnston, 1993) and 1.5- to 6.5-fold differences in maximum unloaded shortening velocity (Johnston and Brill, 1984; Johnston and Salamonski, 1984) have been found previously between fish fast and slow muscle. The changes in V_0 measured in the present study may have been due to undetected minor changes in myosin composition. Sequencing studies at the protein or gene level would be required to determine whether a small number of amino acid substitutions had occurred in the isoforms expressed during growth.

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