ACTA PHYSIOLOGICA

Acta Physiol 2011, **202,** 671–681

There is no slowing of motility speed with increased body size in rat, human, horse and rhinoceros independent on temperature and skeletal muscle myosin isoform

M. Li,[1,2] M. Li,[1] J. O. Marx[3] and L. Larsson[1,4]

1 *Department of Clinical Neurophysiology, Uppsala University Hospital, Uppsala, Sweden*

2 *Department of Neurology, Qilu Hospital, Shandong University, Jinan, Shandong, China*

3 *Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, USA*

4 *Department of Biobehavioral Health, The Pennsylvania State University, University Park, PA, USA*

Received 4 May 2010,
revision requested 20 June 2010,
final revision received 2 March 2011,
accepted 18 March 2011

Correspondence: L. Larsson, Department of Neuroscience, Clinical Neurophysiology, Uppsala University, Uppsala, SE-751 85, Sweden.
E-mail: lars.larsson@neuro.uu.se

Abstract

Aim: The predictions of scaling of skeletal muscle shortening velocity made by A.V. Hill 60-years ago have proven to be remarkably accurate at the cellular level. The current investigation looks to extend the study of scaling of contractile speed to the level of the molecular motor protein myosin at both physiological and unphysiological low temperatures.

Methods: A single muscle cell *in vitro* motility assay to test myosin function, i.e. myosin extracted from short single muscle fibre segments, was used in four species representing a 5 500-fold difference in body mass (rat, man, horse and rhinoceros) at temperatures ranging from 15 to 35 °C.

Results: The *in vitro* motility speed increased as the temperature of the assay increased, but a more profound effect was observed on the slower isoforms, narrowing the relative differences between fast and slow myosin heavy chain (MyHC) isoforms at physiological temperature in all species. The *in vitro* motility speed varied according to MyHC isoform within each species: I < IIa < IIx < IIb, but the expected scaling relationship within orthologous myosin isoforms was not observed at any temperature.

Conclusion: The scaling effect of body size and limb length on shortening velocity at the muscle fibre level, i.e. the decreasing shortening velocity associated with increasing body weight and limb length, was not confirmed at the motor protein level when including mammals of very large size. Thus, other factors than myosin structure and function appear to cause this scaling effect and thin filament isoform expression or myofilament lattice spacing are forwarded as alternative underlying factors.

Keywords *in vitro* motility assay and body size, myosin heavy chain.

Experimental studies carried out in frogs and small rodents have had a significant impact on our understanding of regulation of muscle contraction from the whole muscle, motor unit, and muscle fibre to the motor protein levels and results have typically been generalized to all mammalian species, including humans, because of the similarity in structure and homology of contractile proteins. In spite of the similarities between species in skeletal muscle structure and function, there is reason to be cautious when extrapolating results from small rodents and frogs to humans as there are body size related differences in regulation of skeletal muscle contraction between mammals at which the muscle operates (Iriarte-Diaz 2002). In 1950, A. V. Hill observed that animals of similar body shape and gait characteristic were able to move at similar velocities,

independent of body size. If a small animal with a shorter stride length moves at a comparable running speed as a large animal, then the small animal must move at higher stride frequency (Hill 1950). Therefore, Hill suggested that there would be an inverse relationship between skeletal muscle shortening velocity and body size.

Hill's insight into the scaling of animal locomotion performance has stimulated many experimental studies. Most of the work examining the scaling effect has utilized the skinned muscle fibre preparation, allowing detailed studies of regulation of muscle contraction without confounding factors of multicellular preparations. This method has advanced our understanding of scaling-related differences in regulation of muscle contraction effects on skeletal muscle contractility, confirming many of Hill's hypotheses by demonstrating an inverse relationship between body mass and maximum velocity of unloaded shortening (Seow & Ford 1991, Rome 1992, Widrick *et al.* 1997, Pellegrino *et al.* 2003). A stronger scaling has typically been reported for slow than fast MyHC isoforms both at the muscle fibre and motor protein levels because of the metabolic constraints placed on the slow-twitch muscle fibres because of the high rate of recruitment during muscle activation. Our group has extended these findings (Marx *et al.* 2006) by studying an unprecedented 100 000-fold range of body mass in six mammalian species, including humans, showing that limb length is a more important determinant in the scaling effect on maximum velocity of unloaded shortening than body mass.

The development of a single fibre *in vitro* motility assay has allowed us to extend the study of skeletal muscle scaling from the cellular level to the molecular level. A significant scaling-related slowing of actin filaments propelled by myosin extracted from single muscle fibres suggests that the scaling-related slowing at the single muscle fibre level is, at least in part, caused by altered functional properties of the motor protein itself (Hook *et al.* 2001, Pellegrino *et al.* 2003). Myosin is the molecular motor that converts chemical energy derived from the hydrolysis of ATP into mechanical work and there is good evidence that different myosin heavy chain (MyHC) isoforms confer distinct contractile properties to muscle fibres. In turn, the contractile properties of a whole muscle result from the aggregate biomechanical properties and genetic control of myosin isoform expression of its constituent fibres. The relative proportion of muscle fibres expressing fast MyHC isoforms is typically higher in rodent limb muscles than in muscles from large mammals. This together with the absence of limb muscle fibres expressing the fastest MyHC isoform, the IIb MyHC, in large mammals contribute to the slowing in contractile speed in mammals with a large body mass.

Regulation of muscle contraction is highly dependent on temperature and temperature is accordingly also of critical importance for our understanding of scaling effects (Walker 1951, Close & Hoh 1968, Hill 1972, Ranatunga 1984, Wang & Kawai 2001, Kawai 2003). First, experiments at the single muscle fibre level and motor protein levels are typically conducted at unphysiologically low temperatures, 12–25 °C. The single fibre *in vitro* motility offers the opportunity to study the interaction between specific myosin isoforms and actin over a wide temperature range, including physiological temperatures. We have previously shown that the relative differences in actin motility speed is reduced dramatically between different MyHC isoforms at physiological temperatures and the 7.5-fold difference in motility speed at 15 °C between type I and IIax MyHC extracted from human single fibre segments was reduced to a twofold difference at 35 °C (Lionikas *et al.* 2006). Second, amphibian and fish muscle, as poikilotherms, have adapted physiologically to function at a variety of environmental temperatures, whilst mammalian muscle has evolved to function optimally in a very narrow range of physiological temperatures. The aim of the present study was to investigate the scaling of motility speed in specific myosin isoforms extracted from single muscle fibre segments and its dependence on temperature in mammals representing 5 500-fold differences in body mass.

Materials and methods

Animals and human subjects

Skeletal muscle tissue from male rat, humans, horses and a rhinoceros was collected for this investigation. Except for the rhinoceros, all of the animals and human subjects were young adults free of any musculoskeletal disease that may alter muscle function. The rhinoceros was 26-years old with a history of chronic pododermatitis. The condition had been under clinical control for several years when the muscle biopsy was obtained. Femur length, used as an estimate of limb length, was determined by direct measurement in the rat. Femur lengths for the humans were calculated from the tables of Knight (1996), and for the horse and rhinoceros from Christiansen (1999). A description of the animals and subjects in this investigation is included in Table 1. The ethical committee at Uppsala University (Uppsala, Sweden), the Pennsylvania State University's Institutional Review Board and Institutional Animal Care and Use Committee approved all aspects of this study.

Table 1 Characteristics of the animals and subjects

Species	Strain	Number	Age	Body mass	Femur length†
Rat	Sprague–Dawley	5	6 months	450 g	35 mm
Human	–	4	30 years	80 kg	449 mm
Horse	Quarter Horse	2	1 year	400 kg	414 mm
Rhinoceros	White	1	26 years	2,500 kg	731 mm

Values are means. †Femur lengths were determined by direct measurement in the rat; horse and rhinoceros, equations from Christiansen (1999) and humans from tables by Knight (1996).

Muscle preparation and permeabilization of fibres

In the rat, the soleus, extensor digitorum longus (EDL) and plantaris muscles were removed immediately after euthanasia. In the humans, horses and rhinoceros, muscle biopsy specimens were obtained by means of the percutaneous chonchotome method. In humans, biopsies were obtained from the vastus lateralis muscle under local anaesthesia. In horses, biopsies were obtained from the gluteus medius muscle during sedation and local anaesthesia. In the rhinoceros, muscle biopsies were obtained from the hamstrings muscle group during general anaesthesia. Before the procedure, the human subjects were informed of all risks associated with the procedure and signed and informed consent form. Following the collection of the muscle tissue, all of the samples were then treated identically. The muscle tissue was immediately placed in relax solution at 4 °C. A second biopsy sample was frozen in isopentane chilled with liquid nitrogen and stored at −80 °C for overall protein determination using gel electrophoresis. Bundles of approx. 50–100 fibres were dissected in the chilled relax solution and tied with surgical silk to glass capillaries, then stretched to approx. 110% of their resting length. The bundles were chemically skinned in relax solution containing 50% (vol/vol) glycerol (Relax solution: pCa 9.0, 4 mM ATP, 1 mM free Mg^{2+}, 20 mM imidazole, 14.5 mM creating phosphate and 7 mM EGTA) for 24 h at 4 °C and were subsequently stored in the skinning solution at −20 °C for up to 4 weeks. Because of the value of the tissue, after the skinning procedure, some bundles of human and rhinoceros fibres were sucrose treated for long-term storage at −80 °C. This procedure has been shown to not affect the contractile properties of the muscle fibres (Frontera *et al.* 1997).

In vitro motility assay

The unregulated actin used throughout this study was extracted from rabbit skeletal muscle essentially as described (Pardee & Spudich 1982) and was fluorescently labelled with rhodamine-phalloidin (Molecular Probes). The single fibre *in vitro* motility system has been described in detail elsewhere (Hook *et al.* 1999, Hook & Larsson 2000). In brief, a short muscle fibre segment was placed on a glass slide between two strips of grease, and a mica cover-slip was placed on top, creating a flow cell of approx. 2-μL volume. Myosin was extracted from the fibre segment through the addition of a high salt buffer (0.5 M KCl, 25 mM HEPES, 4 mM $MgCl_2$, 1 mM EGTA and 1% 2-mercaptoethanol, pH 7.6). After 30-min incubation on ice, a low salt buffer (25 mM KCl, 25 mM Hepes, 4 mM $MgCl_2$, 1 mM EGTA and 1% 2-mercaptoethanol, pH 7.6) was applied, followed by BSA (1 mg mL^{-1}). Non-functional myosin molecules were blocked with fragmentized F-actin, and rhodamine-phalloidin labelled actin filaments were subsequently infused into the flow cell, followed by motility buffer (2 mM ATP, 0.1 mg mL^{-1} glucose oxidase, 23 g mL^{-1} catalase, 2.5 mg mL^{-1} glucose and 0.4% methyl cellulose in salt buffer) to initiate the movement. The pH of the buffers was adjusted with KOH, and the final ionic strength of the motility buffer was 71 mM. The flow cell was placed on the stage of an inverted epifluorescence microscope (Olympus IX 70; Olympus America, Center Valley, PA, USA). Actin movements were filmed with an image-intensified SIT camera (SIT 66; DAGE-MIT, USA) and recorded on tape with a video-cassette recorder.

From each single fibre preparation, 13–20 actin filaments moving with constant speed in an oriented motion were selected for speed analysis. Recordings and analysis were only performed from preparations in which >90% of the filaments moved bi-directionally. A filament was tracked from the centre of mass, and the speed was calculated from 10 to 20 frames at an acquisition rate of one or five frame(s) per second, depending on the fibre type, using an image analysis package (OPTIMAS 6.0; Optimas, USA). The average speed and standard deviation of the 13–20 filaments were calculated. The average motility speed of each fibre was taken as representative for the muscle fibre.

The study was carried out at one of the following temperature sequences: (1) 15, 20, 25, 30, 35 °C, (2)

25, 30, 35, 20, 15 °C, (3) 25, 20, 15, 30, 35 °C. The temperature of the flow cell was adjusted to 25 and 15 °C after the records were collected at different temperature sequences. Motility speeds were subsequently analysed at the respective temperatures with regard to temperature hysteresis. Exposure to each temperature took 1–2 min and the whole experiment was completed in about 30 min. Video recordings were performed when the actual temperature of the flow cell was within ±0.2 °C of the target temperature at 15–25 °C temperatures and within ±0.3 °C at 30–35 °C temperatures. Labelled actin was periodically re-applied between temperatures because of rapid bleaching particularly at the high temperatures. After the motility experiment, each fibre segment was placed in sodium dodecyl sulphate (SDS) sample buffer in a plastic microfuge tube and stored at −80 °C pending MyHC analysis on 6% SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Electrophoretic separation of myosin heavy isoforms

The MyHC composition of a muscle fibre was determined by 6% SDS-PAGE. The total acrylamide and Bis concentrations were 4% (wt/vol) in the stacking gel and 6% in the running gel and the gel matrix included 30% glycerol. Electrophoresis was performed at 120 V for 24 h with a Tris-glycine electrode buffer (pH 8.3) at 15 °C (SE 600 vertical slab gel unit; Hoefer Scientific Instruments, USA). The gels were silver stained and subsequently scanned in a soft laser densitometer (Molecular Dynamics, Sunny-vale, CA, USA) with a high spatial resolution (50 μm pixel spacing) and 4 096 optical density levels. The volume integration function (ImageQuant software version 3.3, Molecular Dynamics) was used to quantify the protein amount on the gels to identify the MyHC isoform composition in fibres expressing more than one MyHC isoform, and the hybrid fibres were grouped independent on the relative amount of the two co-expressed isoforms.

MyHCs on the 6% SDS-PAGE from rhinoceros m. biceps femoris cross-sections were transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare UK Limited, Buckinghamshire, UK). Membranes were incubated with MyHC type I (A4840) and II (A474, Developmental Studies Hybridoma Bank, Iowa State University, USA) monoclonal antibodies. The membranes were incubated with secondary antibodies NXA931 (GE Healthcare) and using ECL Advance western blotting detection kit (RPN 2135, GE Healthcare UK Limited, Buckinghamshire, UK) according to manufacturer's instructions. Type I and II MyHC isoforms with a migration order identical to human skeletal muscle type I and IIa MyHCs were observed (Fig. S1 Supporting information).

Statistical analysis

Mean and SEM were calculated by standard procedures. The statistical significance was estimated by the ANOVA. Differences were considered significant at $P < 0.05$. If significant main effects were found, a multiple comparison procedure was employed to isolate, which groups differed from each other. The temperature sensitivity of motility speed expressed as activation energy, E_a, and temperature coefficient, Q_{10}, was analysed in Arrhenius plots. Linear regressions were used to describe the relationships between motility speed and body mass as well as the motility speed and femur length. The exponent of the equation, b, was considered to be the scaling coefficient.

Results

Anthropometric properties and MyHC isoform expression

The average age and anthropometric characteristics of the different species representing a 5 500-fold body mass and 20-fold femur length span are presented in Table 1. A total of 57 membrane permeabilized muscle fibre segments from the four species fulfilled the criteria for acceptance were included in the final analyses, i.e. 17 fibres from rat, 18 fibres from human, 15 fibres from horse and seven fibres from rhinoceros limb muscles. The MyHC isoforms expressed in the different muscle fibres included in the analyses are given in Table 2. Muscle fibres expressing fast (type II) or slow (β/slow or type I) MyHC isoforms were observed in all species. In the rat, soleus fibres expressed mainly the β/slow (type I) MyHC isoform, EDL fibres expressed primarily IIx and IIb MyHC isoforms along with a small amount of the IIa MyHC isoform and plantaris expressed IIa, IIx and IIax MyHC isoforms. In humans, both fast and slow MyHC isoforms were expressed in the vastus laterals muscle. In the horse, MyHC isoform expression in the gluteus medius muscle varied according to the depth in the muscle, i.e. fibres expressing the β/slow HyHC isoform were mainly found in the deep region of the muscle whilst IIa, IIx and IIax MyHCs dominated in the superficial region. In the rhinoceros, an approximate equal number of fast and slow muscle fibres was observed in the hamstrings muscle.

Motility speed vs. temperature

The motility speed of actin filaments moving over extracted myosin from single skeletal muscle fibre segments was measured over temperatures ranging from 15 to 35 °C with 5 °C increments (Table 2). A significant temperature dependent increase in motility speed

Table 2 Motility speed at temperatures varying from 15 to 35 °C in fibres expressing different MyHC isoforms or combination of isoforms in four mammalian species representing a 5 500-fold difference in body mass

Species	MyHC	No. fibres	15°C	20°C	25°C	30°C	35°C	Fold change from 15 to 35°C
Rat	I	5	0.09 ± 0.02	0.39 ± 0.14	1.19 ± 0.3	2.83 ± 0.50	7.14 ± 0.63	79.3
	II a	3	0.16 ± 0.08	0.52 ± 0.18	1.63 ± 0.78	3.5 ± 1.85	12.76 ± 0.26	79.8
	IIx	3	0.6 ± 0.17	2.07 ± 0.70	5.13 ± 1.05	10.57 ± 3.14	15.16 ± 2.94	25.3
	IIxb	3	0.59 ± 0.19	2.05 ± 0.32	5.53 ± 0.08	11.29 ± 0.73	19.52 ± 0.76	33.1
	IIb	3	0.7 ± 0.14	2.42 ± 0.39	5.92 ± 1.22	12.07 ± 1.00	20.13 ± 0.82	28.8
Human	I	6	0.07 ± 0.01	0.31 ± 0.09	0.77 ± 0.13	1.91 ± 0.24	3.9 ± 0.46	55.7
	II a	9	0.38 ± 0.07	1.01 ± 0.18	2.53 ± 0.32	4.93 ± 0.50	8.7 ± 1.06	22.9
	IIax	3	0.94 ± 0.19	1.67 ± 0.43	3.58 ± 0.75	6.52 ± 0.99	9.19 ± 1.51	9.8
Horse	I	5	0.19 ± 0.02	0.41 ± 0.05	0.89 ± 0.08	2.19 ± 0.36	4.41 ± 0.95	23.2
	II a	3	0.38 ± 0.02	1.17 ± 0.23	2.52 ± 0.44	5.8 ± 0.39	10.79 ± 1.03	28.4
	IIax	4	0.62 ± 0.16	1.53 ± 0.20	3.82 ± 0.41	7.14 ± 1.11	12.24 ± 0.56	19.8
	IIx	3	0.99 ± 0.01	2.57 ± 0.16	6.04 ± 0.31	10.12 ± 0.31	16.04 ± 0.77	16.2
Rhinoceros	I	4	0.07 ± 0.01	0.41 ± 0.04	1.05 ± 0.14	2.75 ± 0.17	4.85 ± 0.44	65.4
	II a	3	0.36 ± 0.02	0.94 ± 0.21	2.54 ± 0.62	4.36 ± 0.81	7.91 ± 2.09	22.0

Source of variation	DF	SS	MS	*F*	*P*
MyHC isoform	13	1300	100	199	<0.001
Temperature	4	3711	928	1845	<0.001
MyHC isoform × temperature	52	885	17	34	<0.001

Values are means ± SE. Main effect MyHC (14 levels) $P < 0.001$; Main effect temperature (five levels) $P < 0.001$; MyHC-by-temperature $P < 0.001$. Two-way ANOVA was used to test for MyHC isoform and temperature differences. *P*-values denote the main effect results for MyHC isoform, temperature and MyHC isoform temperature interaction.

of actin filaments propelled by both slow and fast myosin isoforms was observed irrespective species (Fig. 1), and motility speed was highly dependent on MyHC isoform irrespective species and temperature, following the gradient: I < IIa < IIax < IIx < IIxb < IIb. Statistically significant differences between MyHC isoforms were, however, typically restricted to higher temperatures, 25 °C and above (Table 2). All the results were independent of increasing or decreasing the temperatures. The same results were also obtained after lowering the temperature from 25 to 15 °C at the end of the experiment, i.e. there was no temperature hysteresis in any of the different species.

Temperature had a more profound effect on the *in vitro* motility speed of slow than fast MyHC isoforms in all species (Table 2). At 15 °C, the relative differences in motility speed between type IIa and I MyHCs were 5.8- and 5.1-fold in humans and the rhinoceros, respectively, and the corresponding values were 2.2- and 1.6-fold at 35 °C. In the rat, on the other hand, a 1.8-fold difference in motility speed between type IIa and I MyHCs was observed at both 15 and 35 °C, but the relative difference between type IIa and IIx as well as between IIa and IIb MyHC isoforms decreased from 3.2- and 4.4-fold at 15 °C to 1.2- and 1.6-fold at 35 °C (Table 2).

Activation energies (E_a) and temperature coefficients (Q_{10})

The motility speed of actin propelled by slow and fast MyHC isoforms were displayed in Arrhenius plots, i.e. velocity was plotted vs. the reciprocal of temperature, to compare the temperature dependence of the motility speed in different species (Fig. 1). The temperature sensitivity was given as Q_{10} representing the ratio of the speeds of a chemical reaction (here the motility speed) at two temperatures differing by 10 °C. The minimum energy necessary for a specific reaction to occur was given as E_a. According to Ranatunga, Q_{10}, was determined from the slope of the regression line fitted to the Arrhenius plot by using the equation: $Q_{10} = \exp[(E_a/R)\cdot 1/T_1 - 1/T_2]$, where T_1 and T_2 denote the two absolute temperatures (Kelvin) chosen for Q_{10} calculation. E_a/R is the slope of the Arrhenius plot and $T_1 - T_2 = 10$ degrees Kelvin (Ranatunga 1984). A transition in the Arrhenius plot occurred at 25 °C in rats and rhinoceros. The same results were obtained regardless of whether the plots were calculated from experiments, where temperatures were increased or decreased. Activation energy and Q_{10} values were calculated below and above the transition point in all species, in spite of the fact that a single regression line fit

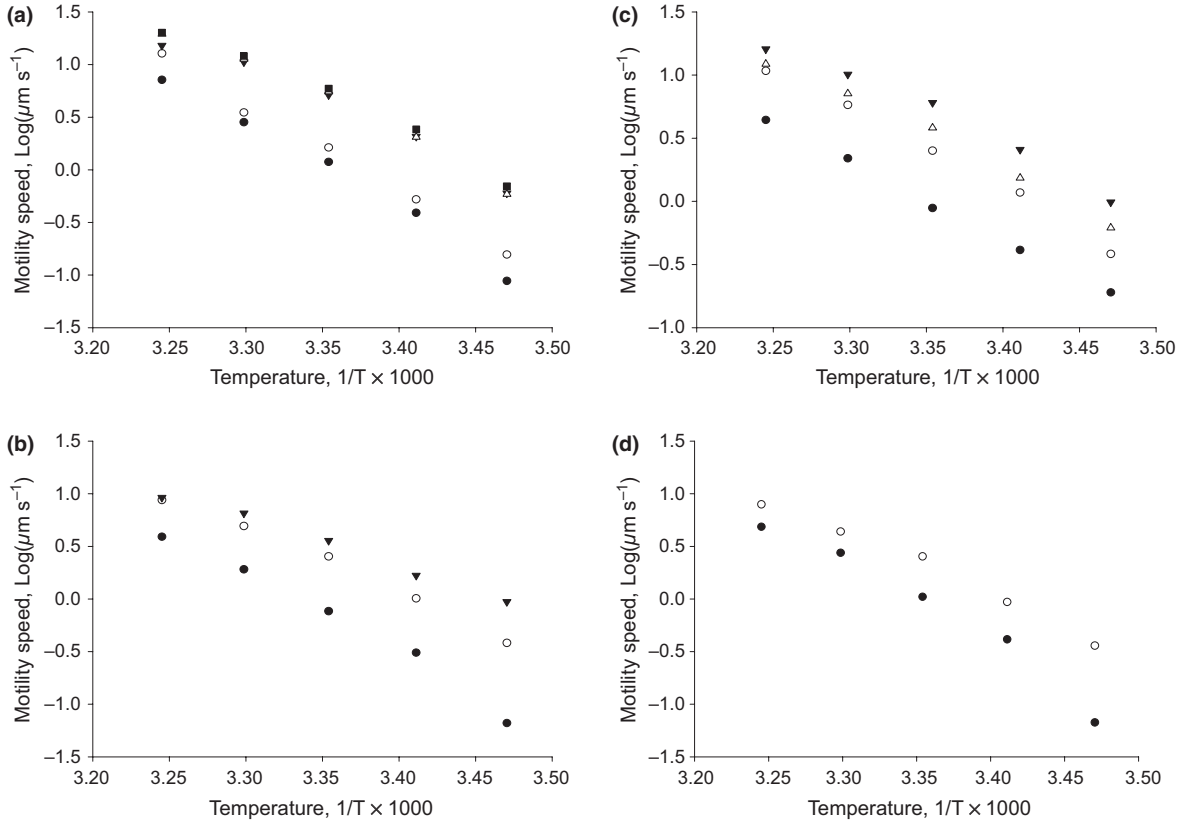

Figure 1 Arrhenius plot for influence of different temperatures on motility speed of myosins expressing various MyHC isoforms from different species (a) rat, (b) human, (c) horse and (d) rhinoceros. Motility speed is expressed as log (μm s^{-1}) and temperature as $1/T \times 1000$, where T is the absolute temperature. In (a), type I, IIa, IIx, IIxb and IIb MyHC isoforms are illustrated as filled circles (●), open circles (○), filled triangles (▼), open triangles (△) and filled square (■) respectively. In (b), type I, IIa, IIx and IIax MyHCs correspond to filled circles (●), open circles (○), filled triangles (▼) and open triangles (△) respectively. In (c), type I, IIa, and IIx MyHCs correspond to filled circles (●), open circles (○) and filled triangles (▼) respectively. In (d), type I and IIa MyHCs correspond to filled circles (●) and open circles (○) respectively.

the whole temperature range well in some species and MyHC isoforms (Fig. 1). In all species, the E_a and Q_{10} in the 10–25 °C range were higher than those in the 25–35 °C range, but significant differences ($P < 0.05$) were restricted to E_a irrespective species and MyHC isoform (Table 3).

Motility speed vs. body mass and femur length

The analysis of scaling is restricted to the type I and IIa MyHC isoforms, as these isoforms were expressed in all species investigated. We have previously reported a significant scaling effect on motility speed when comparing mice, rats and humans in fibres expressing the type I MyHC isoform in both young and old individuals (Hook *et al.* 2001). In this study, similar motility speeds were observed in rat and human single fibre *in vitro* motility preparations as in our previous study (Hook *et al.* 2001). Also, consistent with the previous study, significant differences were observed in both type I and IIa MyHCs when comparing rats and humans, especially at higher temperatures (Table 2). However, there was no difference in motility speed between human, horse and rhinoceros in type I and IIa MyHC preparations (Table 2) at essentially all of the temperatures. Accordingly, motility speed did not scale significantly to body mass or femur length over the 5 500-fold range in the log-transformed plots at almost all temperatures examined (Fig. 2, Table 4). Based on this surprising lack of significance, we re-analysed the data from the Marx *et al.* (2006) for the four species used in the current study and found the relationship between shortening velocity and femur length remained statistically significant ($P < 0.05$) in the type I fibres and a similar relation was observed in the type IIa fibre preparation, albeit not statistically significant ($P < 0.1$). Shortening velocity did not, on the other hand, correlate significantly with body mass in these four species in type I or IIa fibre preparations. Interestingly, the two fibre type and temperature relationships that did achieve

Table 3 Activation energy and temperature coefficients of motility speed before and after the transition point at 25 °C

		Before 25 °C		After 25 °C	
Species	No. fibres	E_a	Q_{10}	E_a	Q_{10}
Rat					
I	5	80.70†,‡	2.99†	60.39‡‡*	2.28†
IIa	3	73.34§,¶	2.79†	70.62††	2.64†
IIx	3	67.12¶,††	2.48†	36.04§,¶*	1.64†
IIxb	3	70.79§,¶	2.62†	41.83‡,§*	1.76†
IIb	3	66.42¶,††	2.46†	41.08‡,§*	1.75†
Human					
I	6	76.22‡,§	2.81†	54.30†*	2.09†
IIa	9	58.85‡‡	2.22†	40.96c*	1.74†
IIax	3	45.85§§	1.87†	37.44§,¶*	1.67†
Horse					
I	5	47.72§§	1.91†	52.78†	2.05†
II a	3	58.10‡‡	2.20†	48.60†,‡*	1.93†
IIax	4	57.28‡‡	2.18†	37.17§,¶*	1.66†
IIx	3	56.19‡‡	2.14†	32.41¶*	1.55†
Rhinoceros					
I	4	85.58†	3.18†	51.03†*	2.00†
II a	3	59.91††,‡‡	2.26†	37.70§,¶*	1.67†

Source of Variation	DF	SS	MS	*F*	*P*
MyHC isoform	13	5342	411	16	<0.001
E_a and Q_{10}	3	152490	50830	1985	<0.001
MyHC isoform × E_a and Q_{10}	39	7852	201	8	<0.001

Values are means. The activation energy, E_a (kJ mol^{-1}) and temperature coefficient, Q_{10}, were calculated according to Ranatunga (1984). Main effect MyHC (14 levels) $P < 0.001$; main effect E_a and Q_{10} (four levels) $P < 0.001$; interaction $P < 0.001$. Two-way ANOVA was used to test the differences in E_a and Q_{10} before and after 25 °C.

Superscripts denote significant statistical differences in E_a or Q_{10} between fibres expressing different MyHC isoforms in the four species. A similar superscript beside two values in each column indicates that there is no significant difference in E_a or Q_{10} between the two fibres.

*significant differences in E_a between temperatures below and above 25 °C ($P < 0.05$). There is no difference in Q_{10} between temperatures before and after 25 °C ($P < 0.05$).

statistical significance based on femur length, the type IIa fibres at 15 and 25 °C both had the reverse relationship of the hypothesized scaling relationship than was expected by Hill's predictions, with an increase in *in vitro* motility speed as the animals' femur length increased.

Discussion

In previous studies, our group has explored the effect of scaling on skeletal muscle shortening velocity at the single-muscle fibre level and at the molecular level using a single fibre *in vitro* motility assay (Hook *et al.* 2001, Marx *et al.* 2006). In these studies, we advanced the hypotheses of Hill by finding that limb length is a more critical factor than body mass in determining shortening velocity and that the scaling effect extended to the molecular level, i.e. in species ranging in size from mouse to humans. The current study advances this by confirming the differences between rat and human myosin ability to propel actin at physiological temperatures and that if large animals were included in the analysis, the anticipated scaling effects in *in vitro* motility speed no longer exists. The two previous studies that examined the effect of scaling in *in vitro* motility speed both supported the idea that myosin would scale in parallel with cellular muscle shortening velocity (Hook *et al.* 2001, Pellegrino *et al.* 2003). The main limitation of these studies was that they did not extend into animals larger than humans. However, this would not be expected to be a significant limitation based on the single fibre shortening velocity preparations, often including larger species and consistently showing a strong scaling effect (Rome *et al.* 1990, Seow & Ford 1991, Marx *et al.* 2006), especially in the slow-twitch fibres. In all species and at all temperatures, the

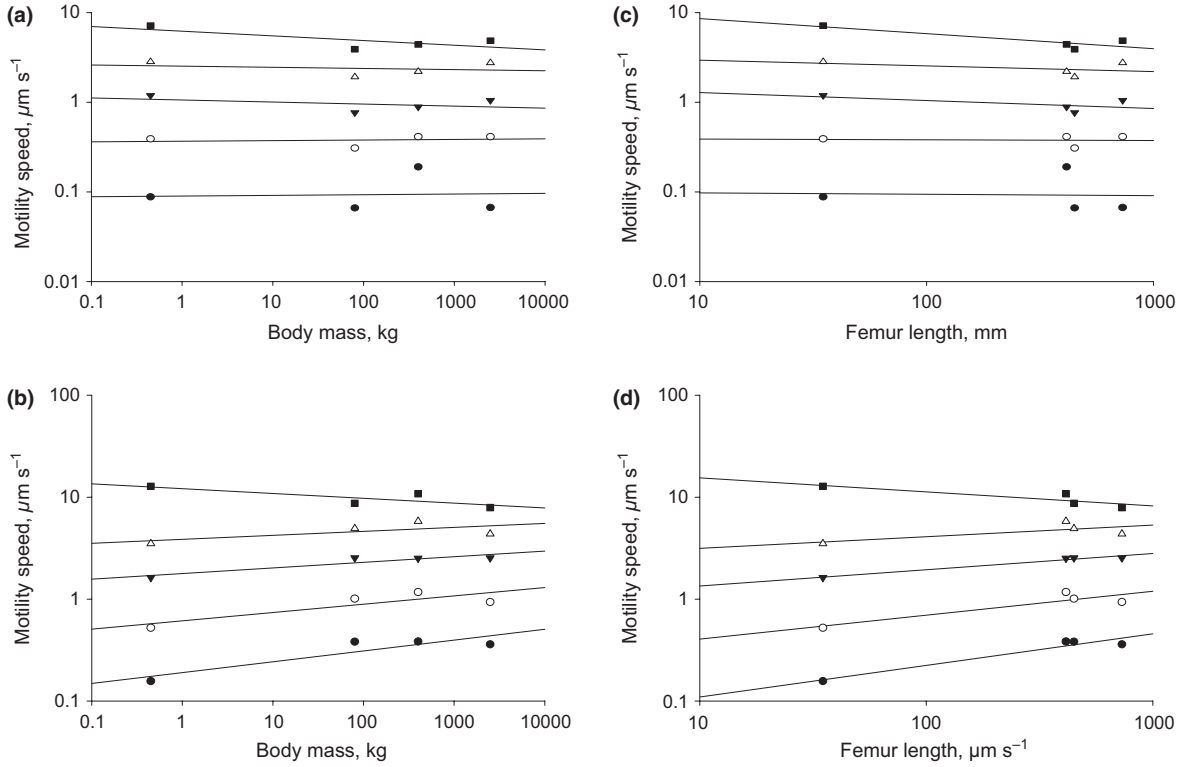

Figure 2 Log–log plots of motility speed vs. body mass in muscle fibres expressing the type I (a) and type IIa (b) MyHC isoform at different temperatures. Symbols in (a and b) denote rats, humans, horses, and rhinoceros from the left to the right. Log-log plots of motility speed vs. femur length in muscle fibres expressing the type I (c) and type IIa (d) MyHC isoform at different temperatures. Symbols in (c and d) denote rats, horses, humans, and rhinoceros from the left to the right. In all graphs, filled circles (●), open circles (○), filled triangles (▼), open triangles (△) and filled squires (■) correspond to motility speeds at temperatures 15 °C, 20 °C, 25 °C, 30 °C and 35 °C respectively.

in vitro motility speed clearly followed the predicted order of type I < IIa < IIx < IIb. However, in the current study, no significant scaling effect was noted for type I fibres at any temperature (Fig. 2) and the only scaling effect was observed in type IIa fibre preparations at 15 and 25 °C, but it was reversed from the expected relationship, with motility speed increasing as the size of the animal increased. The biological significance of this very weak and inconsistent scaling effect remains uncertain. Nevertheless, it is interesting to note that Toniolo *et al.* 2004 reported that type IIa muscle fibres from 140 to 160 kg pigs had faster *in vitro* motility speeds (0.99 ± 0.10 μm s^{-1}) than previously reported in humans (0.841 ± 0.034 μm s^{-1}, Pellegrino *et al.* 2003) using identical *in vitro* motility assays, but different from the assay used in the current study.

The anticipated scaling relationship at the molecular level, between orthologous MyHC isoforms did not prove true, probably because of the modulatory effects of other factors. Myosin light chain isoforms (MyLCs) have been reported to modulate shortening velocity within muscle fibres expressing the same MyHC isoform in both skinned single fibre preparations (Sweeney *et al.* 1986, 1988, Greaser *et al.* 1988, Reiser *et al.* 1988, Bottinelli *et al.* 1994a) and in *in vitro* motility assays (Lowey *et al.* 1993). In previous studies, we have confirmed the modulatory influence of MyLCs on shortening velocity at the single muscle level in young rats, but not in old rats or in human muscle fibres (Larsson & Moss 1993, Li & Larsson 1996). However, it cannot be excluded that the lack of a modulatory influence of MyLCs on shortening velocity is secondary to a deficient precision and resolution by single-dimension SDS-PAGE to separate all MyLC isoforms (Sugiura *et al.* 1992). Bicer & Reiser (2007) reported a body mass-related influence on the apparent molecular mass in fast-type essential MyLCs, especially MyLC1F, among 19 species with a approx. 500 000-fold difference in body mass (from shrew to elephant), i.e. an increase in MyLC1F molecular mass was observed in mammals with a larger body mass, particularly in the number alanine-proline repeats in the actin-binding domain of MyLC1F that may modulate cross-bridge kinetics. However, MyLCs are extracted together with MyHCs in the single fibre *in vitro* motility assay and they are expressed in a similar stoichiometric

Table 4 Linear regression parameters of motility speed vs. body weight or femur length at various temperatures

MyHC	Coefficients	15 °C	20 °C	25 °C	30 °C	35 °C
Body mass vs. motility speed						
I	*b*[0]	−1.04657	−0.43509	0.024223	0.402755	0.792457
	b[1]	0.007442	0.006721	−0.02261	−0.01292	−0.05222
	r^2	0.003102	0.032409	0.190033	0.065529	0.54804
	r	0.0557	0.18	−0.436	−0.256	−0.74
	P-value	0.944	0.82	0.564	0.744	0.26
IIa	*b*[0]	−0.72157	−0.21307	0.250962	0.586446	1.083377
	b[1]	0.106328	0.081634	0.055109	0.038919	−0.04721
	r^2	0.812949	0.731873	0.857603	0.46049	0.662561
	r	0.902	0.855	0.926	0.679	−0.814
	P-value	0.0984	0.145	0.0739	0.321	0.186
Femur length vs. motility speed						
I	*b*[0]	−0.99724	−0.40357	0.196685	0.532268	1.099772
	b[1]	−0.01458	−0.00778	−0.08894	−0.06362	−0.1678
	r^2	0.001617	0.005903	0.399397	0.215693	0.768394
	r	−0.0402	−0.0768	−0.632	−0.464	−0.877
	P-value	0.96	0.923	0.368	0.536	0.123
IIa	*b*[0]	−1.27071	−0.62724	−0.02971	0.384121	1.327052
	b[1]	0.310008	0.234938	0.159046	0.114019	−0.13759
	r^2	0.938422	0.823159	0.96999	0.536706	0.764099
	r	0.969	0.907	0.985	0.733	−0.874
	P-value	0.0313	0.0927	0.0151	0.267	0.126

The equation $y = ax-b$ was used to describe the relationship between motility speed in fibres expressing different MyHC isoforms. The exponent of the equation, *b*, was the scaling factor or allometric coefficient. *b*[0], *b*[1] and r^2 were obtained through linear regression on log-transformed data. *r* and *P*-value were obtained through linear correlation on log-transformed data and *b*[1] denotes the exponent of the equation, *b*.

relationship as in the muscle cell (Hook *et al.* 1999) and other factors than MyHCs and MyLCs may account for the scaling effect at the single muscle fibre level in larger mammals. Mutations and altered isoform expression in the regulatory proteins troponin and tropomyosin have been shown to significantly affect the shortening velocity at the single muscle fibre level and *in vitro* motility speed, independent on myosin structure and function (Sweeney *et al.* 1998, Clemmens *et al.* 2005, Ochala *et al.* 2007). Differences between species in the coordinated expression or mutations of thick and thin filament proteins, affecting single fibre contractility measurements, may account for the unexpected loss of a scaling effect within MyHC isoforms between species observed at the single muscle fibre level but not in this *in vitro* motility assay, using unregulated thin filaments. Further, scaling-related changes in myofilament lattice spacing cannot be ruled out as a mechanism underlying the slowing of contractile speed in large mammals.

Previous studies on scaling at the single fibre and motor protein levels have been confined to measurements at unphysiological low temperatures. Measuring the effects of different temperatures in the same single muscle fibre *in vitro* motility assay imposed a significant challenge and is also the cause for the relatively low number of preparations fulfilling the criteria for acceptance in the various species and muscle fibre types. In accordance with previous studies, the motility speed of all the species demonstrated a high temperature dependence (Rossi *et al.* 2005, Li *et al.* 2006), i.e. motility speed increased significantly with temperature irrespective MyHC isoform expression in the four species. However, the relative and frequently absolute differences in motility speed between different MyHC isoforms decreased with increasing temperature in accordance with previous studies in rat, rabbit and humans (Rossi *et al.* 2005, Li *et al.* 2006).

E_a and Q_{10} characterize the temperature sensitivity of the different MyHC isoforms and a transition point occurred at 25 °C in all MyHC isoforms in the rat and rhinoceros as well as in horse IIx and IIax MyHC. This transition point was, on the other hand, not observed in human muscle fibres indicating a species-related temperature dependent difference in actomyosin interactions (Lionikas *et al.* 2006). It has been suggested that the differences in temperature sensitivity of skeletal muscle contractile properties contribute to differences in the temperature range at which muscle work under physiological conditions and the capacity to regulate body temperature (Bottinelli *et al.* 1996). This is further

supported by the differences in temperature sensitivity between amphibians, small animals and humans (Bennett 1984, Ranatunga 1984). The best fit was accordingly made with a single linear regression line and the same trend was observed in horse type I and IIa MyHC preparations. The primary interest of the current study was to compare the differences in temperature sensitivity among species, E_a and Q_{10} were calculated separately before and after 25 °C in all four species. Both E_a and Q_{10} values in fibres expressing the slow MyHC isoform were higher than in fibres expressing fast MyHC isoforms, except in horse fibres suggesting a species difference in MyHC isoform temperature sensitivity. The E_a and Q_{10} in the 10–25 °C range were higher than those in the 25–35 °C range indicating a change in the catalytic activity at 25 °C. This is in accordance with the previous observations at the whole muscle, single fibre level as well as in biochemical studies at the actomyosin ATPase level (Ranatunga 1984, Ranatunga *et al.* 1987, Bottinelli *et al.* 1994b, 1996, Stienen *et al.* 1996). The variation in the E_a and Q_{10} within the fibres expressing the same MyHC isoform illustrated different temperature sensitivity between species.

In conclusion, scaling effects at the motor protein level in mammals covering a 5 500-fold difference in body mass indicate more complex mechanisms from studies derived from smaller mammals. That is, there was no significant scaling effect noted in skeletal muscle function at the molecular level in type I fibres, and at some temperatures, a reverse of the anticipated scaling effect was noted in type IIa fibres. These findings indicate that other factors than the structure and function of the motor protein myosin are responsible for the scaling effect in large mammals. A strong impact of temperature on myosin function was observed in all species investigated, but the effects were species and MyHC isoform specific.

Conflict of interest

The authors report no conflicts of interest.

We would like to thank Dr Robert Gilmour and 'the Wilds' Zoo in Cumberland, Ohio for their role in providing muscle tissue for this investigation and Dr John Hermanson for providing bones for analysis. This study was supported by grants from the NIAMS (AR45627 and AR47318) and the Swedish Research Council (8651) to L. Larsson. We are grateful to Yvette Hedström and Ann-Marie Gustafson for the gel electrophoresis analyses. We thank the human subjects for providing human muscle tissue, making this work possible.

References

Bennett, A.F. 1984. Thermal dependence of muscle function. *Am J Physiol* **247**, R217–R229.

Bicer, S. & Reiser, P.J. 2007. Variations in apparent mass of mammalian fast-type myosin light chains correlate with species body size, from shrew to elephant. *Am J Physiol Regul Integr Comp Physiol* **292**, R527–R534.

Bottinelli, R., Canepari, M., Reggiani, C. & Stienen, G.J. 1994a. Myofibrillar ATPase activity during isometric contraction and isomyosin composition in rat single skinned muscle fibres. *J Physiol (Lond)* **481**, 663–675.

Bottinelli, R., Canepari, M., Reggiani, C. & Stienen, G.J. 1994b. Myofibrillar ATPase activity during isometric contraction and isomyosin composition in rat single skinned muscle fibres. *J Physiol* **481**(Pt 3), 663–675.

Bottinelli, R., Canepari, M., Pellegrino, M.A. & Reggiani, C. 1996. Force-velocity properties of human skeletal muscle fibres: myosin heavy chain isoform and temperature dependence. *J Physiol* **495**(Pt 2), 573–586.

Christiansen, P. 1999. Scaling of the limb long bones to body mass in terrestrial mammals. *J Morphol* **239**, 167–190.

Clemmens, E.W., Entezari, M., Martyn, D.A. & Regnier, M. 2005. Different effects of cardiac vs. skeletal muscle regulatory proteins on *in vitro* measures of actin filament speed and force. *J Physiol* **566**, 737–746.

Close, R. & Hoh, J.F. 1968. Influence of temperature on isometric contractions of rat skeletal muscles. *Nature* **217**, 1179–1180.

Frontera, W.R., Grimby, L. & Larsson, L. 1997. Firing rate of the lower motoneuron and contractile properties of its muscle fibers after upper motoneuron lesion in man. *Muscle Nerve* **20**, 938–947.

Greaser, M.L., Moss, R.L. & Reiser, P.J. 1988. Variations in contractile properties of rabbit single muscle fibres in relation to troponin T isoforms and myosin light chains. *J Physiol (Lond)* **406**, 85–98.

Hill, A. 1950. The dimensions of animals and their muscular dynamics. *Sci Prog* **38**, 209–230.

Hill, D.K. 1972. Resting tension and the form of the twitch of rat skeletal muscle at low temperature. *J Physiol* **221**, 161–171.

Hook, P. & Larsson, L. 2000. Actomyosin interactions in a novel single fiber *in vitro* motility assay. *J Muscle Res Cell Motil* **21**, 357–365.

Hook, P., Li, X., Sleep, J., Hughes, S. & Larsson, L. 1999. *In vitro* motility speed of slow myosin extracted from single soleus fibres from young and old rats. *J Physiol (Lond)* **520**(Pt 2), 463–471.

Hook, P., Sriramoju, V. & Larsson, L. 2001. Effects of aging on actin sliding speed on myosin from single mouse, rat and human skeletal muscle cells. *Am J Physiol Cell Physiol* **280**, C782–C788.

Iriarte-Diaz, J. 2002. Differential scaling of locomotor performance in small and large terrestrial mammals. *J Exp Biol* **205**, 2897–2908.

Kawai, M. 2003. What do we learn by studying the temperature effect on isometric tension and tension transients in mammalian striated muscle fibres? *J Muscle Res Cell Motil* **24**, 127–138.

Knight, B. 1996. *Forensic Pathology*. Arnold, Landon.

Larsson, L. & Moss, R.L. 1993. Maximum velocity of shortening in relation to myosin isoform composition in single fibres from human skeletal muscles. *J Physiol* **472**, 595–614.

Li, X. & Larsson, L. 1996. Maximum shortening velocity and myosin isoforms in single muscle fibers from young and old rats. *Am J Physiol* **270**, C352–C360.

Li, M., Lionikas, A., Yu, F., Tajsharghi, H., Oldfors, A. & Larsson, L. 2006. Muscle cell and motor protein function in patients with a IIa myosin missense mutation (Glu-706 to Lys). *Neuromuscul Disord* **16**, 782–791.

Lionikas, A., Li, M. & Larsson, L. 2006. Human skeletal muscle myosin function at physiological and non-physiological temperatures. *Acta Physiol (Oxf)* **186**, 151–158.

Lowey, S., Waller, G.S. & Trybus, K.M. 1993. Skeletal muscle myosin light chains are essential for physiological speeds of shortening. *Nature* **365**, 454–456.

Marx, J.O., Olsson, M.C. & Larsson, L. 2006. Scaling of skeletal muscle shortening velocity in mammals representing a 100 000-fold difference in body size. *Pflugers Arch* **452**, 222–230. Epub 2005 Dec 2007.

Ochala, J., Li, M., Tajsharghi, H., Kimber, E., Tulinius, M., Oldfors, A. & Larsson, L. 2007. Effects of a R133W beta-tropomyosin mutation on regulation of muscle contraction in single human muscle fibres. *J Physiol* **581**, 1283–1292. Epub 2007 Apr 1212.

Pardee, J.D. & Spudich, J.A. 1982. Purification of muscle actin. *Methods Enzymol* **85**(Pt B), 164–181.

Pellegrino, M.A., Canepari, M., Rossi, R., D'Antona, G., Reggiani, C. & Bottinelli, R. 2003. Orthologous myosin isoforms and scaling of shortening velocity with body size in mouse, rat, rabbit and human muscles. *J Physiol* **546**, 677–689.

Ranatunga, K.W. 1984. The force-velocity relation of rat fast- and slow-twitch muscles examined at different temperatures. *J Physiol* **351**, 517–529.

Ranatunga, K.W., Sharpe, B. & Turnbull, B. 1987. Contractions of a human skeletal muscle at different temperatures. *J Physiol* **390**, 383–395.

Reiser, P.J., Greaser, M.L. & Moss, R.L. 1988. Myosin heavy chain composition of single cells from avian slow skeletal muscle is strongly correlated with velocity of shortening during development. *Dev Biol* **129**, 400–407.

Rome, L.C. 1992. Scaling of muscle fibres and locomotion. *J Exp Biol* **168**, 243–252.

Rome, L.C., Sosnicki, A.A. & Goble, D.O. 1990. Maximum velocity of shortening of three fibre types from horse soleus muscle: implications for scaling with body size. *J Physiol (Lond)* **431**, 173–185.

Rossi, R., Maffei, M., Bottinelli, R. & Canepari, M. 2005. Temperature dependence of speed of actin filaments propelled by slow and fast skeletal myosin isoforms. *J Appl Physiol* **99**, 2239–2245.

Seow, C.Y. & Ford, L.E. 1991. Shortening velocity and power output of skinned muscle fibers from mammals having a 25 000-fold range of body mass. *J Gen Physiol* **97**, 541–560.

Stienen, G.J., Kiers, J.L., Bottinelli, R. & Reggiani, C. 1996. Myofibrillar ATPase activity in skinned human skeletal muscle fibres: fibre type and temperature dependence. *J Physiol* **493**(Pt 2), 299–307.

Sugiura, T., Matoba, H., Miyata, H., Kawai, Y. & Murakami, N. 1992. Myosin heavy chain isoform transition in ageing fast and slow muscles of the rat. *Acta Physiol Scand* **144**, 419–423.

Sweeney, H.L., Kushmerick, M.J., Mabuchi, K., Gergely, J. & Sreter, F.A. 1986. Velocity of shortening and myosin isozymes in two types of rabbit fast-twitch muscle fibers. *Am J Physiol* **251**, C431–C434.

Sweeney, H.L., Kushmerick, M.J., Mabuchi, K., Sreter, F.A. & Gergely, J. 1988. Myosin alkali light chain and heavy chain variations correlate with altered shortening velocity of isolated skeletal muscle fibers. *J Biol Chem* **263**, 9034–9039.

Sweeney, H.L., Feng, H.S., Yang, Z. & Watkins, H. 1998. Functional analyses of troponin T mutations that cause hypertrophic cardiomyopathy: insights into disease pathogenesis and troponin function. *Proc Natl Acad Sci USA* **95**, 14406–14410.

Toniolo, L., Patruno, M., Maccatrozzo, L., Pellegrino, M.A., Canepari, M., Rossi, R., D'Antona, G., Bottinelli, R., Reggiani, C. & Mascarello, F. 2004. Fast fibres in a large animal: fibre types, contractile properties and myosin expression in pig skeletal muscles. *J Exp Biol* **207**, 1875–1886.

Walker, S.M. 1951. Failure of potentiation in successive, post-tetanic, and summated twitches in cooled skeletal muscle of the rat. *Am J Physiol* **166**, 480–484.

Wang, G. & Kawai, M. 2001. Effect of temperature on elementary steps of the cross-bridge cycle in rabbit soleus slow-twitch muscle fibres. *J Physiol* **531**, 219–234.

Widrick, J.J., Romatowski, J.G., Karhanek, M. & Fitts, R.H. 1997. Contractile properties of rat, rhesus monkey, and human type I muscle fibers. *Am J Physiol* **272**, R34–R42.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Electrophoretic separation (6% SDS-PAGE) of type I, IIa and IIx MyHC isoforms from rhinoceros (R) and human (H) muscle biopsy cross-sections.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.