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RESEARCH ARTICLE

Energy turnover in mammalian skeletal muscle in contractions mimicking locomotion: effects of stimulus pattern on work, impulse and energetic cost and efficiency

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ABSTRACT

Active muscle performs various mechanical functions during locomotion: work output during shortening, work absorption when resisting (but not preventing) lengthening, and impulse (force–time integral) whenever there is active force. The energetic costs of these functions are important components in the energy budget during locomotion. We investigated how the pattern of stimulation and movement affects the mechanics and energetics of muscle fibre bundles isolated from wild rabbits (*Oryctolagus cuniculus*). The fibres were from muscles consisting of mainly fast-twitch, type 2 fibres. Fibre length was held constant (isometric) or a sinusoidal pattern of movement was imposed at a frequency similar to the stride frequency of running wild rabbits. Duty cycle (stimulation duration×movement frequency) and phase (timing of stimulation relative to movement) were varied. Work and impulse were measured as well as energy produced as heat. The sum of net work (work output–work input) and heat was taken as a measure of energetic cost. Maximum work output was produced with a long duty cycle and stimulation starting slightly before shortening, and was produced quite efficiently. However, efficiency was even higher with other stimulation patterns that produced less work. The highest impulse (considerably higher than isometric impulse) was produced when stimulation started while the muscle fibres were being lengthened. High impulse was produced very economically because of the low cost of producing force during lengthening. Thus, locomotion demanding high work, high impulse or economical work output or impulse requires a distinct pattern of stimulation and movement.

KEY WORDS: Energy, Heat, Cost of transport, Performance, Rabbit

INTRODUCTION

Locomotion claims a substantial proportion of the energy budget of many animals. The energy required to move a 1 kg body mass a unit distance (cost of transport, COT) is remarkably independent of speed and gait but is highly dependent on stride length and leg length/body size, with large animals having a lower COT (Taylor et al., 1982; Nudds et al., 2009). All direct energy use for locomotion is via the locomotor muscles (ignoring ancillary costs like ventilation and circulation of blood). Thus, evolutionary pressure has probably resulted in economical operation of these muscles over a wide range of speeds and gaits. For example, a racehorse will walk at 0.9 m s^{-1} with a stride frequency of 0.5 Hz and a limb stance time of 600 ms. It can also move 20 times faster at 18 m s^{-1} with a similar COT but a stance time of only 80 ms and a stride frequency of 2.4 Hz (Witte et al., 2004). The arrangement of the musculo-skeletal system is likely to contribute to this versatility of function (Wilson and Lichtwark, 2011) whilst maintaining efficient conversion of metabolic energy to mechanical work.

In a moving animal, a muscle is subjected to a length change once per stride and it experiences a tensile force. The interplay between force, length and the rate of change of length is defined by the muscle's force versus velocity curve and the timing and duration of muscle activation. If a muscle is shortening when under load, it will perform mechanical work on the animal; if it is lengthening, it will absorb mechanical work; and if it is isometric, there will be no net work.

Different muscles operate under all these conditions during locomotion depending on the role they are playing, and muscle tissue must be able to meet all these demands. Some optimisation is due to muscles containing fibres of different length, cross-sectional area and distribution of fibre types, with, for instance, force-generating postural muscles having short fibres and muscles that produce rapid movement having long fibres. Optimisation of structure has been widely explored through experimental measurement and modelling in a variety of locomotor systems (Carrier et al., 2011). In addition, motor unit recruitment patterns and the timing and duration of activation vary with the demands on the muscle. Whilst mathematical modelling can provide dynamic predictions for movement that result from activation patterns (or vice versa), knowledge of how much energy is used by the muscles is based on limited data. Model outputs that aim to generalise the determinants of economical locomotion across species are dependent on measurements on muscle from a range of animal sizes and lifestyles. Here, we directly measured heat production and mechanical work production (and absorption) by skeletal muscle from wild rabbits. We explored a wide range of activation conditions to define optimum conditions in terms of work, impulse and associated costs and, importantly, the energetic and performance implications of a muscle operating under suboptimum activation conditions. This is critical for understanding the diversity of muscle function under varying demands of locomotion.

MATERIALS AND METHODS

Wild rabbits (*Oryctolagus cuniculus* Linnaeus) were live-trapped on the Hawkshead Campus of the Royal Veterinary College with ethical approval from the Royal Veterinary College Ethics

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Committee (RVC 2012 1183). They were killed according to Schedule 1 (revised 1997) of the Animals (Scientific Procedures) Act 1986 (UK). Experiments were done on muscle fibre bundles dissected from extensor digiti-V (ED-V) and peroneus longus (PL). These two muscles are in the distal part of the hindlimb and are representative of most limb muscles in that they consist almost entirely of type 2, fast-twitch fibres (see Results).

The fibre typing and the energetics experiments were done on muscles from different rabbits from the same wild population.

Fibre typing using immunohistochemical staining

A small piece of muscle was snap frozen in isopentane cooled in liquid nitrogen. Sections were cut (8 µm thick) from frozen samples using a cryostat and mounted on glass slides. Serial sections were collected on four slides and allowed to dry at room temperature for 30 min before storage at −80°C.

The slides were allowed to reach room temperature and the sections were then circled with a hydrophobic PAP pen (H4000, Vector Laboratories Ltd, Peterborough, UK). The sections were fixed in 1:1 methanol:acetone at −20°C for 15 min then washed in phosphate-buffered saline pH 7.4 (PBS, product number 10209252, Fisher Scientific, Loughborough, UK), with three changes in 10 min.

Goat anti-collagen V antibody (product number 135001, Bio-Rad Laboratories Ltd, Watford, UK; 1:20 dilution) was incubated with the sections for 1 h at room temperature before washing with PBS, three changes in 10 min, followed by donkey anti-goat AlexaFluor 488-conjugated secondary antibody (A-11055, Invitrogen, Bracknell, UK; 1:1000 dilution) for 1 h at room temperature.

After another wash in PBS, the sections were incubated with one of the following four anti-myosin heavy chain (MHC) antibodies: type 2A [A4.74, Developmental Studies Hybridoma Bank (DSHB), University of Iowa, East Iowa City, IA, USA; 1:6 dilution) overnight at 4°C; type 2B (10F5, DSHB; 1:20 dilution) overnight at 4°C; type 2X (6H1, DSHB; 1:20 dilution) for 1 h at room temperature; type 1 (MAB1628, Millipore UK Ltd, Watford, UK; 1:50 dilution) for 1 h at room temperature. After the MHC antibody treatment, the sections were washed with PBS, three changes in 10 min.

The sections were then incubated with the relevant secondary antibody diluted 1:1000 for 1 h at room temperature: AlexaFluor 594-conjugated goat anti-mouse IgG (A-21125, Invitrogen) for type 2A, AlexaFluor 594-conjugated goat anti-mouse IgM (A-21044, Invitrogen) for type 2B, AlexaFluor 594-conjugated goat antimouse IgM for type 2X and AlexaFluor 594-conjugated goat anti-mouse IgG for type 1.

After being washed with PBS, three changes in 10 min, the sections were cover-slipped with Vectashield DAPI (H1200, Vector Laboratories Ltd) to stain the nuclei before being examined with a Leica DM4000 fluorescence microscope fitted with TX2 (594 nm), L5 (488 nm) and A (350 nm) filter sets and using a 10× objective. The L5 filter was used for the collagen image and the TX2 filter for the muscle fibre type image. The images were captured using Axiovision LE software (Zeiss) and exported in .Zvi format to Volocity software (PerkinElmer) for background correction and conversion into grey-level .tiff format. Background correction used blank images captured with the same objective for each filter block used. QWin Quips image analysis software (Leica Microsystems) macro was then used to count the fibres and measure the minimum Feret diameter for each fibre. The system was calibrated for the microscope and ×10 objective (1 pixel=1.01 µm). The collagen grey-level image was used to outline the periphery of each individual fibre and the intensity of pixels from the fibre was used to determine whether a particular MHC immunofluorescence was positive.

Energetic experiments

Experiments were done on eight fibre bundles from n=7 wild rabbits (3 female, 4 male; mass range 1.164–1.595 kg, mean±s.e.m. 1.414±0.051 kg; two of the fibre bundles were from one rabbit). Three fibre bundles from ED-V and five from PL muscles were used. As the number of muscles was so small, comparison of the two muscles was not informative; we combined the results for ED-V and PL based on an earlier study (Curtin et al., 2015) of a larger group of ED-V and PL muscles from the same population of wild rabbits, which showed that their mechanical properties were similar. Experiments were performed at 25°C. The composition of the saline was (mmol l^{-1}): NaCl 135, KCl 4.0, $CaCl_2$ 2.35, $MgCl_2$ 0.85, NaH_2PO_4 1, $NaHCO_3$ 20 and glucose 5.5, equilibrated with 95% O_2+5% CO_2. Small bundles of fibres were dissected from the muscle, and a fragment of tendon at each end of the fibre bundle was held in a platinum foil clip, which served as a stimulus electrode and as the attachment to the motor and force transducer (Series 300B Lever System and Series 400A Force Transducer System, Cambridge Technology, Inc., Watertown, MA, USA). The fibre bundle was stimulated electrically (Isolated Stimulator Model DS2, Digitimer Ltd, Welwyn Garden City, Herts, UK). Muscle temperature was measured by a custom-made thermopile consisting of constantan–chromel thermocouples (Seebeck coefficient 42.3 µV K^{-1} per thermocouple). The output from each 1 mm section, containing four couples, was recorded separately. A LabView program controlled the stimulator and motor, and recorded force, motor position and the output from the thermopile sections.

Supra-maximal stimulus strength (V at 80 Hz, 2 ms pulse^{-1}) and L_o, the fibre length giving maximum active force, were found for each fibre bundle at the start of the experiment. In the main protocol (Fig. 1A), the fibre bundle was subjected to sinusoidal movement of 1.5 mm peak-to-peak amplitude, which was on average about 12% L_o, at a frequency of 1 or 2 Hz. The lower frequency was used for two of the eight fibre bundles to allow enough time for active force to relax completely to the baseline before the next cycle began. Stimulation consisted of three tetani lasting 0.1, 0.2, 0.3 or 0.4 of the movement cycle duration, referred to as duty cycle (DC) 0.1, etc. (Fig. 1B). The stimulation phase, or timing of the tetanus within the movement cycle, was varied in steps of 0.1 of the movement cycle through the whole range of possible phases; that is, between 0 (stimulation starts when shortening starts) and −0.1 (Fig. 1C). Passive force was recorded during sinusoidal movement without stimulation and was subtracted from that with stimulation to give the active force. Standard isometric contractions were performed at regular intervals. The duration of stimulation in these standard contractions corresponded to 0.4 of the movement cycle duration for the fibre bundle (Fig. 1D).

Work was calculated as the integral of active force and movement. Impulse, defined as the integral of active force and time, was used as a quantitative measure of the muscle's production of active force in a given period of time. *In vivo* active muscle produces impulse while performing all its functions: holding a position (isometric, force without length change), motor (force during muscle shortening), resisting but not preventing lengthening (force while being stretched). Heat production was evaluated from the thermopile output using the heat capacity of the fibre bundle and the time constant for heat loss, which were evaluated by the Peltier method (Kretzschmar and Wilkie, 1975). Stimulus heat was measured after the muscle fibres had been made unexcitable with procaine (30 mmol l^{-1}). The heat values are reported net of stimulus heat.

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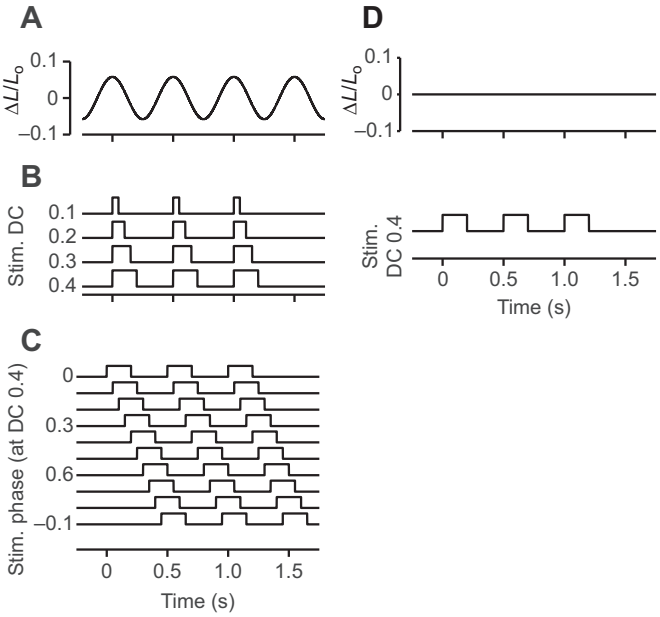

Fig. 1. Diagram of the pattern of movement and stimulus patterns imposed on the fibre bundle. (A) Sinusoidal movement at 2 Hz. $\Delta L/L_o$ is fibre length change from its initial value, expressed relative to L_o, the fibre length that gave maximum isometric force. (B) One tetanic stimulation per movement cycle. Four different tetanus durations were used: 0.1, 0.2, 0.3 or 0.4 of the movement cycle duration. Stimulation duty cycle (DC) is the tetanus duration per movement cycle duration. (C) Tetanic stimulation was given at 10 different times relative to the movement. Stimulation phase is the time of the first stimulus relative to the start of shortening. (D) Pattern of length and stimulation used during isometric contractions.

At the end of the experiment, the fibre bundle was photographed on the thermopile at L_o and the clip-to-clip length was measured from the image. The fibre bundle was pinned at L_o in the Sylgard-lined Petri dish and fixed in ethanol. The fibre length was measured under the stereomicroscope (mean±s.e.m. 12.8±1.1 mm, n=8). The bundle was rinsed and returned to saline; clips and tendon were removed and the blotted mass measured (mean±s.e.m. 10.51±1.43 mg, n=8). Cross-sectional area was calculated from mass and fibre length, assuming a density of 1 mg mm^{-3} (mean±s.e.m. 0.875±0.179 mm^2, n=8).

Units

Force, impulse, work and heat are all extensive properties that depend on the amount of contracting material and its contractility. To take account of variations in the contractility of a fibre bundle within an experiment, the measurements made in a set of three contractions (Fig. 1) were divided by a factor, k, which is specific to that set of contractions. The value of k was calculated by linear interpolation (for simplicity) on the basis of the set's number between the isometric values before and after that set. The isometric values were calculated as follows:

$$k_{IS} = \frac{\left(\frac{f}{\bar{F}} + \frac{h}{\bar{H}} + \frac{i}{\bar{I}}\right)}{3}, \quad (1)$$

where f, h and i are the peak force, heat and impulse values for an isometric set (IS), and $\bar{F}$, $\bar{H}$ and $\bar{I}$ are the averages of all the isometric sets for that fibre bundle.

A similar method was used to normalize for the variation in contractility of the eight different fibre bundles we used. The measurements made in all the sets of contractions by a particular fibre bundle were divided by a factor, R_{FB}, specific to that fibre bundle (FB):

$$R_{FB} = \frac{\left(\frac{\bar{f}}{\bar{F}} + \frac{\bar{h}}{\bar{H}} + \frac{\bar{i}}{\bar{I}}\right)}{3}, \quad (2)$$

where $\bar{f}$, $\bar{h}$ and $\bar{i}$ are the averages for all the isometric sets for that fibre bundle, and $\bar{F}$, $\bar{H}$ and $\bar{I}$ are the averages for all the fibre bundles.

Values shown in Tables S1–S4 and in the figures are values normalized for variation in contractility and expressed relative to the appropriate aspect of muscle size. Isometric force and impulse are expressed relative to cross-sectional area, where area=blotted mass/L_o, assuming a density of 1 mg blotted mass per mm^3. All values of work (positive, negative and net) and heat are expressed relative to blotted muscle mass.

We measured the work, heat and impulse produced in three complete cycles of movement. The average value per cycle (total/3) is reported in the figures.

RESULTS

Fibre typing

Immunostaining of fibre bundles from ED-V and PL muscles of wild rabbits showed that they contained only a small proportion of type 1 (slow-twitch) fibres (Fig. 2). Among the 14 muscles tested, the maximum proportion of type 1 fibres was 0.095 and the mean±s.e.m. value was 0.057±0.006. All the subgroups of type 2 (fast-twitch) fibres were present, in the following proportions:

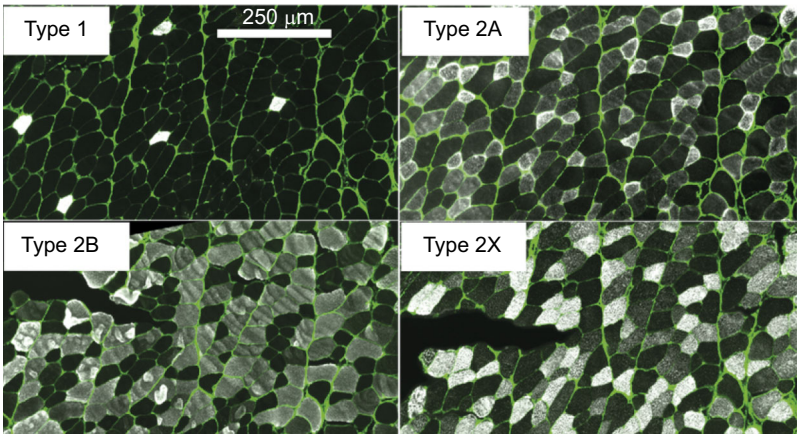

Fig. 2. Examples of immunohistochemically stained fibres. Sections were stained for myosin heavy chain isoforms 1, 2A, 2B and 2X. Immunostained fibres appear bright; collagen staining is green. All images are from the same region of the same muscle in serial sections.

type 2A, 0.432±0.038; type 2B, 0.440±0.043; type 2X, 0.374±0.052. These results are based on fibre counts. The number of fibres per section ranged from 196 to 1310, with a mean of 623.

Mechanical performance and cost

Both the duration of stimulation (duty cycle) and its timing relative to movement (phase) affected the mechanical performance and energetic cost of contractions. Fig. 3 shows the length change, stimulation, active force, cumulative impulse and energy produced (or absorbed) by a bundle of fibres from PL muscle during three cycles of movement at 2 Hz and a stimulus duty cycle 0.3 for two different stimulus phases, +0.1 and −0.4. In these two sets of records from the same fibre bundle, the direction of movement during stimulation was different, but the range of speeds was the same. Fig. 3A shows example records for stimulus phase +0.1 that gave net positive work (work done by the muscle on the motor). Fig. 3B shows the corresponding records for stimulus phase −0.4 that gave net negative work. In this case, the muscle produced force as it resisted being lengthened, and absorbed the work done on it by the motor. The peak active force is much higher when stimulation occurs during lengthening than during shortening. Impulse (integral of active force and time) and net work (integral of force and length change) show corresponding differences.

The sum of net work and heat production is the total energy output during the time period shown in Fig. 3. This output, or expenditure of energy, represents the immediate cost of producing the net work and impulse; it must be paid for by ATP hydrolysis (immediately coupled to the creatine kinase reaction). Subsequently, ATP regeneration/resynthesis will occur. Comparing records in Fig. 3A,B shows that when peak force is higher, impulse is also higher, whereas cost is similar.

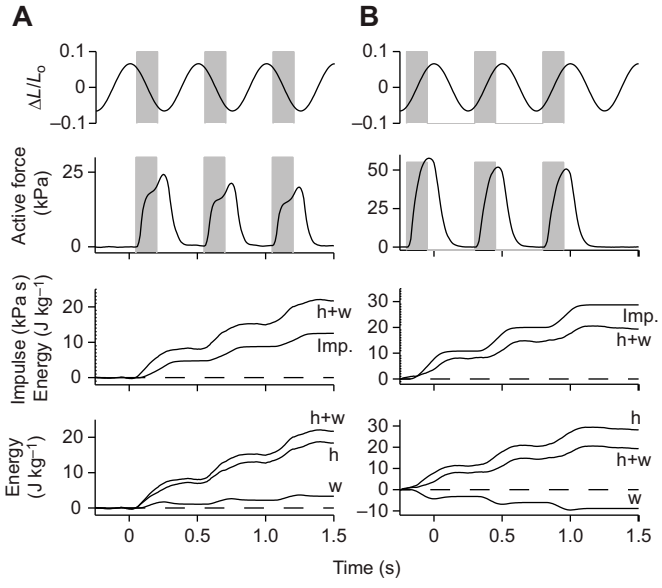

Fig. 3. Example recordings. Net positive work (A) and net negative work (B) for a peroneus longus (PL) fibre bundle (blotted mass, 13.37 mg; L_o, 11.5 mm). Three cycles of sinusoidal movement at 2 Hz and peak-to-peak amplitude 1.5 mm (=13.0% L_o), stimulus duty cycle 0.3 and stimulus phase +0.1 in A and −0.4 in B. $\Delta L/L_o$ is length change expressed as a fraction of L_o, the length giving maximum isometric force. Active force is expressed relative to cross-sectional area. The shading indicates the period of stimulation. Impulse is the integral of active force and time (kPa s). Three forms of energy (J kg^{-1} muscle fibre) are shown: h, cumulative heat output; w, cumulative net work, where net work is the sum of positive and negative work; and h+w, the cumulative sum of heat output and net work. h+w is a measure of cost and is included in the graph with impulse to ease comparison of the results shown in A and B.

The relationship of positive, negative and net work to stimulus phase and duty cycle is shown in Fig. 4A,B, which summarises the results for the eight fibre bundles. Fig. 4C shows the same values in contour graphs. At all duty cycles, the variation of work with stimulus phase was similar. The largest positive work was produced at stimulus phases −0.1, 0 or 0.1; that is, with stimulation and shortening starting at about the same time. Negative work was close to zero with these stimulus phases. The most negative work was produced at stimulus phases in the range 0.4–0.6 where stimulation and lengthening start at about the same time; positive work was close to zero at these stimulus phases. The net work reached its maximum positive value at stimulus phases that gave high positive work, and net work reached is maximum negative value at phases where negative work dominated. With increasing stimulus duty cycle, particularly at the low end of the range (0.1–0.2), the magnitude of the work increased. In addition, the peak positive and negative components of work shifted to somewhat more negative stimulus phases as the duty cycle increased, which shows as the slight southeast to northwest tilt of the ridge and valley in each of the contour graphs (Fig. 4C).

Fig. 5 shows how impulse, energetic cost and cost per unit impulse varied with stimulus phase and duty cycle. Note that the variation in net work with stimulus phase (Fig. 4B) was almost the mirror image of the variation in impulse (Fig. 5A): stimulus phases that gave high net positive work gave low impulse, and large net negative work was produced when impulse was high. The energetic cost generally increased with duty cycle. With the isometric condition ($\Delta L/L_o$=0), we used only one stimulation pattern: duration corresponding to a duty cycle of 0.4. The results are shown in the DC 0.4 graphs in Fig. 5. Isometric impulse was considerably less than the maximum value during movement, which was produced when stimulation occurred during most of the lengthening (phases near 0.5), but isometric impulse was greater than that during movement with stimulus phases in the range −0.1 to +0.1. In contrast, the cost of isometric contractions was always lower than the cost of contraction during movement, regardless of the stimulus phase. The observation that the metabolic cost was consistently lower in isometric contractions than in shortening contractions is another example of the long-established 'Fenn effect' (Fenn, 1923).

DISCUSSION

Fibre types

The muscles we used have only small proportions of type 1 (slow-twitch) fibres, so the mechanical and energetic properties we report are mainly those of the type 2 (fast-twitch) fibres. This distribution of fibre types matches with rabbits' characteristic behaviour of sprinting to the burrow when a predator appears. Most mammalian muscles contain a mixture of fast and slow motor units. These can be differentially recruited and can shorten at different speeds. Experiments on mouse muscles have shown that during sinusoidal movement higher power can be produced by fast-twitch fibres than by slow-twitch fibres, but at higher immediate cost (Barclay, 1994). Here, we have made a more extensive study of the energetics of fast-twitch mammalian fibres, by using a wider range of stimulation patterns that include stimulation during lengthening.

Mechanics and energetic cost

In an intact animal, muscle fibres are functionally flexible enough to perform different roles during locomotion: motor, brake and support. The actual role can vary across the continuum from

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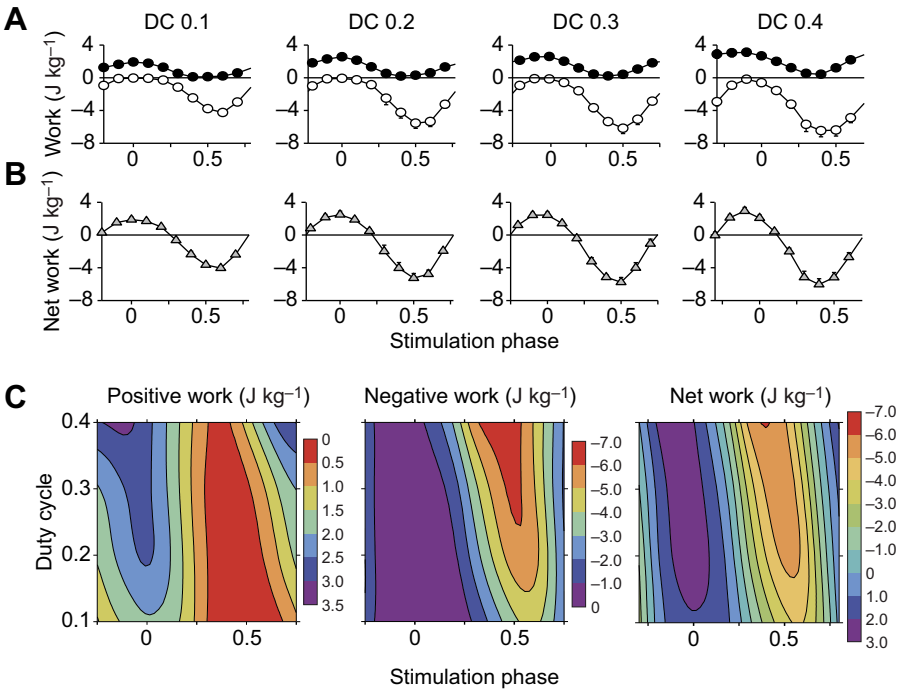

Fig. 4. Effects of stimulus duty cycle and phase on work. (A) Positive work (filled circles) and negative work (open circles). (B) Net work, the sum of positive and negative work. Duty cycle (DC) is the fraction of the movement cycle during which the fibre bundle was stimulated. Stimulation phase indicates the fraction of cycle time between the start of shortening and the start of stimulation: 0 corresponds to stimulation and shortening starting simultaneously; 0.5 corresponds to stimulation and lengthening starting simultaneously. (C) Contour graphs of the relationship between stimulation parameters and positive work, negative work and net work. All values are the average per cycle. Results from eight fibre bundles. Raw data including *n* values are given in Table S1.

motor, shortening against a load and delivering mechanical work; to brake/damper, lengthening under load and absorbing energy; to support, holding constant length with force. For instance, walking upstairs, walking downstairs and standing mid-stair. The muscle uses energy at different rates for these different roles and the interplay between how much energy (ATP) is used and how much work and force the muscle delivers determines the efficiency of the activity at a muscle level. Efficiency at a muscle level is important as it defines how much energy the muscle uses and how much work and heat are produced delivering that particular role. Whilst operating at maximum efficiency might seem ideal, experimental evidence has shown that muscles can and do operate away from a single set of optimal conditions (Curtin and Woledge, 1996; Carrier et al., 2011). The energetic implications of moving away from the optimum efficiency condition are a key component of what was explored here. The area of the high plateaux on the contour plots represents optimum efficiency and the slopes into the valleys show how steeply efficiency falls as contraction conditions change (Fig. 6D). It is apparent that in an imposed sinusoidal movement, a muscle can operate as motor or brake by simply changing the phase of activation. Increasing the duration of activation generally delivers more work but with a lower efficiency (Fig. 6C,D).

When functioning as a motor, muscle fibres convert chemical energy from ATP hydrolysis into mechanical work. The work is

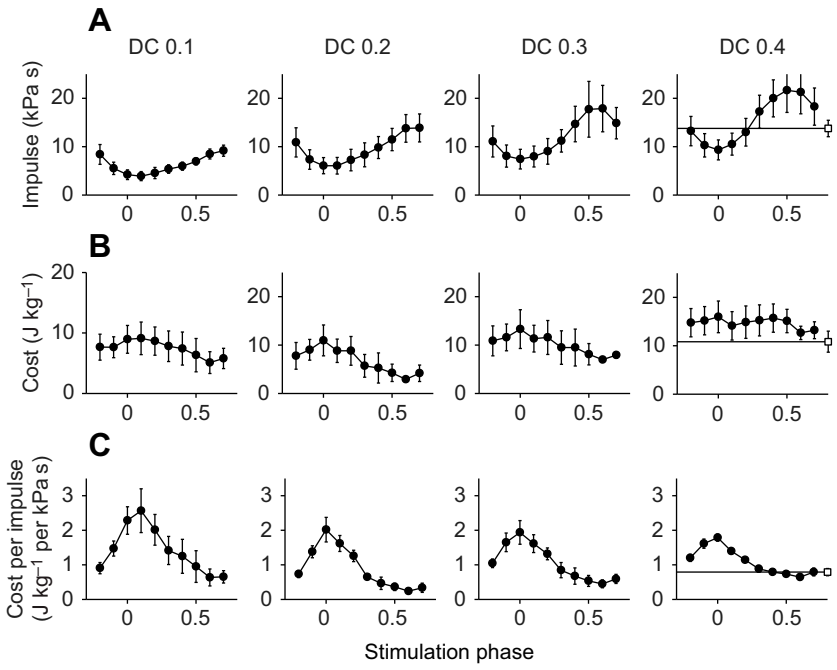

Fig. 5. Effects of stimulus duty cycle and phase on impulse, cost and cost per unit impulse. (A) Impulse, the integral of force and time, during contractions with movement. (B) Cost of contraction, which is the sum of net work and heat. (C) Cost per unit of impulse. The values for stimulation duration corresponding to DC 0.4 and without movement (isometric) are shown as an open square and horizontal line in the DC 0.4 graphs. All values are the means±s.e.m. per cycle. Raw data including *n* values are given in Table S2.

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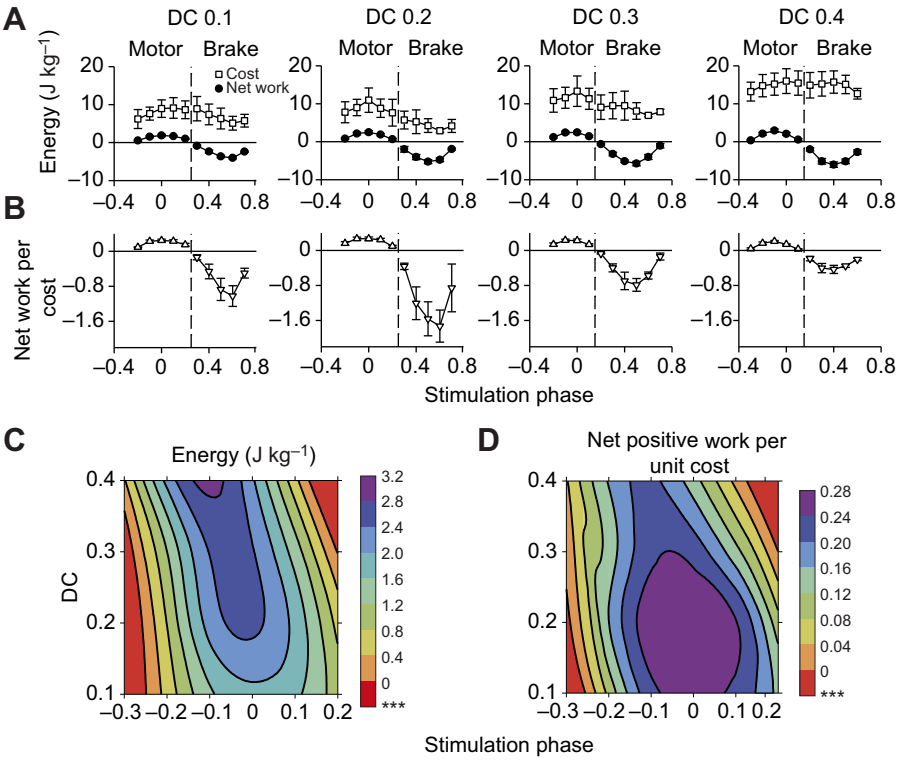

Fig. 6. Net work and cost of muscle fibres functioning as a motor and a brake. (A) The immediate cost (heat+net work) and the net work per cycle of movement for four different durations (duty cycles, DC) of stimulation and 10 phases of stimulation. The 'motor' values are averages of results with net positive work, and the 'brake' values are averages of results with net negative work. (B) Means of the ratio of net work (J)/cost (J). The 'motor' values are the same as conventional initial mechanical efficiency values. All values are means ±s.e.m. (C,D) Contour graphs showing the relationship of stimulus parameters to (C) net positive work and (D) initial mechanical efficiency [net positive work (J kg^{-1}) per unit cost (J kg^{-1})]. *** indicates no data in this area. Raw data including *n* values are given in Table S3.

done on structures external to the muscle fibres as they shorten while producing force. This function is most obvious for the whole animal when moving up hill and in the part of a single stride when muscle fibres shorten while producing force. When functioning as a brake, active muscle fibres resist the external force that is lengthening them; the muscle fibres absorb the work being done on them (negative work). This occurs more extensively when an animal moves down hill or when muscle fibres lengthen in the landing part of a single stride. When acting as a support, active muscle supports external structures while remaining at a constant length. Muscle functions in this way when it prevents joint movement due to gravity during standing. While carrying out all these functions, muscle is producing impulse; that is, producing active force for a period of time (see below). During locomotion, all three functions may be performed at the same time by different muscles and by the same muscle at different parts of the locomotor cycle. The muscle uses ATP in all these cases. Here we measured the net energy released from the muscle as heat and net work and used it as a measure of the immediately incurred cost of the contraction.

Energetic cost and net energy release

Net energy release is calculated on the basis of the law of conservation of energy. In this context, it is the sum of the following: (1) work done by the muscle, positive work, which is energy output from the muscle, (2) work done on the muscle by the motor, negative work, which is energy input into the muscle (the fate of this energy will be discussed later), and (3) heat, which is energy output from the muscle. Thus, net energy release is the sum of the two outputs and one input of energy. In the experiments reported here, the net energy is always a positive quantity, that is, energy release from the muscle. The law of conservation of energy requires that there is a source of this energy; we assume that the source is the energy from concurrent chemical reactions (ATP hydrolysis, creatine kinase reaction, ion binding, unbinding, etc.).

Motor function: cost and efficiency of energy conversion in muscle

The sections labelled 'motor' in Fig. 6A show the results for stimulus phases that gave net positive work. The ratio of net positive work to the cost of these contractions (=net positive work/cost) is the initial enthalpy efficiency (Fig. 6B,D): 'enthalpy' because it is based on heat and work (enthalpy), not on free energy, and 'initial' because the cost is the ATP and the immediate buffering by the creatine kinase reaction during the contraction. This cost does not include subsequent 'recovery' processes that involve oxygen consumption and resynthesis of ATP. The variation in initial enthalpy efficiency with stimulus phase is mainly due to variation in the amount of work rather than cost (compare Fig. 6B with Fig. 5B). The cost of contraction is mainly due to ATP use by two intracellular processes: activation and the cross-bridge cycle. Activation includes heat changes from Ca^{2+} binding and unbinding reactions and ATP use by active transport of Ca^{2+} into the sarcoplasmic reticulum (SR). From existing evidence, it appears that the amount of Ca^{2+} released from the SR is not affected by sarcomere lengths in the range used here (Barclay et al., 2007; Pham et al., 2017). Therefore, we assumed that the cost of pumping it back into the SR would be the same at all stimulation phases for each duty cycle. In other words, the contribution of the cost of activation to the total energy cost is the same within each panel of Fig. 5B. Thus, the variation in cost is due to ATP hydrolysis by the cross-bridges. The cross-bridge cycle is a network of reactions involving ATP and the thin, actin-containing filaments and thick, myosin-containing filaments. When the filaments are sliding in the direction that shortens muscle (motor function), every cross-bridge cycle (attachment, force production and filament sliding) hydrolyses one molecule of ATP. Variation in the work per ATP molecule is due to the amount of force the attached cross-bridge produces and the extent of filament sliding that occurs while the cross-bridge remains attached (Smith et al., 2005; Barclay, 2018).

The maximum efficiency (work/cost) we measured here was relatively low, 0.207, compared with values measured in muscles from other species (see extended data table 5 in Curtin et al., 2018). This could be due to an intrinsic property of the muscle fibres from wild rabbit, such as unusually high activation costs, and/or to cross-bridge attachments that are relatively brief and/or produce relatively low force. Alternatively, the conditions we used may not be optimal for efficient conversion of chemical energy into work. We have tested a wide range of stimulus patterns, but only sinusoidal movement at one frequency and amplitude. In our experiments, shortening velocity ranged from 0 (isometric) to approximately 0.75 L_o s^{-1} which is less than the velocity (1.5 L_o s^{-1}) that gave maximum power in the fully active rabbit muscle (Curtin et al., 2015). Combinations of movement frequency and amplitude giving higher velocities, and non-sinusoidal movement patterns, particularly based on patterns that occur in the intact animal seem worth exploring.

Braking function and cost of negative work

The term negative work may seem obscure; it is the amount of work done on an actively contracting muscle to lengthen it. The muscle absorbs this work while producing active force resisting (but not preventing) lengthening. The results labelled 'brake' in Fig. 6 are for cases where the net work was negative; that is, the sum of the work done on the muscle exceeded that done by the muscle or, in other words, work input exceeded work output. What happens to this net energy input into the muscle? There are two possibilities: energy is stored in the muscle or it is released as energy output (heat and/or positive work).

There is evidence from experiments on whole muscle, fibre bundles and single fibres that when a lengthening is fast enough, some of the energy put into the fibre is stored (Hill and Howarth, 1959; Constable et al., 1997; Linari et al., 2003). The nature or mechanism of this storage is unclear, but at least one possible mechanism has been ruled out. Some ATPases, such as the ATP synthase in mitochondria, are reversible; is ATP resynthesised during muscle lengthening? The idea is appealing and evidence for it has been searched for extensively but has never been found (Loiselle et al., 2010). Thus, it must be concluded that the ATPases of muscle contraction cannot be reversed by lengthening an active muscle. Other possible storage mechanisms, particularly in higher-energy cross-bridge states, are discussed by Linari et al. (2003), and the possibility of the elastic energy storage in stretched titin producing positive work is discussed by Linke (2018).

What happens to stored energy? Following lengthening, in single frog fibres held at constant length, stored energy is released as heat with a time constant of about 50 ms; thus, the release is complete by 200 ms (Linari et al., 2003). The release may be even quicker in the experiments reported here where the temperature was higher than that used by Linari et al. (2003) (25 versus 1°C). Thus, it seems likely that release of stored energy as heat would be complete in each of the 500 ms movement cycles used in our experiments. Similarly, if stored energy is released as positive work in subsequent shortening, it is likely to be complete in each movement cycle. Therefore, the net energy released by the muscle when acting as a brake is the energy output as heat+positive work output−energy input as work done on the muscle; in other words, heat+net work (when the muscle is acting as a brake, the net work is a negative quantity). The net energy is referred to as 'cost' in Fig. 5B and represents the energy that is supplied by ATP hydrolysis.

The low cost per impulse (Fig. 5C) during lengthening and the low cost of negative work (Fig. 6A) agree with the results of experiments using two different, chemical methods to measure costs during lengthening (Curtin and Davies, 1973; Bickham et al., 2011). Our results and these earlier studies all show that it is inexpensive for muscle to act as a 'brake'.

Muscle as a motor and as a brake: why work per unit cost is different

At all duty cycles, the maximum net positive work per cost is less than the maximum net negative work per cost (Fig. 6). This reflects the fact that positive work is more expensive than negative work, i.e. the motor function of muscle is costlier than its braking function. The low ATP use by active muscle during lengthening (braking function) points to the fact that the cross-bridges operate in a fundamentally different way during lengthening from that during shortening (motor function). During lengthening, force remains high over distances of filament sliding that are greater than cross-bridge length, so bridges must break and reattach. The low rate of ATP use indicates that each forced breaking and reattachment does not require the use of an ATP molecule, whereas during shortening, cross-bridge detachment and reattachment does (Piazzesi and Lombardi, 1995; Bickham et al., 2011).

Cost of impulse

In the special case of an animal moving at constant speed on the level, little net work is done. For muscle–tendon units containing muscle fibres that are short compared with their in-series tendons, most of the length change can occur in the tendons, producing what has been called a 'bouncing' gait. The storage and release of elastic energy in tendons in this situation is envisaged as meeting the mechanical requirements (length and force changes). The tendons are passive springs and would thus incur only a small metabolic cost (only that needed to make up for their imperfect elasticity) (Wilson and Goodship, 1994; Dimery, 1985). In this scenario, the role of the muscle fibres is to match the force of the in-series tendon with ideally no change in length; the increase in force as the tendon stretches would be matched by the increase in force as the isometric contraction progresses, followed by isometric force declining in relaxation as the tendon shortens. Thus, the muscle fibres' costs would be solely due to isometric force production (impulse, integral of force and time) rather than to the production of work. It has been supposed that the energetic cost of such an action by the muscle–tendon unit would be less than for an equivalent movement produced by the same muscle fibres in series with an infinitely stiff tendon where all of length change is in the muscle, doing work during shortening (Lichtwark et al., 2007; Lichtwark and Wilson, 2007, 2008). In an experimental study, Holt et al. (2014) tested this idea using frog (*Xenopus laevis*) iliofibularis muscle with minimal in-series tendon. Contrary to expectation, they found that the impulse was not produced more economically under isometric conditions than in the stretch–shortening protocol that produced close to zero net work. We used isometric and zero net work protocols similar to theirs, but with a wider range of stimulus patterns and sinusoidal movement rather than constant speed shortening and lengthening. Our results (Fig. 7C) agree with theirs in showing that the cost per unit of impulse in an isometric contraction is very similar to that for a stretch followed by shortening when the stimulation phase was chosen such that net work is close to zero (Fig. 7B) and the stimulation duration was same as the isometric condition. Thus, their conclusion that isometric contraction does not provide an economic benefit in

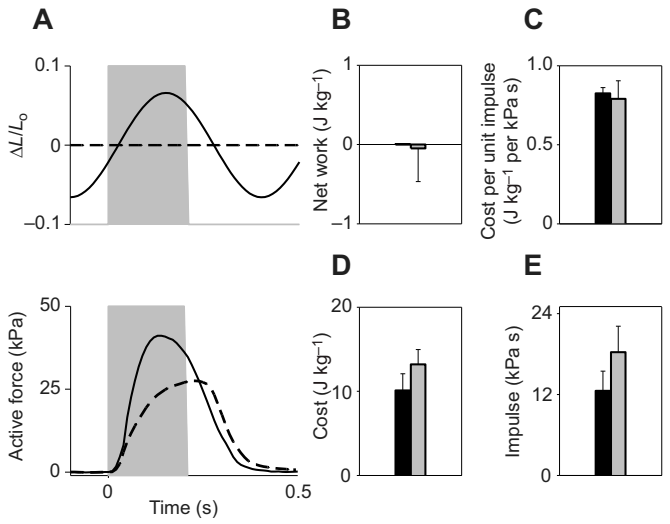

Fig. 7. Comparison of contractions that produce zero net work. Isometric contractions versus movement with stimulation during lengthening followed by shortening. (A) Example records of movement ($\Delta L/L_o$ is length change expressed as a fraction of L_o) and active force during one cycle at 2 Hz. Shading indicates the period of stimulation (DC 0.4, phase –0.3). Dashed lines indicate isometric contractions; solid lines indicate lengthening–shortening. (B–E) Net work (B), cost per unit impulse (C), cost (D) and impulse (E). Black bars indicate isometric contraction, grey bars indicate stretch–shortening. Values are given for one cycle (average of three cycles). Means±s.e.m, n=4 fibre bundles, each from a different rabbit. Raw data are given in Table S4.

terms of cost per unit impulse, compared with a stretch–shortening cycle giving zero net work, also applies in mammalian muscle performing sinusoidal movements. Although the two contraction patterns have matching net work and cost per unit impulse, it is worth noting some differences. Fig. 7A shows the time courses of force production where the peak forces and the rates of rise of force do not match. In addition, compliant tendons have energetic benefits during cyclic contractions that produce net positive work. Average power output, instantaneous power and efficiency can all be enhanced by using artificial tendons to vary the compliance in series with muscle fibres (Lichtwark and Barclay, 2010).

Efficiency of muscle versus oxygen consumption by running animals

How does the initial enthalpy efficiency of muscle contraction that we report here relate to the values of the net efficiency of a running animal measured in terms of power and rate of oxygen consumption? The most important point is that the two are not equivalent, and direct comparison of the values is not meaningful. Smith et al. (2005) and Barclay (2018) give clear and thorough explanations; three of the relevant points will be summarised here.

First, in a running animal, net efficiency is measured as work/energy from metabolic oxidation, where energy from metabolic oxidation=ml O_2 used×20.1 J ml^{-1} O_2 (or can be expressed in terms of power and rate of oxygen consumption). The net efficiency does not depend on the muscle's 'initial efficiency' (=work/initial energy produced while the work is being done). This may seem paradoxical because 'net efficiency' is work/(initial energy+recovery energy). The key point is that recovery energy does not equal the energy from metabolic oxidation; instead, recovery energy equals energy from metabolic oxidation minus initial energy. The 'minus initial energy' term expresses the fact that the reaction(s) that produces the initial energy during contraction is reversed during recovery (see eqn 24 in Smith et al., 2005). In summary:

$$\begin{aligned}\text{Net efficiency} &= \text{work/(initial energy + recovery energy)},\\ &= \text{work/(initial energy + energy from metabolic oxidation} - \text{initial energy)},\\ &= \text{work/energy from metabolic oxidation}.\end{aligned} \quad (3)$$

Thus, unless the ratio of recovery heat to initial energy is known, it is not possible to interconvert muscle initial enthalpy efficiency and net efficiency based on work and oxygen consumption (Barclay, 2018).

Even when the value of recovery heat and initial energy are known, other information is needed to relate initial enthalpy efficiency of muscle contraction to the net efficiency from oxygen consumption by a running animal. Two particularly important values are the baseline O_2 consumption and the muscle work. What 'baseline' O_2 consumption by a running animal should be subtracted from the total to give that component of O_2 consumption by muscle? The appropriate baseline would continue unchanged during running with the O_2 consumption by muscle simply adding to it. Oxygen consumption at rest does not meet this criterion (Barclay, 2018). The total work done during running consists of that done by muscle and that done by non-muscle elastic elements that store and release work during each stride (the so-called bouncing component). The component of the work done by the muscle in the running animal must be evaluated for a meaningful comparison of muscle efficiency with net efficiency of a running animal (Barclay, 2018).

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Competing interests
The authors declare no competing or financial interests.

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Summary: Muscle can be a motor (work output) or a brake (work absorption), or can simply exert force; these functions and their costs are relevant to locomotion. This versatility is revealed by a varying stimulation pattern.

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